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Molecular Population Genetics of Accessory Gland Protein Genes and Testis-Expressed Genes in *Drosophila mojavensis* and *D. arizonae*

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ABSTRACT

Molecular population genetic investigation of Drosophila male reproductive genes has focused primarily on *melanogaster* subgroup accessory gland protein genes (*Acp*'s). Consistent with observations from male reproductive genes of numerous taxa, *Acp*'s evolve more rapidly than nonreproductive genes. However, within the Drosophila genus, large data sets from additional types of male reproductive genes and from different species groups are lacking. Here we report findings from a molecular population genetics analysis of male reproductive genes of the *repleta* group species, *Drosophila arizonae* and *D. mojavensis*. We find that *Acp*'s have dramatically higher average pairwise K_a/K_s (0.93) than testis-enriched genes (0.19) and previously reported *melanogaster* subgroup *Acp*'s (0.42). Overall, 10 of 19 *Acp*'s have $K_a/K_s > 1$ either in nonpolarized analyses or in at least one lineage of polarized analyses. Of the nine *Acp*'s for which outgroup data were available, average K_a/K_s was considerably higher in *D. mojavensis* (2.08) than in *D. arizonae* (0.87). Contrasts of polymorphism and divergence suggest that adaptive protein evolution at *Acp*'s is more common in *D. mojavensis* than in *D. arizonae*.

MOLECULAR studies in a diverse array of animal taxa suggest that genes involved in reproduction evolve at an accelerated rate relative to other genes (reviewed in Swanson and Vacquier 2002). Positive selection has been inferred for some proteins (Swanson and Vacquier 1995; Metz and Palumbi 1996; Sutton and Wilkinson 1997; Wyckoff *et al.* 2000; Torgerson *et al.* 2002), although population genetic data are sufficiently sparse to leave unresolved the question of the relative importance of directional selection *vs.* genetic drift in reproduction-related proteins compared to other protein classes. In any case, rapid phenotypic/molecular evolution of reproductive characters/genes is consistent with the notion that male-male and male-female interactions may contribute to the rapid divergence between populations and the evolution of reproductive isolation (Eberhard 1996; Rice 1998).

Molecular evolutionary investigation of Drosophila reproduction has focused on male accessory gland protein genes (*Acp*'s) of *melanogaster* subgroup species. The number of putative *Acp*'s in these species is on the order of 83 (Swanson *et al.* 2001), although <20 have extensive experimental support (Schäfer 1986; DiBenedetto *et al.* 1987; Chen *et al.* 1988; Monsma and Wolfner 1988; Wolfner *et al.* 1997). Genetic analysis has shown that *Acp*'s contribute to proper sperm storage (Neubaum and Wolfner 1999; Tram and Wolfner 1999; Chapman *et al.* 2000), normal ovulation and oviposition (Herndon and Wolfner 1995; Heifetz *et al.* 2000), increased egg-laying rates, and reduced female receptivity (Chen *et al.* 1988; Aigaki *et al.* 1991; Kalb *et al.* 1993; Chapman *et al.* 2003; Liu and Kubli 2003). *Acp*'s show higher rates of protein divergence (Aguadé 1997, 1998, 1999; Tsaur and Wu 1997; Tsaur *et al.* 1998; Begun *et al.* 2000; Swanson *et al.* 2001) and protein polymorphism (Coulthart and Singh 1988; Begun *et al.* 2000) compared to "average" proteins in *Drosophila melanogaster* and *D. simulans* (*e.g.*, Begun *et al.* 2000). Less energy has been devoted to population genetic investigation of male reproductive genes primarily expressed in testes (but see Duvernell and Eanes 2000; Parsch *et al.* 2001a). However, a few analyses suggest that Drosophila testis-expressed genes evolve quickly (Parsch *et al.* 2001b; Meiklejohn *et al.* 2004; Richards *et al.* 2005) and may sometimes be associated with evolution of novel function (Long and Langley 1993; Nurminsky *et al.* 1998; Betrán and Long 2003).

Because our current population genetic understanding of Drosophila is dominated by data from *melanogaster* subgroup species, we have no way of knowing whether the patterns of polymorphism and divergence or the functional biology of reproduction-related proteins will be similar in other Drosophila species (Wagstaff and Begun 2005). Given the hypothesis that the dynamics of certain male reproduction-related proteins may be driven by male-male and male-female postcopulatory

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interactions, one strategy for furthering our understanding of the evolution of these proteins is to investigate Drosophila species having different reproductive biology from *D. melanogaster* and *D. simulans*. *D. arizonae* and *D. mojavensis* are cactophilic fly species within the *mulleri* complex of the *repleta* group. As members of the subgenus Drosophila, these desert Drosophila are ~40–60 million years diverged from *D. melanogaster* and other Sophophora subgenus flies (Powell and DeSalle 1995).

A major difference in the reproductive biology of desert Drosophila *vs. D. melanogaster* is that remating occurs more frequently in desert Drosophila. Within 24 hr of an initial mating, 95% of *D. arizonae* and *D. mojavensis* females tend to remate, while only 2% of *D. melanogaster* females remate in this same time period (Markow 1982, 1996). Frequent remating favors competition between male ejaculates, whereas infrequent remating would be more likely to favor genotypes successfully obtaining initial access to females (*e.g.*, Markow 2002). Data from Drosophila species suggest that there is a positive correlation between high female remating rates and exaggerated ejaculates in the form of either sperm gigantism or excessive ejaculate donation to female tissues (Markow 2002). Although both desert Drosophila species discussed here contribute large ejaculate donations to ovaries, *D. arizonae* and *D. mojavensis* contribute small and large donations, respectively, to female somatic tissues (Pitnick *et al.* 1997). Experiments in *D. melanogaster* revealed no detectable incorporation of ejaculate-derived material into female somatic or ovarian tissues (Pitnick *et al.* 1997). While ejaculate donations are often perceived to be of nutritive value, a cost to remating has been observed in *D. mojavensis* females, suggesting the possibility of sexual conflict (Etges and Heed 1992). Another major difference in the reproductive biology of *repleta* group *vs. melanogaster* subgroup flies is that *repleta* group males require significantly more time to reach sexual maturity. For example, *D. arizonae* and *D. mojavensis* require 4–5 days posteclosion to reach maturity, compared to 1–2 days for *D. melanogaster* males (Pitnick *et al.* 1995).

Data on natural variation in reproductive traits suggest a more dynamic postmating interaction between the sexes in desert Drosophila compared to *melanogaster* subgroup flies. Immediately after mating, a pronounced insemination reaction occurs in the female reproductive tract of *D. arizonae* and *D. mojavensis* (Patterson 1947; Patterson and Stone 1952) but is absent in *D. melanogaster* (Wheeler 1947; Markow and Ankney 1988). The reaction manifests itself as a large mass within the vaginal pouch and acts as a barrier that prevents remating for the several hours that it persists (Patterson 1947; Knowles and Markow 2001). Seminal fluid proteins may be the primary male contributor to this phenotype, as it is triggered in the absence of live spermatozoa (Patterson 1947). Comparisons between desert Drosophila species, as well as between different populations within species, show that postcopulatory male-female interactions change across short evolutionary time periods. For example, heterospecific matings between *D. arizonae* and *D. mojavensis* trigger an exaggerated insemination reaction that is both harder and longer lasting than that of the respective conspecific matings of either species (Patterson 1947). Moreover, both *D. arizonae* and *D. mojavensis* show larger and longer insemination reactions in interpopulation *vs.* intrapopulation crosses (Knowles and Markow 2001) within species. Further evidence of rapid evolution of reproductive traits comes from the observation that *D. mojavensis* shows significant among-population variation in the correlated traits of male sperm size and female sperm-storage organ length (Pitnick *et al.* 2003).

These data support the idea that properties of ejaculates or ejaculate-female interactions evolve very quickly in desert Drosophila, possibly as a result of antagonistic coevolution between the sexes (Rice 1996, 1998) and/or cryptic female choice (Eberhard 1996). We should expect such elaboration of ejaculate characteristics to extend to the molecular level. The purpose of this study is to add a molecular framework to investigation of desert Drosophila reproduction. First, we report the composition of *D. mojavensis* male reproductive tract cDNA libraries relative to the gene annotations of *D. melanogaster*. Many of these data are presented as supplementary online material (http://www.genetics.org/supplemental). Second, we report results from molecular and evolutionary analyses of genes expressed in male reproductive tracts in *D. mojavensis* and *D. arizonae* and compare these results to those previously reported from *D. melanogaster*/*D. simulans*.

MATERIALS AND METHODS

***D. mojavensis* reproductive tract library:** Poly(A)-enriched mRNA was prepared with the MicroPoly(A)-Pure kit (Ambion, Austin, TX) from 50 whole reproductive tracts of adult male *D. mojavensis* flies. First-strand cDNA was reverse transcribed with the SMART PCR cDNA synthesis system reagents and protocol (CLONTECH, Palo Alto, CA). Second-strand product was produced with the Expand high-fidelity polymerase system (Roche Molecular Biochemicals, Indianapolis). Cycling parameters were programmed as instructed by the manufacturer, including a 4-min extension step for 10 total cycles. The second-strand product was cloned into the TOPO vector (Invitrogen, San Diego) and used for bacterial transformations according to the manufacturer's instructions. Colony PCR was carried out using cloning-vector-derived primers (M13 reverse and T7) on 480 colonies (*i.e.*, five 96-well plates). The resulting PCR products were purified prior to sequencing with M13R and T7 primers on an Applied Biosystems (Foster City, CA) 377 automated sequencer (ABI, Columbia, MD). These sequences included 54 unique transcripts. Expressed sequence tags (ESTs) from this library can be found under accession nos. DR033184–DR033386 and DR033894–DR033895.

Preliminary expression analysis and *D. mojavensis* testis cDNA library production: Dot blots prepared from PCR products of the 54 unique clones were hybridized separately

to ^{32}P-labeled cDNAs derived from *D. mojavensis* accessory glands and testes. Hybridizations were carried out at 65° in a buffer consisting of 0.5 M NaPi (pH 7.2), 7% SDS, 1 mM EDTA. Filters were washed at 60° with buffer at 40 mM NaPi, 1% SDS, and 1 mM EDTA. Comparison of signal intensities from hybridization of labeled accessory gland *vs.* testis cDNA suggested that the majority of the clones represented accessory gland transcripts.

To increase the sample size of testis-enriched transcripts we made a testis cDNA library. This library was produced as described above for whole reproductive tracts, but with 50 *D. mojavensis* dissected testes as the source tissue. This library was sequenced to the point of producing 118 unique ESTs. ESTs from the testis library can be found under accession nos. DR033387–DR033542.

BLAST methodology and characterization of amino acid sequences: All unique ESTs were compared to *D. melanogaster* through a pipeline of BLAST analyses to one or more FlyBase Release 3.1 databases (Altschul *et al.* 1997). Default BLAST parameters were used except that the cutoff value for significance was set to $E = 0.01$. The pipeline started with BLASTp (protein to predicted *D. melanogaster* proteins) queries of all ESTs for which an open reading frame (ORF) was well established (as described below). ESTs that returned significant ($E < 1e\text{-}8$) *D. melanogaster* sequences were not queried further. The remaining ESTs were BLASTx (nucleotide to protein) queried to the same *D. melanogaster* database. Once again, ESTs that returned small *E*-values were not queried further. This pipeline continued through tBLASTx (nucleotide to nucleotide query, using all six possible protein translations of the sequences) and BLASTn (nucleotide to nucleotide) queries of predicted *D. melanogaster* genes and chromosome arms. For the ESTs that returned no *D. melanogaster* sequences at $E < 0.0001$, the NCBI whole-genome shotgun (wgs) database was tBLASTx queried with the same default parameters (Altschul *et al.* 1997). The NCBI wgs database includes many complete genomes, including *D. pseudoobscura* and the mosquito, *Anopheles gambiae*. All *D. mojavensis* ESTs were also tBLASTx or BLASTn queried (BLASTn was used only if tBLASTx failed to return sequences of $E < 0.0001$) to the *D. melanogaster* dbEST database using default BLAST parameters and an *E*-score cutoff of 0.01. Finally, we queried the SignalP 3.0 (Nielsen and Krogh 1998; Bendtsen *et al.* 2004) and NCBI CDD (Marchler-Bauer *et al.* 2003) servers with amino acid sequences corresponding to ESTs with identifiable ORFs to identify the presence of signal peptides and conserved domains, respectively.

A subset of genes isolated from both libraries was scrutinized in greater detail to winnow candidates for population genetic analysis. Each clone sequence was subjected to an ORF analysis by the GeneJockey software program (Biosoft, Ferguson, MO). If a putative initiation codon followed by an ORF covering at least 70% of the EST could not be identified, we used RACE to gather additional cDNA sequence data.

Reproductively mature *D. mojavensis* adults of both sexes served as the tissue source for RACE-ready template. mRNA was isolated using the MicroPoly(A)-Pure kit (Ambion, Austin, TX). RACE-ready cDNA was prepared and target molecules were PCR amplified and isolated using the GeneRacer (Invitrogen) protocol, which preferentially selects full-length transcripts for first-strand cDNA synthesis. RACE products derived from such a library should provide high-quality information on the 5′ ends of transcripts. Several criteria were used to identify the set of ORFs ultimately used in molecular evolutionary analysis: (i) size and position of candidate ORFs within an EST, (ii) presence of a predicted signal peptide sequence for putative *Acp*'s (Wolfner *et al.* 1997; Swanson *et al.* 2001), (iii) tBLASTx homology to genes in public databases (*e.g.*, *D. melanogaster* genome release 3.1), and (iv) presence/absence of INDEL mutations and/or premature termination codons in polymorphism data from genomic DNA. Only strongly supported ORFs were used in evolutionary analysis.

Quantitative PCR evaluation of ESTs: Genes targeted for population genetic analyses as accessory gland *vs.* testis-enriched in expression on the basis of dot blots were subjected to more rigorous quantification of transcript distribution and abundance by real-time quantitative PCR. For the subset of genes in which a related *D. melanogaster* gene was identified, quantitative PCR was also carried out in *D. melanogaster* to provide comparisons of expression between lineages. The purpose of this analysis was to assign genes to three expression classes: *Acp*, testis enriched, and other tissues. A total of 58 and 33 genes were investigated in *D. mojavensis* and *D. melanogaster*, respectively.

Tissue dissections consisted of 80 *D. mojavensis* and 40 *D. melanogaster* male flies. All flies were reproductively mature and were dissected in RNA*later* (Ambion) into three tissue categories: accessory glands, testes, and carcasses without the reproductive tracts. Each collection of dissected tissues was divided equally into two replicate samples for RNA isolation. Likewise, whole, reproductively mature female flies from each species ($n = 40$) were evenly split into two replicate RNA preps. Total RNA was extracted using Trizol Reagent (Invitrogen), purified through RNeasy (QIAGEN) columns, and treated with DNase according to manufacturer's instructions (QIAGEN). RNAs were then reverse transcribed at a concentration of 20 ng/μl using TaqMan reverse transcription (RT) reagents (Applied Biosystems). These first-strand cDNAs served as the templates for quantitative PCR analysis.

Quantitative PCR was performed using an ABI Prism 7700 sequence detector and SYBR green PCR core reagents (Applied Biosystems). Amplification primers were designed with Primer Express (Applied Biosystems). For every 20-μl PCR reaction, 0.5 μl of first-strand cDNA was used. Quantitative PCR conditions were 94° for 10 min followed by 40 cycles of 94° for 20 sec, 59° for 30 sec, and 72° for 30 sec. A dissociation step was added to the end of the run to ensure that only a single amplicon was produced in each reaction. All primer pairs produced a single product. A total of 13 quantitative PCRs were processed for each gene. Three reactions were run for each of the four tissues: one for each of the two replicate RT reactions as well as a single minus-RT reaction derived by drawing equally from the minus-RT templates of paired replicates. The 13th reaction was a no-template control. We found no evidence of genomic contamination or primer-by-reagent interactions.

Quantitative PCR quantification: Quantification followed the $2^{-\Delta\Delta C_T}$ methods of Livak and Schmittgen (2001). Quantitative PCR provides an estimate of C_T, the cycle at which the quantity of amplified product exceeds a predetermined threshold. Therefore, more abundant transcripts should yield lower C_T scores. To control for different first-strand cDNA concentrations across templates, as well as run and reagent effects, our ΔC_T scores were calculated by subtracting experimental gene C_T scores from housekeeping gene C_T scores derived from the same tissue and experimental microtiter plate. The housekeeping control for both species was the ribosomal protein gene *CG7808*, which was identified in the *D. mojavensis* reproductive tract cDNA library (*moj12*) and is highly conserved between *D. mojavensis* and *D. melanogaster* (96% protein similarity).

Our calculation of $2^{-\Delta\Delta C_T}$ reflects fold change in gene expression of the most abundant tissue template (lowest ΔC_T score) relative to the second most abundant tissue template for any given gene. There were two justifications for this approach. First, we observed several instances in which quantitative PCR product was detected in only two of the four templates. Second, compared to approaches estimating fold

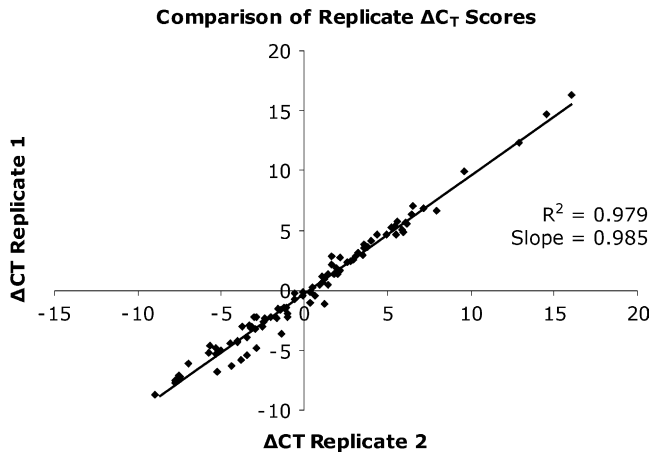

FIGURE 1.—Comparison of replicate ΔC_T scores. Each point represents a pair of replicates. Perfect replication would generate slope and R^2 scores of 1.0.

differences across all tissues, our approach minimizes fold difference values, thereby providing conservative lower-bound estimates for actual differences between tissue transcriptome profiles. The two replicate $2^{-\Delta\Delta C_T}$ scores for each gene were always independently calculated and then averaged for the reported values.

Quantitative PCR statistics: Replicate $2^{-\Delta\Delta C_T}$ scores for every gene and for each of the four templates can be used to determine the amount of experimental error. A scatter plot of replicate ΔC_T scores for the most abundant tissue of each surveyed gene ($n = 91$, Figure 1) reveals a high degree of similarity between replicate pairs ($R^2 = 0.979$). The slope of this line ($m = 0.985$) is very close to $m = 1$, showing that the high repeatability of our assays holds across a large range of expression estimates.

We used our replicate $2^{-\Delta\Delta C_T}$ scores to determine threshold fold differences that are sufficiently disparate to represent significant differences. To approximate a gamma distribution, we calculated ratios of replicate pairs by dividing the higher $2^{-\Delta\Delta C_T}$ score by its counterpart and then subtracting one. A total of 91 replicate reaction pairs generated a distribution ranging from 0.0 to 18.24. We then used the x_0 value at which the area under the frequency distribution ($0 \le x \le x_0$) is equal to 0.95 to establish a critical threshold for significant differences between $2^{-\Delta\Delta C_T}$ scores. For the complete data set, $2^{-\Delta\Delta C_T}$ scores >7.84 represent significant differences between tissues ($P < 0.05$). This is a conservative critical threshold estimate because genes that are highly tissue specific (those with high $2^{-\Delta\Delta C_T}$ scores) are susceptible to larger error in terms of relative expression differences. This is a consequence of fold differences being derived by comparing the most abundant tissue (lowest ΔC_T) to the second most abundant tissue. Thus, fold difference for a gene that is highly tissue specific in expression is estimated by comparison to a tissue showing very low transcript abundance. In such cases, experimental error associated with the less abundant tissue expression will affect $2^{-\Delta\Delta C_T}$ scores of highly tissue-specific genes. Many of our genes have large $2^{-\Delta\Delta C_T}$ scores (see supplementary Table S2; http://www.genetics.org/supplemental), which indicate high tissue specificity. Restricting our statistical analysis to genes with $2^{-\Delta\Delta C_T} < 50$ ($n = 28$), the critical threshold for significance is reduced to 3.25 ($P < 0.05$). Further narrowing the analysis to genes with $2^{-\Delta\Delta C_T} < 15$ ($n = 24$) reduces the critical threshold to 2.10 ($P < 0.05$).

The different critical values for different subsets of the data support the idea that error variance of relative expression levels is greater for genes with the highest $2^{-\Delta\Delta C_T}$ scores. Therefore, we view the critical threshold of 2.10 as most informative because it is derived from the very data whose relative expression patterns are most in doubt. Even so, we choose a conservative critical threshold of $2^{-\Delta\Delta C_T} = 5.0$ (fivefold difference in relative expression for the most abundant *vs.* next most abundant tissue) for the purpose of categorizing genes as either *Acp*'s or testis enriched. Though somewhat arbitrary, we note that categorization of genes would not be substantially altered by choosing a more conservative threshold. For example, a critical threshold of 18 would only recategorize three testis-enriched genes as genes showing no strong pattern of tissue enrichment.

***D. mojavensis* genomic library:** A genomic library was constructed to provide flanking data around gene sequences to help identify regions of homology between *D. melanogaster* and *D. mojavensis* (*e.g.*, WAGSTAFF and BEGUN 2005; see supplementary material, http://www.genetics.org/supplemental). *D. mojavensis* genomic DNA was partially digested with *Sau*3A and size fractionated by electrophoresis through a 0.6% agarose gel. DNA fragments between 9 and 23 kb were selected via gel extraction (QIAGEN), ligated to λ-DASH II/*Bam*HI vector (Stratagene, La Jolla, CA), and packaged using the Lambda DASH II/Gigapack II cloning kit (Stratagene). The resultant library consisted of $\sim 2.3 \times 10^6$ plaque-forming units. Plaques were screened with ^{32}P-labeled *D. mojavensis* target DNA. Lambda DNA was purified from selected plaques and *D. mojavensis* genomic inserts were amplified using T3/T7 vector primers and LA-Taq long PCR polymerase (TaKaRa, Shiga, Japan). The resulting PCR products were sheared by sonication and the fragments were blunt ended using Klenow fragment of DNA polymerase and T4 DNA polymerase. Fragments of 1–2 kb were isolated from a low-melting agarose electrophoresis gel and cloned into the pUC18/*Sma*I/BAP vector with a Ready-to-Go kit (Amerisham Biosciences, Piscataway, NJ). Sequencing of the phage through ~7× coverage was performed on an ABI Prism 3700 sequencer. Consensus sequences were assembled using the SeqMan program of the DNASTAR software package (Lasergene, Madison, WI).

Nomenclature: Unique ESTs were assigned numbers (1–54 for reproductive tract library ESTs, 100–217 for testis library ESTs). Genes from the quantitative PCR analysis showing at least fivefold greater expression ($2^{-\Delta\Delta C_T} > 5$) in either accessory glands or testes were categorized as *Acp*'s and testis enriched in expression (hereafter referred to as testis-enriched genes), respectively. Prefixes for numbered EST names were added according to these expression patterns, with *Acp* preceding accessory gland genes and *Tes* preceding testis-enriched genes. Those genes that did not exceed this threshold (*moj9*, *moj29*, *moj30*, *moj32*, *moj137*, and *moj152*) were given the *moj* prefix to avoid a connotation of tissue specificity. Four *Acp*'s (*Acp5*, *Acp16*, *Acp21*, and *Acp27*) are members of recently duplicated gene families (B. J. WAGSTAFF, unpublished data) and are given an additional *-a* or *-b* suffix to differentiate between members. Five genes were named as *Acp*'s (*Acp4*, *Acp15*, *Acp17*, *Acp23*, and *Acp36*) on the basis of very strong evidence from our dot blot data rather than from quantitative PCR experiments. The remaining ESTs were given the *moj* prefix, as no relative expression data were gathered for the associated genes.

Stocks and DNA sequencing: A total of 15 fly stocks from the Drosophila Species Stock Center (Tucson, AZ) were used for collection of most population genetic data. *D. arizonae* (15081-1271.00, 15081-1271.04, 15081-1271.05, 15081-1271.08, 15081-1271.12, 15081-1271.13, and 15081-1271.14; various locations, mainland Mexico) and *D. mojavensis* were represented by seven lines each, while a single *D. mulleri* stock (15081-1371.00; Lake Travis, TX) provided outgroup data. Of the seven

D. mojavensis stocks, four were *D. mojavensis baja* (15081-1351.03, 15081-1351.09, 15081-1351.12, and 15081-1351.14; various locations, Baja, Mexico) and three were *D. mojavensis mojavensis* (15081-1352.00, 15081-1352.01, and 15081-1352.02; various locations, southern California). Primers used for amplification of genomic DNA were designed from ESTs or from extended sequences identified by RACE analysis. Expand High-Fidelity polymerase (Roche Molecular Biochemicals) was used for PCR amplification. Single alleles for sequencing were isolated by cloning PCR products into the TOPO vector (Invitrogen) and selecting one bacterial colony for PCR amplification for each allele. Amplified colony-PCR products and their associated sequences were obtained using M13 reverse and T7 primers. A second collection of *D. mojavensis* mainland and Baja strains (kindly provided by W. Etges, University of Arkansas) was used for additional population sequencing of *Acp7*. PCR products from the Etges strains were directly sequenced. All sequencing was done on an Applied Biosystems 377 automated sequencer (ABI). Sequences were aligned and edited using the DNASTAR software package (Lasergene). Generally, the small, predicted size of most *Acp*'s resulted in survey data for most codons. Compared to *Acp*'s, testis-enriched genes, on average, provided lower coverage of codons on a per gene basis (see Table 1).

Statistical analysis of aligned sequences: The DnaSP program (Rozas and Rozas 1999) was used for most of the population genetic analyses. Average levels of polymorphism or divergence for different groups of genes (*e.g.*, *Acp vs.* testis enriched) refer to means weighted according to sequence length. For genes sampled for multiple alleles, replacement and synonymous divergence represent the average pairwise difference. Fixations for polarized McDonald-Kreitman tests were assigned using parsimony. Only codons with single mutations that could be clearly assigned to either the *D. arizonae* or *D. mojavensis* lineage were considered.

Lineage-specific synonymous and replacement divergences were estimated using the free-ratio maximum-likelihood model of the PAML computer program (Yang 1997). For most of these analyses we used one randomly selected allele from each of three species: *D. arizonae*, *D. mojavensis*, and *D. mulleri*. In some cases for which *D. mulleri* data were unavailable, we used a duplicated gene that predated the *D. arizonae/D. mojavensis* speciation event (B. J. Wagstaff, unpublished data). We used only duplicated genes showing synonymous divergence that was comparable to or less than the average *D. mulleri* synonymous divergence (see Table 3). Hypothesis testing was carried out using likelihood-ratio tests (Goldman and Yang 1994; Yang 1998). To determine whether or not K_a significantly exceeds K_s in a particular lineage, the likelihood value for the null hypothesis ($K_a = K_s$; *i.e.*, the one-ratio model) was also calculated. Twice the log-likelihood difference between the two models is then compared to a χ^2-distribution with one d.f. to determine the level of significance.

RESULTS

Content and characterization of *D. mojavensis* male reproductive tract libraries

The content and basic characteristics of the *D. mojavensis* male reproductive tract (ESTs 1–54) and testis (ESTs 100–217) libraries are listed in supplementary Table S1 (see http://www.genetics.org/supplemental). Genes with measured accessory gland or testis tissue enrichment are given the *Acp* and *Tes* prefixes, respectively. Six *moj* genes (*moj9*, *moj29*, *moj30*, *moj32*, *moj137*, and *moj152*; see supplementary Table S2, http://www.genetics.org/supplemental) are expressed in multiple tissues. No relative expression analyses were performed on genes corresponding to the remaining *moj* ESTs (see quantitative PCR section below).

Library content: Minimal sequencing of the *D. mojavensis* male reproductive tract library revealed that most of the ESTs corresponded to just a few genes. Preliminary dot blot analysis of an initial set of clones revealed that most ESTs were accessory gland rather than testis derived. Of the first 139 sequenced clones, 35 corresponded to *Acp1*, 27 to *Acp5*, and 18 to *Acp17*. The 139 clones also contained 13 singletons and 10 transcripts represented by 2–9 clones each. The preponderance of *Acp*'s in the reproductive tract library cannot be easily explained by size differences between accessory glands and testes, as *D. mojavensis* testes appear to be considerably larger than accessory glands (B. J. Wagstaff, personal observation). Thus, per unit of tissue, accessory glands likely produce much more mRNA than the testis. We conclude that the *D. mojavensis* accessory gland transcriptome has low complexity and high transcript abundance relative to that of the testis transcriptome. To increase the discovery rate of new transcripts, additional clones were screened by multiplexed PCR reactions that included primer pairs specific to *Acp1*, *Acp5*, and *Acp17*. Clones not corresponding to any of these three genes were then sequenced. This multiplex PCR strategy revealed 28 new ESTs from only 66 additional sequencing reactions. In total, 54 unique ESTs were revealed from the reproductive tract library. The average length of all 205 ESTs was 438 bp.

We constructed and screened a *D. mojavensis* testis cDNA library to increase our sample size of testis-expressed genes. The distribution of replicate ESTs differs dramatically from the original reproductive tract library (supplementary Table S1, http://www.genetics.org/supplemental). The testis library has a much higher complexity than the reproductive tract library, with 105 of 156 clones present as single-copy sequences. Similarly high complexity of a testis cDNA library was previously observed in *D. melanogaster* (Andrews *et al.* 2000), suggesting that this might be a general property of the Drosophila testis transcriptome. In total, 156 sequencing reactions returned an average EST length of 451 bp and produced 118 unique ESTs.

The whole reproductive tract library contains a higher percentage of unique ESTs with potential signal peptide sequences, which is to be expected of a library derived primarily from accessory gland transcripts (Wolfner *et al.* 1997; Swanson *et al.* 2001). Of library sequences subjected to SignalP analysis, 64% (32/50) of whole reproductive tract-derived unique sequences and 10.3% (7/68) of testis-derived unique sequences contain putative signal sequences (those with hidden Markov model $P > 0.75$, supplementary Table S1, http://www.genetics.org/supplemental).

TABLE 1

Polymorphism and divergence at individual *Acp*, *Tes*, and *moj* genes

Gene	No. alleles *a, mo, mu*[a]	No. sites analyzed	ORF size	No. coding analyzed	Sample	θ_{syn}	θ_{rep}	K_s	K_a	K_a/K_s[b]
Acp1	7, 7, 1	326	354	288	*ari*	0.0000	0.0131	0.0463	0.0636	1.3744
					moj	0.0291	0.0056			
Acp2	7, 7, 1	237	354	234	*ari*	0.0218	0.0000	0.0638	0.0619	0.9705
					moj	0.0218	0.0184			
Acp3	7, 5, 0	305	207	150	*ari*	0.0342	0.0036	0.0799	0.0744	0.9316
					moj	0.0000	0.0168			
Acp5a	7, 7, 0	571	105	99	*ari*	0.0151	0.0057	0.1110	0.1099	0.9896
					moj	0.0000	0.0170			
Acp7	7, 7, 1	561	465	453	*ari*	0.0205	0.0086	0.0468	0.0378	0.8079
					moj	0.0068	0.0086			
Acp8	7, 7, 0	275	144	123	*ari*	0.0128	0.0179	0.1621	0.1214	0.7492
					moj	0.0128	0.0179			
Acp11	1, 1, 0	156	201	156		—	—	0.1600	0.0392	0.2450
Acp16a	7, 6, 0	151	189	141	*ari*	0.0000	0.0159	0.0596	0.1315	2.2049
					moj	0.0000	0.0299			
Acp16b	7, 4, 0	214	216	204	*ari*	0.0251	0.0184	0.0618	0.0499	0.8080
					moj	0.0336	0.0070			
Acp19	7, 7, 1	570	687+	510	*ari*	0.0107	0.0041	0.0267	0.0332	1.2424
					moj	0.0107	0.0031			
Acp21a	6, 7, 0	228	207	180	*ari*	0.0092	0.0066	0.0552	0.2274	4.1209
					moj	0.0086	0.0278			
Acp22	1, 2, 0	78	81	78		—	—	0.0000	0.0000	—
Acp24	6, 7, 0	135	129	120	*ari*	0.0000	0.0094	0.0559	0.0325	0.5825
					moj	0.0308	0.0175			
Acp25	7, 7, 1	324	354	294	*ari*	0.0346	0.0018	0.0582	0.0314	0.5386
					moj	0.0173	0.0018			
Acp27a	7, 7, 0	348	291	282	*ari*	0.0000	0.0019	0.0063	0.0135	2.1379
					moj	0.0120	0.0076			
Acp42	7, 7, 0	477	597+	363	*ari*	0.0104	0.0043	0.0724	0.0445	0.6146
					moj	0.0260	0.0043			
Acp45	1, 1, 0	372	408	372		—	—	0.0353	0.0323	0.9150
Acp48	7, 7, 0	516	630+	513	*ari*	0.0075	0.0040	0.1504	0.0861	0.5726
					moj	0.0187	0.0051			
Acp54	1, 1, 0	102	111	102		—	—	0.0000	0.0970	$K_a > K_s$
moj9	7, 7, 1	517	786+	447	*ari*	0.0228	0.0048	0.0495	0.0046	0.0938
					moj	0.0228	0.0024			
moj29	1, 1, 0	492	615	492		—	—	0.0374	0.0026	0.0695
moj30	7, 7, 1	631	621+	498	*ari*	0.0350	0.0043	0.0842	0.0056	0.0670
					moj	0.0455	0.0064			
moj32	1, 1, 0	180	429+	180		—	—	0.0000	0.0000	—
moj137	1, 1, 0	198	246+	198		—	—	0.0000	0.0000	—
moj152	1, 1, 0	303	396+	303		—	—	0.0893	0.0219	0.2452
Tes14	7, 7, 1	491	240	240	*ari*	0.0071	0.0000	0.0134	0.0000	0.0000
					moj	0.0153	0.0000			
Tes31	1, 1, 0	204	228	204		—	—	0.1280	0.0199	0.1555
Tes33	7, 7, 1	524	639+	468	*ari*	0.0606	0.0056	0.1169	0.0047	0.0401
					moj	0.0404	0.0022			
Tes39	1, 1, 0	210	219	210		—	—	0.0682	0.0000	0.0000
Tes40	1, 1, 0	393	505+	393		—	—	0.1217	0.0033	0.0271
Tes41	1, 1, 0	384	510	384		—	—	0.1274	0.0101	0.0793
Tes100	7, 7, 1	507	168	168	*ari*	0.0000	0.0153	0.0423	0.0273	0.6453
					moj	0.0353	0.0061			
Tes101	7, 7, 1	293	387	153	*ari*	0.0114	0.0000	0.0327	0.0012	0.0373
					moj	0.0000	0.0035			
Tes104	7, 7, 1	726	738+	663	*ari*	0.0239	0.0016	0.0725	0.0006	0.0077
					moj	0.0159	0.0000			

(*continued*)

TABLE 1

(Continued)

Gene	No. alleles *a, mo, mu*[a]	No. sites analyzed	ORF size	No. coding analyzed	Sample	θ_{syn}	θ_{rep}	K_s	K_a	K_a/K_s[b]
Tes105	7, 7, 1	363	234	231	*ari*	0.0145	0.0047	0.0206	0.0066	0.3185
					moj	0.0145	0.0047			
Tes106	7, 7, 1	368	207	207	*ari*	0.0184	0.0050	0.1611	0.0062	0.0383
					moj	0.0368	0.0050			
Tes107	7, 7, 1	501	126	126	*ari*	0.0389	0.0000	0.0815	0.0000	0.0000
					moj	0.0260	0.0000			
Tes109	7, 6, 0	234	927+	228	*ari*	0.0290	0.0132	0.0346	0.0311	0.8992
					moj	0.0000	0.0094			
Tes110	7, 7, 1	826	399	390	*ari*	0.0085	0.0014	0.0765	0.0029	0.0382
					moj	0.0000	0.0028			
Tes112	5, 7, 0	428	276	273	*ari*	0.0153	0.0000	0.0417	0.0048	0.1145
					moj	0.0325	0.0000			
Tes113	7, 7, 0	335	624	282	*ari*	0.0065	0.0037	0.0512	0.0072	0.1412
					moj	0.0194	0.0019			
Tes114	2, 7, 1	250	132+	96	*ari*	0.0000	0.0000	0.0633	0.0000	0.0000
					moj	0.0193	0.0000			
Tes115	6, 7, 1	321	204	207	*ari*	0.0000	0.0054	0.0448	0.0166	0.3706
					moj	0.0000	0.0025			
Tes118	4, 6, 0	729	936+	555	*ari*	0.0089	0.0076	0.0367	0.0151	0.4114
					moj	0.0142	0.0020			
Tes120	1, 1, 0	363	423+	363		—	—	0.0958	0.0106	0.1106
Tes122	1, 1, 0	267	267+	267		—	—	0.0172	0.0146	0.8488
Tes123	1, 1, 0	486	621+	486		—	—	0.1574	0.0768	0.4879
Tes124	1, 1, 0	159	651+	159		—	—	0.0277	0.0000	0.0000
Tes127	1, 1, 0	285	309+	285		—	—	0.0452	0.0282	0.6239
Tes129	1, 1, 0	405	525	405		—	—	0.0109	0.0032	0.2936
Tes130	1, 1, 0	150	174	150		—	—	0.0905	0.0125	0.1381
Tes131	1, 1, 0	528	603+	528		—	—	0.0407	0.0176	0.4324
Tes133	1, 1, 0	333	414+	333		—	—	0.0650	0.0160	0.2462
Tes134	7, 7, 1	805	609	558	*ari*	0.0238	0.0010	0.0540	0.0103	0.1897
					moj	0.0030	0.0039			
Tes140	1, 1, 0	240	240	240		—	—	0.0881	0.0169	0.1918
Tes154	7, 7, 1	696	579+	507	*ari*	0.0033	0.0011	0.0439	0.0019	0.0426
					moj	0.0263	0.0021			

ari, *D. arizonae*; *moj*, *D. mojavensis*; θ_{syn}, synonymous heterozygosity; θ_{rep}, replacement heterozygosity.
[a] Number of alleles corresponding to *D. arizonae*, *D. mojavensis*, and *D. mulleri*, respectively.
[b] Ratios with positive K_a and zero K_s are designated by $K_a > K_s$.

Library quality: Completeness of 5′ cDNA ends was assessed by two methods on a total of 155 ESTs. First, for transcripts represented by more than one clone, we compared the similarity of 5′ ends among clones, with the assumption that the longest clone is likely to include the complete 5′ end of a gene. Second, several transcripts were subjected to 5′ RACE verification. RACE analysis showed that all 20 of the *Tes100* ESTs were truncated products, each ~113 bp shorter than the reference 5′ sequence. Thus, *Tes100* clones appear to be outliers in terms of assessment of library quality. Using the multiple-clone method, we estimate 79.7% (63/79) of our ESTs to be complete at the 5′ end. For ESTs compared to a reference 5′ RACE sequence, ~62.5% (60/96) contain the complete 5′ end. However, removing the *Tes100* outliers increases the estimate to 78.9% (60/76), a ratio that is consistent with the multiple-clone estimate. Therefore, our estimates suggest that approximately four-fifths of cDNA clones were complete at the 5′ end.

BLAST analyses *vs. D. melanogaster*: Results of *D. mojavensis* EST BLAST analyses to *D. melanogaster* databases, including the closest-matching genes and secondary *E*-scores, are listed in supplementary Table S1 (http://www.genetics.org/supplemental; $E < 0.01$ was the BLAST score threshold for inclusion). None of the ESTs that failed to match *D. melanogaster* sequences matched any other NCBI database sequences. Approximately 61% (33/54) and 58% (68/118) of whole reproductive tract and testis library unique ESTs, respectively, showed BLAST similarity to *D. melanogaster* sequences. However, there were major differences between accessory gland- *vs.* testis-derived sequences, with *Acp*'s showing a much lower level of conservation between species

than testis-enriched genes. Only 33% (8/24) of *Acp*'s generated significant hits, compared to 82% (27/33) for testis-enriched genes. A 2×2 contingency table is significantly heterogeneous ($P \ll 0.01$). Furthermore, the median *E*-value of the eight *Acp*'s with $E < 0.01$ ($E = 1e\text{-}3$, a value too high to reliably indicate orthology) is much greater than the median for testis-enriched genes ($E = 2e\text{-}21$). The six genes that are more ubiquitously expressed on the basis of quantitative PCR data (*moj9*, *moj29*, *moj30*, *moj32*, *moj137*, and *moj152*) had highly significant BLAST matches to *D. melanogaster* sequences (median $E = 5e\text{-}42$). The remaining *moj* sequences are similar to the testis-enriched genes, with 55% (59/108) returning $E < 0.01$ *vs. D. melanogaster* (median $E = 1e\text{-}27$). This is not surprising, given that most *moj* sequences are from the testis cDNA library.

Twenty of the 27 *D. mojavensis* testis-enriched genes that appear to have *D. melanogaster* homologs have BLAST hits to the *D. melanogaster* testis EST collection (Andrews *et al.* 2000), suggesting that testis expression patterns between species are generally conserved. Our quantitative PCR data from 6 of the remaining 7 genes isolated from the *D. mojavensis* testis library (see below) suggest that they too show testis-enriched expression in *D. melanogaster* in spite of their absence from the *D. melanogaster* testis EST collection, further supporting the notion for a generally conserved Drosophila testis transcriptome.

Certain biochemical functions, including proteases, protease inhibitors, and lipases, appear to be common in *melanogaster* subgroup *Acp*'s, as inferred from sequence similarity to protein databases (Swanson *et al.* 2001). This is in contrast to our results from the 54 unique *D. mojavensis* reproductive tract ESTs (most of which are likely *Acp*'s), which revealed evidence for two protease inhibitors, *Acp36* and *Acp48*, and a single lipase gene, *moj37*. None of the predicted 54 proteins contain putative protease domains. The proportion of known *D. mojavensis Acp*'s (2/24) that contain any of these three domains is significantly different from the proportion (21/57) from the Swanson *et al.* (2001) set of *melanogaster* subgroup *Acp*'s (*G*-test, $P = 0.026$). This is suggestive of a fundamental, functional divergence in seminal fluid function in the two species, although more work, including direct biochemical assays, would be necessary to put this conclusion on firmer ground.

***D. melanogaster-D. mojavensis* orthology:** The existence of gene families and shared protein domains can yield small BLAST *E*-scores, yet obscure inferences regarding orthology between *D. melanogaster* and *D. mojavensis*. Alternatively, conserved intron-exon structure is expected for genes of shared ancestry (Meyer and Durbin 2004) but not for unrelated genes that share only a particular protein domain. For example, human-mouse orthologs have the same number of coding exons ~86% of the time (Mouse Genome Sequencing Consortium 2002). Thus, genes showing conserved intron-exon structure and large *E*-score differences (*e.g.*, $E > 1e\text{-}10$) between primary and secondary BLAST hits are probably orthologs.

Comparison of genomic sequence from our population genetic data to our EST sequences allowed us to determine intron-exon structure for a subset of *D. mojavensis* genes (*i.e.*, genes from Table 1). We used this information in concert with comparisons of primary *vs.* secondary BLAST *E*-values and protein size, to investigate putative *D. melanogaster* orthologs for many of our *D. mojavensis* genes (indicated by an asterisk, supplementary Table S1; http://www.genetics.org/supplemental). For the remaining ESTs we have data only on primary *vs.* secondary BLAST *E*-values, many of which are suggestive of orthology.

Acp's: Of the eight *Acp*'s that show BLAST similarity to *D. melanogaster* genes ($E < 0.01$), only *Acp36* and *CG16713* (supplementary Table S1; http://www.genetics.org/supplemental) are likely orthologs. Both consist of 82 residues and possess a Kunitz domain that covers 59 of those residues. The aligned predicted proteins are 57.3% identical (47/82) and require no gaps. Although *Acp36* also returns a significant BLAST hit to another protein with a Kunitz domain (*CG16712*), its amino acid sequence is more similar to *CG16713*. Three additional *Acp*'s (*Acp1*, *Acp2*, and *Acp25*) are part of a gene family and are clearly homologous to the *Acp53* gene family (Holloway and Begun 2004) in *D. melanogaster* (supplementary Table S1; http://www.genetics.org/supplemental). However, a protein distance tree clusters the three *D. mojavensis* genes together, rather than generating the three interspecific pairs expected under one-to-one orthology and homogeneous rates of protein evolution. Thus, although the proteins appear homologous, orthology is uncertain. The remaining *Acp*'s show no compelling evidence for orthology for several reasons, including poor BLAST scores, radically different protein lengths or intron-exon organization, or very different expression patterns between species (described below).

Testis-enriched and moj genes from the population genetics survey: Most testis-enriched and all six *moj* genes from the population genetics survey have clear *D. melanogaster* orthologs on the basis of primary and secondary BLAST *E*-scores and gene organizations inferred from comparison of cDNA and genomic sequence (supplementary Table S1; http://www.genetics.org/supplemental). However, there are some exceptions. *Tes33* and *Tes104* are part of an SCP-related gene family and have no obvious orthologs among the many *D. melanogaster* SCP-related genes. *Tes114*, *Tes120*, and *Tes123*, are also part of gene families that obscure interspecific relationships. Finally, *Tes101* and *Tes109* are too dissimilar to their *D. melanogaster* primary BLAST hits ($E = 6e\text{-}03$ and $E = 7e\text{-}04$, respectively) to conclude that they represent orthologous pairs.

Of the 41 genes in our quantitative PCR analyses (see below) that return significant BLAST matches to

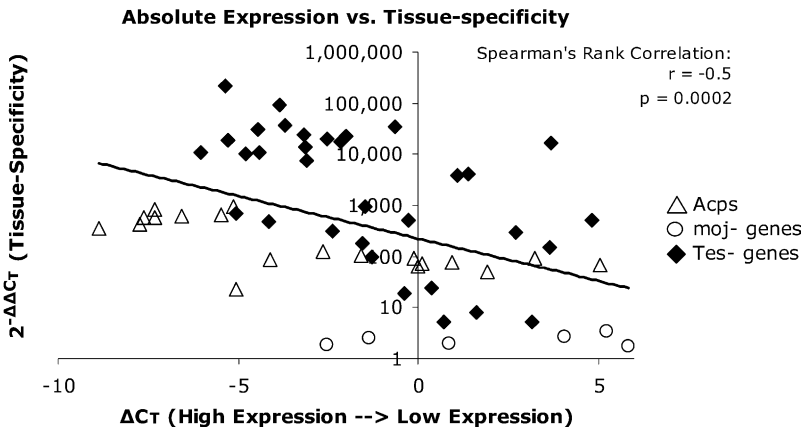

FIGURE 2.—Correlation between absolute levels of expression and degree of tissue specificity. The more tissue-specific genes (high $2^{-\Delta\Delta C_T}$) also tend to show higher absolute levels of expression (low ΔC_T). Testis-enriched genes are indicated by solid diamonds, *Acp*'s by open triangles, and *moj* genes by open circles.

D. melanogaster sequences, only *Tes14* and *Tes118* correspond to putative unannotated genes. This supports the observation that the *D. melanogaster* genome annotation is of high quality (MISRA *et al.* 2002; DRYSDALE 2003; YANDELL *et al.* 2005). Details regarding *Tes14*, *Tes118*, and other data bearing on *D. mojavensis-D. melanogaster* orthology are presented as supplementary material (http://www.genetics.org/supplemental).

Relative quantification of *D. mojavensis* gene expression: Supplementary Table S2 (http://www.genetics.org/supplemental) summarizes the expression quantification results for all *D. mojavensis* genes surveyed, as well as several *D. melanogaster* genes that are discussed in the next section. Of the 58 total *D. mojavensis* genes selected for quantitative PCR, 19 are expressed primarily in the accessory glands, 33 are expressed primarily in the testis, and the remaining six (*moj9*, *moj29*, *moj30*, *moj32*, *moj137*, and *moj152*) are more evenly expressed, as indicated by $2^{-\Delta\Delta C_T} < 5$. The vast majority of the 58 genes appear to be either tissue specific or highly tissue enriched in expression, with 46 of 58 genes being at least 50 times more abundant in one tissue than in any other. All 19 *Acp*'s contain putative signal peptide sequences (supplementary Table S1; http://www.genetics.org/supplemental). Furthermore, the ΔC_T scores indicate that the six most abundantly expressed genes are *Acp*'s. These data, as well as the preponderance of putative accessory gland transcripts in the *D. mojavensis* reproductive tract library, support the conclusion that *Acp*'s are typically abundantly expressed, secreted peptides (WOLFNER 1997).

Figure 2 depicts the relationship between ΔC_T and $2^{-\Delta\Delta C_T}$ scores. The highly significant negative correlation ($R = -0.5$, $P = 0.0002$) suggests that genes showing greater degrees of tissue specificity (high $2^{-\Delta\Delta C_T}$ scores) tend to have greater transcript abundance (lower ΔC_T). The $2^{-\Delta\Delta C_T}$ scores suggest that the 19 most tissue-specific genes are testis rather than accessory gland enriched. Although this could be genuine, we suspect that it is an artifact of trace accessory gland contamination of testis tissue dissections. The transparent and fragile nature of accessory gland tissue should lead to this type of contamination rather than the converse. However, low levels of this one-way contamination should not dramatically affect our conclusions. The fact that several putative *Acp*'s clearly show very large fold differences suggests that this trace contamination is negligible. For example, *Acp2* ranks as the most tissue-specific *Acp*, with transcript abundance in accessory glands estimated as 933 times greater than that in the testis (supplementary Table S2; http://www.genetics.org/supplemental). Conservatively assuming this gene is not transcribed in the testis, this would suggest that there are 933 parts accessory gland material in the accessory gland tissue preparation for every 1 part of contaminating accessory gland material in the testis tissue preparation. Thus, we would not conclude, for example, that *Tes101* ($2^{-\Delta\Delta C_T} = 36{,}656$) is more tissue specific than *Acp2* ($2^{-\Delta\Delta C_T} = 933$). On the other hand, *Acp2* is certainly more tissue specific than *Acp25* ($2^{-\Delta\Delta C_T} = 51$) since contamination would affect every *Acp* gene $2^{-\Delta\Delta C_T}$ score in a similar manner.

Comparison of *D. melanogaster* and *D. mojavensis* expression patterns: Our quantitative PCR data suggest that putative orthologs of *D. mojavensis* testis-enriched genes are also testis enriched in *D. melanogaster*. Nevertheless, the relative amount of testis specificity varies across genes. At the most extreme, *D. melanogaster CG3708* is ~164-fold more testis specific than *Tes129*. There are also large fold differences between *Tes106/CG30334* (97-fold), *Tes110/CG15219* (24-fold), and *Tes127/CG10090* (53-fold). These comparisons reflect significant differences between *D. mojavensis-D. melanogaster* expression profiles at these genes ($P < 0.05$). Several additional testis-enriched genes are borderline significant with fold differences >5. These conclusions are all based on the premise that the housekeeping ribosomal protein gene used as an internal standard has not evolved substantial gene expression differences in *D. melanogaster vs. D. mojavensis*. Moreover, fold differences can be dramatically different between orthologous pairs solely because of regulatory changes in the secondary tissue and, as such, misrepresent actual differences between species in primary tissues. In this

sense, ΔC_T scores are more revealing because they are correlated with absolute expression levels. Five *moj* and eight *Tes* orthologous gene pairs have sizable differences between ΔC_T scores (>4), with *Tes124-CG14079* at the most extreme (11.35) (supplementary Table S2; http://www.genetics.org/supplemental). Because of the uncertainty associated with housekeeping gene regulation and primer efficiency (see MATERIALS AND METHODS), strong, individual gene pairwise *D. melanogaster vs. D. mojavensis* conclusions are not warranted beyond the rank order of tissue enrichment. However, these data suggest that there have been gene regulation changes between lineages. Further discussion of expression differences between individual *D. melanogaster-D. mojavensis* pairs can be found in the supplementary material (http://www.genetics.org/supplemental).

Genome-wide assays of expression differences between *melanogaster* subgroup species suggest that male-biased genes show greater interspecific expression differences compared to other genes (MEIKLEJOHN *et al.* 2003; RANZ *et al.* 2003; RIFKIN *et al.* 2003). Our data, although consistent with these reports, suggest that despite rapid evolution of male-biased expression, wholesale shifts in tissue specificity are uncommon. A potential caveat is that the apparent interspecific conservation of tissue specificity could be inflated by the fact that we focused on genes coding for more highly conserved proteins. If more highly conserved orthologs are less likely to change tissue specificity, then we have clearly underestimated the frequency of such changes. Comparative genomic analyses of more highly diverged orthologous genes will help address this question (WAGSTAFF and BEGUN 2005).

Molecular population genetics analysis

We surveyed a total of 56 genes (19 *Acp*'s, 31 testis enriched, and 6 ubiquitously expressed) for our molecular population genetics analysis (see Table 1). Up to seven lines each of *D. arizonae* and *D. mojavensis* were analyzed for several genes. However, many genes are represented by only a single allele each from *D. arizonae* and *D. mojavensis*. A *D. mulleri* allele was sequenced whenever possible as an outgroup. An average of 9.3 alleles and 376 bp were sequenced for each gene surveyed.

Evidence of *D. m. baja-D. m. mojavensis* population substructure: Our *D. mojavensis* data consist of up to four alleles of *D. m. baja* and three alleles of *D. m. mojavensis* from various locations of Baja, Mexico and southern California, respectively. Supplementary Table S3 (http://www.genetics.org/supplemental) shows our analysis of population substructure between *D. m. baja* and *D. m. mojavensis*. We use the fixation index, F_{ST}, to estimate genetic differentiation between subspecies. The small size of most surveyed regions and the small number of alleles make inferences from individual genes unreliable. A more accurate view of differentiation can be obtained by examining average F_{ST}-values, weighted according to sequence length. The average for all genes is 0.150, with the *Acp* subset of genes slightly higher at 0.168. These results are within the observed range for genetic differentiation between African and non-African *D. melanogaster* populations (CARACRISTI and SCHLÖTTERER 2003; BAUDRY *et al.* 2004). *Acp7* appeared to be something of an outlier with estimated F_{ST} of 0.864. Therefore, we included additional *D. m. baja* ($n = 5$) and *D. m. mojavensis* ($n = 7$) *Acp7* alleles to the analysis. Our revised estimate showed that differentiation ($F_{ST} = 0.429$) at this locus, although at the high end compared to most loci, was not an obvious outlier.

We also investigate genetic differentiation by estimating divergence between subspecies (K_a and K_s) and comparing those values to nucleotide diversity (π) within subspecies (supplementary Table S3; http://www.genetics.org/supplemental). Since both measurements represent the probability that a particular nucleotide site drawn from two individuals is different, they can be directly compared. Again, our analysis shows some evidence of population substructure. Averaged across all genes, K_a (0.006) is higher than both replacement *D. m. baja* (0.005) and *D. m. mojavensis* (0.004) nucleotide diversities. However, there are no significant differences between sets of K_a and replacement π measurements (Mann-Whitney *U*-test, $P = 0.77$ and $P = 0.41$ for *D. m. baja* and *D. m. mojavensis*, respectively). There is also no evidence for differentiation at synonymous sites, with *D. m. baja* synonymous π at 0.016, K_s at 0.015, and *D. m. mojavensis* synonymous π at 0.013.

Given these results, we do not distinguish between *D. m. baja* and *D. m. mojavensis* alleles in our population genetics analyses. Although our estimates of polymorphism may be slightly inflated relative to those measured from a single population, our tests of adaptive evolution compare nucleotide substitution patterns at synonymous *vs.* replacement sites. Under neutrality, population substructure is expected to have little effect on rejecting the null in the direction of adaptive protein evolution.

Levels of synonymous and replacement polymorphism and divergence: Summary statistics for heterozygosity and divergence for individual genes and for gene categories are presented in Tables 1–3. As suggested by previously published molecular population genetics data from these species (*e.g.*, BEGUN 1997; BEGUN and WHITLEY 2002; MATZKIN and EANES 2003), they are highly variable (Table 1). Average synonymous heterozygosities for *D. mojavensis* and *D. arizonae* are 0.0181 and 0.0170, respectively (Table 2). Synonymous heterozygosity for *D. mojavensis* and *D. arizonae* is marginally lower for *Acp*'s (0.0156 and 0.0135, respectively) compared to testis-enriched genes (0.0170 and 0.0175, respectively). Synonymous divergence between *D. arizonae* and *D. mojavensis* is similar across gene categories as well (Table 2, but see the polarized analysis below for between-species differences). Testis-enriched genes are the most

TABLE 2

Polymorphism and divergence of gene classes

		Polymorphism			Divergence[a]		
Gene class	Sample	θ_{syn}	θ_{rep}	$\theta_{rep}/\theta_{syn}$	K_s	K_a	K_a/K_s
Acp's	*ari*	0.0135	0.0066	0.4866	0.0643	0.0595	0.9257
	moj	0.0156	0.0093	0.5991			
Tes	*ari*	0.0175	0.0037	0.2095	0.0682	0.0128	0.1873
	moj	0.0170	0.0025	0.1476			
moj	*ari*	0.0292	0.0045	0.1553	0.0518	0.0060	0.1164
	moj	0.0346	0.0045	0.1308			
All genes	*ari*	0.0170	0.0049	0.2851	0.0650	0.0250	0.3842
	moj	0.0181	0.0053	0.2935			
sim Acp's[b]		0.0280	0.0074	0.2643	0.1170	0.0497	0.4248
sim 3R[b]		0.0350	0.0013	0.0371	0.1080	0.0107	0.0991

[a] *D. simulans* genes divergence estimates are with respect to *D. melanogaster*.
[b] Data are from Begun *et al.* (2000).

divergent at 0.0682, followed by *Acp*'s at 0.0643 and *moj* genes at 0.0518. None of the variation statistics are significantly different between gene classes or species by Mann-Whitney *U*-tests.

Patterns for replacement variation are quite different. First, mean replacement heterozygosity of *Acp*'s in both species is greater than that of testis-enriched or *moj* genes (Table 2). This is especially striking for *Acp vs.* testis-enriched genes of *D. mojavensis*, with *Acp*'s ~3.7 times more variable than testis-enriched genes in *D. mojavensis* compared to 1.8 times more variable than testis-enriched genes in *D. arizonae*. *D. mojavensis Acp*'s have the highest ratio of replacement to synonymous heterozygosity (0.5991), followed by *D. arizonae Acp*'s at 0.4866 (Table 2). This observation is not attributable to population substructure in *D. mojavensis*, as the ratios of replacement to synonymous *Acp* nucleotide diversity (π) are 0.6429 and 0.6667 in *D. m. baja* and *D. m. mojavensis*, respectively (see supplementary Table S3; http://www.genetics.org/supplemental). The ratios of replacement to synonymous heterozygosity for testis-enriched genes (*D. arizonae*, 0.2095; *D. mojavensis*, 0.1476) and *moj* genes (*D. arizonae*, 0.1553; *D. mojavensis*, 0.1308) are much lower in both species. Average *Acp* replacement divergence between *D. arizonae* and *D. mojavensis* is also considerably higher (0.0595) than that observed at testis-enriched (0.0128) or *moj* genes (0.0060). The ratio of replacement to synonymous divergence for *Acp*'s (0.9257) is 4.9 times greater than the corresponding *Tes* genes ratio (0.1873). Six genes, all *Acp*'s, have $K_a/K_s > 1$ (Table 1). Several other pairwise *Acp* divergence estimates revealed unusually high K_a/K_s values (*i.e.*, >0.5). In contrast, the highest K_a/K_s ratio among nonpolarized *Tes* and *moj* genes is 0.8992 for *Tes109*, with that of most genes being considerably lower (*i.e.*, <0.5). A survey of *Acp* polymorphism and divergence in *D. simulans* and *D. melanogaster* also suggested that these genes evolve unusually quickly at replacement sites relative to other genes (Begun *et al.* 2000). However, the relative amount of replacement to synonymous variation at *Acp*'s in *D. arizonae* and *D. mojavensis* is much greater than that observed in *D. simulans* and *D. melanogaster*. For example, the ratio of replacement to synonymous polymorphism for desert Drosophila *Acp*'s (0.5991 for *D. mojavensis*, 0.4866 for *D. arizonae*, Table 2) is about twofold greater than the corresponding ratio in *D. simulans* (0.2643). The same is true for replacement to synonymous divergence—the K_a/K_s ratio for desert Drosophila (0.9257) is more than twofold greater than the K_a/K_s ratio for *D. melanogaster*/*D. simulans* (0.4248). Thus, levels of both protein polymorphism and divergence are considerably greater at *Acp*'s in *D. arizonae*/*D. mojavensis* than in *D. melanogaster*/*D. simulans*. Although ratios of replacement to silent *Acp* polymorphism appear to be heterogeneous across *melanogaster* subgroup species (Begun *et al.* 2000; Kern *et al.* 2004), we observed no such heterogeneity for *D. mojavensis vs. D. arizonae Acp* polymorphism (see Table 6; *G*-test, $P = 0.574$).

Polarized divergence: The divergence estimates presented in Tables 1 and 2 result from pairwise comparisons and so provide no insight into evolution along the *D. arizonae vs. D. mojavensis* lineage. We investigated evolution along these two lineages using both parsimony and likelihood-based approaches. Table 3 shows the results for all genes for which an outgroup sequence was available. As one might expect from previous analyses, the rank order of K_a/K_s ratios is *Acp* > *Tes* > *moj* in each of the three lineages. Eight of nine *Acp*'s have $K_a/K_s > 1$ in at least one of the three lineages in polarized analyses (Table 3). *Tes* genes contain just two examples of $K_a/K_s > 1$, *Tes105* along the *D. mojavensis*

TABLE 3

Polarized *D. arizonae vs. D. mojavensis* divergence

	D. arizonae			*D. mojavensis*			Outgroup			
Gene/group	K_a	K_s	K_a/K_s	K_a	K_s	K_a/K_s	K_a	K_s	K_a/K_s	Outgroup?
Acp1	0.0226	0.0139	1.6269	0.0480	0.0406	1.1808	0.1429	0.1616	0.8839	*D. mulleri*
Acp2	0.0366	0.0221	1.6559	0.0247	0.0300	0.8232	0.1513	0.2932	0.5160	*D. mulleri*
Acp5a	0.0714	0.0962	0.7426	0.0391	0.0000	$K_a > K_s$	—	—	—	*5b* duplicate
Acp7	0.0159	0.0245	0.6483	0.0275	0.0000	$K_a > K_s$[a]	0.2560	0.1200	2.1337	*D. mulleri*
Acp16a	0.0095	0.0244	0.3868	0.1538	0.0169	9.1017[a]	—	—	—	*16c* duplicate
Acp16b	0.0406	0.0396	1.0248	0.0000	0.0000	—	—	—	—	*16a* duplicate
Acp19	0.0184	0.0167	1.0981	0.0163	0.0000	$K_a > K_s$	0.0953	0.0842	1.1313	*D. mulleri*
Acp25	0.0125	0.0458	0.2732	0.0207	0.0250	0.8265	0.1627	0.4233	0.3842	*D. mulleri*
Acp27a	0.0144	0.0000	$K_a > K_s$	0.0000	0.0134	0.0001	—	—	—	*27b* duplicate
moj9	0.0029	0.0440	0.0653	0.0000	0.0298	0.0001	0.0145	0.0955	0.1516	*D. mulleri*
moj30	0.0000	0.0336	0.0001	0.0027	0.0498	0.0540	0.0109	0.1928	0.0564	*D. mulleri*
Tes14	0.0000	0.0152	0.0001	0.0000	0.0000	—	0.0186	0.1485	0.1254	*D. mulleri*
Tes33	0.0028	0.1064	0.0259	0.0028	0.0492	0.0574	0.0084	0.2142	0.0391	*D. mulleri*
Tes100	0.0000	0.0430	0.0001	0.0141	0.0420	0.3365	0.0219	0.2624	0.0836	*D. mulleri*
Tes101	0.0000	0.0000	—	0.0000	0.0191	0.0001	0.0102	0.0859	0.1191	*D. mulleri*
Tes104	0.0000	0.0302	0.0001	0.0000	0.0327	0.0001	0.0125	0.1529	0.0817	*D. mulleri*
Tes105	0.0000	0.0000	0.0000	0.0060	0.0000	$K_a > K_s$	0.0305	0.2418	0.1259	*D. mulleri*
Tes106	0.0122	0.1532	0.0796	0.0000	0.0192	0.0001	0.0060	0.3648	0.0165	*D. mulleri*
Tes107	0.0000	0.0181	0.0001	0.0000	0.0179	0.0001	0.0000	0.0832	0.0001	*D. mulleri*
Tes110	0.0000	0.0000	—	0.0035	0.0630	0.0548	0.0139	0.0640	0.2173	*D. mulleri*
Tes114	0.0000	0.0611	0.0001	0.0000	0.0000	—	0.0264	0.0000	$K_a > K_s$	*D. mulleri*
Tes115	0.0162	0.0164	0.9889	0.0058	0.0166	0.3508	0.0702	0.0880	0.7979	*D. mulleri*
Tes134	0.0023	0.0356	0.0649	0.0098	0.0354	0.2760	0.0474	0.1407	0.3367	*D. mulleri*
Tes154	0.0000	0.0251	0.0001	0.0000	0.0233	0.0001	0.0082	0.1278	0.0640	*D. mulleri*
All *Acp*'s	0.0220	0.0253	0.8715	0.0273	0.0131	2.0776	0.1525	0.1798	0.8484	*D. mulleri*
All *Tes*	0.0020	0.0345	0.0578	0.0034	0.0306	0.1096	0.0199	0.1501	0.1326	*D. mulleri*
All *moj*	0.0014	0.0348	0.0407	0.0014	0.0382	0.0375	0.0130	0.1364	0.0951	*D. mulleri*

[a] K_a/K_s ratios significantly >1 ($P < 0.05$). Ratios with positive K_a and zero K_s are designated by $K_a > K_s$.

lineage and *Tes114* along the *D. mulleri* lineage. In each case, however, $K_a/K_s > 1$ is largely due to negligible K_s divergence (zero in both cases) rather than unusually rapid protein divergence. K_a/K_s ratios for polarized *Acp*'s *vs. Tes* genes are highly significantly different (Mann-Whitney *U*-test, $P \ll 0.01$).

The *D. mojavensis* lineage has a considerably greater average *Acp* K_a/K_s ratio than either the *D. arizonae* or *D. mulleri* lineage. Across all nine *Acp*'s, the K_a/K_s ratio for *D. mojavensis* (2.0776) is 2.4 times greater than the ratio for *D. arizonae* (0.8715). Although *Acp* replacement divergence is higher in *D. mojavensis* (0.0273) than in *D. arizonae* (0.0220), the much lower K_s in *D. mojavensis vs. D. arizonae Acp*'s makes a significant contribution to the higher *D. mojavensis Acp* K_a/K_s ratio. One possible reason for the low *D. mojavensis* K_s relative to the *D. arizonae* K_s could be different intensities of selection for codon bias between lineages. However, our estimates of effective number of codons (ENC) (Wright 1990) show no major differences between lineages. The average ENCs for *D. mojavensis Acp*'s and testis-enriched genes, weighted according to size, are 51.8 and 50.8, respectively. The corresponding values for *D. arizonae* are 50.7 and 51.6, respectively. Thus, codon bias of *D. mojavensis Acp*'s is actually slightly lower than that of *D. arizonae Acp*'s, contrary to expectations if stronger selection at synonymous sites in *D. mojavensis* were contributing to the lower *D. mojavensis* K_s values.

Unfortunately, we have *D. mulleri* data from only five *Acp*'s (Table 3). This limits our ability to directly compare *Acp* K_a/K_s across the three lineages in a comparable set of analyses. For these five genes the K_a/Ks average ratio is similar for *D. arizonae* and *D. mulleri* (0.8273 and 0.8484, respectively), while the *D. mojavensis* K_a/K_s ratio (1.7163) is roughly twofold greater. Note that the *D. mulleri* data are potentially biased because genes that are evolving more quickly would tend to be underrepresented as a result of PCR failure using primers designed from *D. mojavensis* sequence.

Two *Acp*'s, *Acp7* and *Acp16a*, have K_a/K_s significantly >1 in the *D. mojavensis* lineage, while neither gene is significant in the *D. arizonae* lineage. The significant K_a/K_s for *D. mojavensis Acp7* reflects a contribution from low synonymous divergence (0.0000), as replacement

divergence is similar in *D. mojavensis* (0.0275) to the *Acp* mean (0.0273) for the *D. mojavensis* lineage (Table 3). On the other hand, the high K_a/K_s ratio for *D. mojavensis Acp16a* is primarily attributable to the atypically high replacement divergence (0.1538) relative to the lineage mean (0.0273). *D. mulleri* provides a solitary example, *Acp7*, of K_a/K_s significantly >1 ($P < 0.05$).

Joint analysis of polymorphism and divergence: The neutral theory of molecular evolution predicts that the ratio of replacement to synonymous substitutions should be similar to the ratio of replacement to synonymous polymorphisms (Kimura 1983). The McDonald-Kreitman (MK) test uses a 2×2 contingency table to detect differences in these ratios (McDonald and Kreitman 1991). Table 4 shows the polymorphism and fixation data for individual genes at synonymous and replacement sites. For cases in which an outgroup sequence was available (outgroups identical to those in Table 3), fixed differences between *D. arizonae* and *D. mojavensis* were polarized using parsimony. None of the 54 tests are significant after Bonferroni correction of critical values. The small sizes and large number of genes motivate analysis of pooled data (Table 5). The 2×2 table for *Acp*'s is significantly heterogeneous in a direction consistent with adaptive protein evolution and remains marginally significant if *Acp25* (the single *Acp* with $P < 0.05$) is removed from the analysis. Another individual gene that warrants mention is *Acp48*. With a total of 60 mutations to contribute to the 2×2 contingency table, one might speculate that it has a major effect on the overall conclusion. However, removing the *Acp48* data increases the significance of the heterogeneity of the remaining *Acp*'s. Overall, the analysis of pooled polymorphic and fixed mutations supports the notion that directional selection plays a role in accessory gland protein divergence. Data from testis-enriched and *moj* genes show no significant deviations from neutral expectation in 2×2 contingency tables.

Further evidence for different evolutionary processes among gene classes can be found in the ratios of replacement fixations to polymorphisms (Tables 4 and 5). While a total of seven *Acp*'s have more replacement fixations than polymorphisms, no *Tes* or *moj* genes do, with the exception of *Tes112*, which has no replacement polymorphisms and just a single fixation. The ratio of fixed to polymorphic replacement mutations for *Acp*'s (139:115) is highly significantly different from the ratio for testis-enriched genes (15:60; *G*-test, $P \ll 0.01$), a result that cannot be explained by different neutral mutation rates for the two protein classes. The *moj* genes ratio (0:16) is more testis-like, although with so few data, strong conclusions are unwarranted.

Polarized McDonald-Kreitman analyses: Investigation of polarized fixations provides more insight into evolutionary process in the *D. arizonae vs. D. mojavensis* lineages, although at a cost of reduced number of loci and substitutions included in the analysis (numbers of polarized *vs.* nonpolarized individual gene tests are 9:15, 13:17, and 2:2 for *Acp*'s, *Tes* genes, and *moj* genes, respectively). The data for different gene classes, polarized using parsimony, are presented in Table 6. *D. mojavensis Acp*'s show a highly significant ($P = 0.004$) deviation from neutral expectations. It is formally possible that the *D. mojavensis* data could be explained by too few silent fixations. However, such an explanation would require a change in silent neutral mutation at precisely the correct moment in time. Moreover, since the ratio of silent to replacement substitutions is similar in the two lineages in other gene categories, this explanation would require a bizarre perturbation of silent neutral mutation rate only in *Acp*'s, which seems highly improbable. Thus, the *D. mojavensis Acp* data are more easily interpreted as a large excess of replacement fixations. Interestingly, however, the *D. arizonae Acp* data are not significantly heterogeneous ($P = 0.181$). The lineage differences in polarized MK tests, which are consistent with the greater K_a/K_s ratio in *D. mojavensis Acp*'s noted earlier, support the idea that directional selection has greater effects on *Acp* divergence in *D. mojavensis* than in *D. arizonae*. Note that the number of fixed replacement *vs.* synonymous mutations (24:2) in *D. mojavensis* corresponds to a K_a/K_s ratio for fixed sites of $\sim$4 (assuming a ratio of replacement to silent sites of $\sim$3:1), providing additional support for the interpretation that the 2×2 table for *D. mojavensis Acp*'s can plausibly be explained only by adaptive protein evolution. Polarized data from *moj* genes in both lineages and testis-enriched genes in *D. mojavensis* are not significantly heterogeneous. Data from *D. arizonae* testis-enriched genes are marginally significant (Fisher's exact test, $P = 0.056$; *G*-test, $P = 0.026$), but not in the direction of excess replacement fixations. Additional population genetic data will be required to investigate this pattern.

DISCUSSION

Population genetic investigation of accessory gland protein genes has previously focused on *D. melanogaster* and *D. simulans* (Aguadé 1997, 1998, 1999; Tsaur and Wu 1997; Tsaur *et al.* 1998; Begun *et al.* 2000; Swanson *et al.* 2001; Kern *et al.* 2004). Our study of *Acp*'s and testis-enriched genes of desert Drosophila from the *repleta* group was motivated by our interest in understanding whether the highly diverged mating system of these flies (relative to *D. melanogaster* and *D. simulans*) is associated with different population genetic patterns and mechanisms for male reproduction-related genes. This question may be especially germane to the issue of *Acp*'s (rather than testis-enriched genes).

Desert Drosophila from the *repleta* group remate much more frequently than do *D. melanogaster* or *D. simulans*, opening up the possibility for stronger or fundamentally different selection on male-male and male-female

TABLE 4

Individual gene MK tests

Gene	Polymorphic		Fixed		P^a
	Syn	Repl	Syn	Repl	
Acp1	5	10	1	10	0.130
arizonae	0	7	1	3	0.364
mojavensis	5	3	0	6	0.031*
Acp2	6	8	1	8	0.090
arizonae	3	0	0	5	0.018*
mojavensis	3	8	0	2	1.000
Acp3	3	5	2	6	0.589
arizonae	3	1	—	—	—
mojavensis	0	4	—	—	—
Acp5a	1	4	3	6	0.590
arizonae	1	1	3	3	1.000
mojavensis	0	3	0	2	—
Acp7	8	14	4	7	1.000
arizonae	6	7	2	3	0.813
mojavensis	2	7	0	3	1.000
Acp8	2	8	4	8	0.481
arizonae	1	4	—	—	—
mojavensis	1	4	—	—	—
Acp16a	0	11	2	7	0.189
arizonae	0	4	1	1	0.333
mojavensis	0	7	1	4	0.417
Acp16b	6	9	1	6	0.207
arizonae	3	7	1	4	0.675
mojavensis	3	2	0	0	—
Acp19	5	7	2	11	0.139
arizonae	3	4	2	7	0.377
mojavensis	3	3	0	4	0.200
Acp21a	1	11	2	21	0.971
arizonae	1	2	—	—	—
mojavensis	1	9	—	—	—
Acp24	2	6	1	1	0.504
arizonae	0	2	—	—	—
mojavensis	2	4	—	—	—
Acp25	8	2	2	6	0.017*
arizonae	6	1	1	2	0.103
mojavensis	3	1	1	3	0.148
Acp27a	2	5	0	1	1.000
arizonae	0	1	0	1	—
mojavensis	2	4	0	0	—
Acp42	7	6	3	11	0.078
arizonae	2	3	—	—	—
mojavensis	5	3	—	—	—
Acp48	7	9	14	30	0.396
arizonae	2	4	—	—	—
mojavensis	5	5	—	—	—
moj9	12	6	3	0	0.526
arizonae	6	4	1	0	1.000
mojavensis	6	2	1	0	1.000
moj30	21	10	3	0	0.539
arizonae	10	4	1	0	1.000
mojavensis	13	6	2	0	1.000
Tes14	3	0	0	0	—
arizonae	1	0	0	0	—
mojavensis	2	0	0	0	—
Tes33	24	7	3	0	0.589
arizonae	15	5	1	0	1.000
mojavensis	10	2	2	0	1.000
Tes100	3	7	1	1	0.592
arizonae	0	5	1	0	—
mojavensis	3	2	0	1	—
Tes101	1	1	1	0	—
arizonae	1	0	0	0	—
mojavensis	0	1	1	0	—
Tes104	14	2	7	0	0.557
arizonae	9	2	3	0	1.000
mojavensis	6	0	4	0	—
Tes105	4	4	0	0	—
arizonae	2	2	0	0	—
mojavensis	2	2	0	0	—
Tes106	6	4	5	0	0.231
arizonae	2	2	3	0	0.429
mojavensis	4	2	2	0	1.000
Tes107	5	0	1	0	—
arizonae	3	0	0	0	—
mojavensis	2	0	1	0	—
Tes109	3	10	1	4	0.887
arizonae	3	6	—	—	—
mojavensis	0	4	—	—	—
Tes110	2	3	6	0	0.061
arizonae	2	1	0	0	—
mojavensis	0	2	6	0	—
Tes112	7	0	0	1	—
arizonae	2	0	—	—	—
mojavensis	5	0	—	—	—
Tes113	3	3	2	1	0.633
arizonae	1	2	—	—	—
mojavensis	3	1	—	—	—
Tes114	1	0	1	0	—
arizonae	0	0	1	0	—
mojavensis	1	0	0	0	—
Tes115	0	3	2	2	0.429
arizonae	0	2	1	1	—
mojavensis	0	1	1	1	—
Tes118	6	8	2	4	0.688
arizonae	2	6	—	—	—
mojavensis	4	2	—	—	—
Tes134	9	5	5	2	0.742
arizonae	8	1	2	0	1.000
mojavensis	1	4	3	2	0.189
Tes154	9	3	4	0	0.529
arizonae	1	1	2	0	—
mojavensis	8	2	1	0	1.000

Syn, synonomous; Repl, replacement.

[a] *P*-values are from *G*-tests; Fisher's exact test is used when zero values are present. An asterisk indicates a significant result ($P < 0.05$). Tests were not carried out for loci with very few observations.

interactions in the *repleta* group. Previous results from within- and between-species matings of desert Drosophila (Patterson and Stone 1952; Knowles and Markow 2001; Pitnick *et al.* 2003) support the idea of rapid evolution of ejaculate-female interactions. The

TABLE 5

MK tests for gene classes

	Synonymous	Replacement	Probability
	moj genes		
Polymorphic (*ari:moj*)	33 (16:19)	16 (8:8)	Fisher's exact test:
Fixed	6	0	$P = 0.165$
	All testis-enriched genes		
Polymorphic (*ari:moj*)	100 (52:51)	60 (35:25)	$G = 2.162$
Fixed	41	15	$P = 0.142$
	All *Acp*'s		
Polymorphic (*ari:moj*)	63 (31:35)	115 (48:67)	$G = 6.474$
Fixed	42	139	$P = 0.011$
	All *Acp*'s except *Acp*25		
Polymorphic (*ari:moj*)	55 (25:32)	113 (47:66)	$G = 3.91$
Fixed	40	133	$P = 0.047$

Probability is determined by a *G*-test when all cells contain nonzero values; Fisher's exact test is shown otherwise. Individual species polymorphisms are included in parentheses (which are not guaranteed to add up to the total number of polymorphisms since polymorphic sites can overlap).

fact that *D. mojavensis* males make detectable postmating donations to females whereas *D. melanogaster* and *D. simulans* do not (Markow and Ankney 1984; Pitnick *et al.* 1997) is another interesting biological difference. If *Acp*'s are major players in postcopulatory male-male and male-female interactions (Wolfner 1997, 2002; Chapman 2001), we might expect to observe different functions and patterns of evolution in desert Drosophila *Acp*'s compared to *melanogaster* subgroup *Acp*'s.

Our data do not directly address functional divergence of *D. mojavensis*/*D. arizonae vs. D. melanogaster*/*D. simulans* seminal fluid. However, our BLAST results to

TABLE 6

Polarized MK tests for gene classes

	Synonymous	Replacement	Probability
	D. mojavensis moj genes		
Polymorphic	19	8	Fisher's exact test:
Fixed	3	0	$P = 0.545$
	D. mojavensis testis-enriched genes		
Polymorphic	39	18	$G = 2.295$
Fixed	21	4	$P = 0.130$
	D. mojavensis Acp's		
Polymorphic	21	38	$G = 8.329$
Fixed	2	24	$P = 0.004$
	D. arizonae moj genes		
Polymorphic	16	8	Fisher's exact test:
Fixed	2	0	$P = 0.557$
	D. arizonae testis-enriched genes		
Polymorphic	44	21	$G = 4.967$
Fixed	14	1	$P = 0.026$
	D. arizonae Acp's		
Polymorphic	22	32	$G = 1.792$
Fixed	11	29	$P = 0.181$

Probability is determined by a *G*-test when all cells contain nonzero values; Fisher's exact test is shown otherwise.

protein databases for *D. mojavensis vs. D. melanogaster/D. simulans Acp*'s are suggestive of divergent functional biology (*e.g.*, Wagstaff and Begun 2005), with *D. mojavensis* proteins enriched for unknown functions and depauperate of lipases, proteases, and protease inhibitors compared to those of *D. melanogaster*. Additional support for this inference and its possible connections to mating system variation await future investigation.

The population genetics of desert Drosophila *Acp*'s showed some similarities and several important differences with respect to *D. melanogaster/D. simulans*. *D. melanogaster* and *D. simulans Acp*'s are highly polymorphic and divergent at replacement sites compared to "typical" genes in these two species (Begun *et al.* 2000; Swanson *et al.* 2001). *Acp*'s from *D. arizonae* and *D. mojavensis* showed a similar pattern in that they were much more polymorphic and divergent at replacement sites, at least compared to the non-*Acp* genes (mostly testis-enriched genes) surveyed here. However, *D. arizonae/D. mojavensis Acp*'s are proportionally much more polymorphic and divergent in terms of protein variation compared to *D. melanogaster/D. simulans Acp*'s (Table 2). One interpretation is that *Acp*'s tend to be under less functional constraint in desert Drosophila compared to *melanogaster* subgroup flies. Alternatively, *Acp*'s could be under stronger directional selection in desert Drosophila.

Two types of results support the idea that *Acp*'s experience directional selection in desert Drosophila. First, the K_a/K_s ratio is significantly >1 for two of nine *D. mojavensis Acp*'s. Given the small number of bases surveyed per gene and the fact that the K_a/K_s test is an extremely conservative test for directional selection, observing two of nine genes as individually significant is remarkable. The mean K_a/K_s for *D. mojavensis Acp*'s is 2.078, an extremely high value for any class of genes. Second, the MK tests provide strong evidence for adaptive protein evolution in *Acp*'s, but not in other genes.

Interestingly, *Acp* data strongly deviate from neutral expectations in *D. mojavensis*, but not in *D. arizonae*. Moreover, Table 4 suggests that the highly significant result from the pooled polymorphic and fixed mutations presented in Table 6 is attributable to a consistent excess of replacement fixations across most *D. mojavensis Acp*'s rather than to unusual observations from one or two genes. In fact, almost all *D. mojavensis Acp* substitutions are amino acid changes. Note that polarized analyses of polymorphic and fixed, synonymous and replacement variation have not been carried out for the *D. melanogaster/D. simulans* comparison, as outgroup data are generally lacking. In this respect, the population genetic inferences for desert Drosophila are more incisive than those for *D. melanogaster* and *D. simulans*. These results support the notion that lineage differences in sexual selection may have detectable effects on patterns of protein evolution (Tsaur *et al.* 2001; Dorus *et al.* 2004).

Given their close evolutionary relationship and similar mating systems, the inference of directional selection on *D. mojavensis Acp*'s and the lack of such an inference for *D. arizonae* are interesting. A notable distinction between mating systems is that the *D. mojavensis* ejaculate donation to female somatic tissues is three- to fourfold higher than that in *D. arizonae*, representing a far greater absolute difference than that observed for other sister species pairs from a large phylogenetic survey (Pitnick *et al.* 1997). This suggests the possibility that this difference should be a focus of our attempts to understand effects of mating system variation on protein variation. Perhaps large somatic donations are correlated with more or stronger *Acp*-mediated postcopulatory male-female interactions. An intriguing possibility is that the somatic donation from the male is associated with mechanisms that provide males with direct access to the female soma, thereby allowing more direct manipulation of female physiology. In this sense, donation to the female soma could be a Trojan horse that exposes females to exploitation by males, thereby driving male-female conflict and associated *Acp* divergence. Data from other species pairs with differences in ejaculate donation will shed light on the role of variation in male somatic donations to females in *Acp* evolution.

An alternative explanation of the differences between *D. arizonae* and *D. mojavensis Acp* protein evolution is that our sampling of *Acp* loci has compromised our ability to make an unbiased comparison between lineages. Because our *Acp*'s were isolated from a *D. mojavensis* accessory gland cDNA library, we are biased toward isolating genes that are more abundantly expressed in *D. mojavensis* than in *D. arizonae*. Therefore, a possible explanation for the differential importance of adaptive protein evolution in *D. arizonae vs. D. mojavensis* is that more abundantly expressed *Acp*'s are under stronger directional selection. This possibility is easily addressed through additional quantitative analysis (for both expression and population genetics) of larger numbers of *Acp*'s in both species.

There has been much speculation regarding the potential importance of adaptive protein evolution for male-reproduction-related genes. However, the data presented here are the first molecular population genetic analysis of a sample of Drosophila genes expressed primarily in testes. Our results show that in *D. arizonae/D. mojavensis*, testis-enriched genes evolve much more slowly than *Acp*'s and show no evidence of adaptive protein divergence. Why might *Acp*'s experience more directional selection than testis-enriched genes? Spermatogenesis requires several genes (Fuller 1993; Poccia 1994; Eddy 1998), many of which are unlikely to function directly in male-male and male-female postcopulatory interactions. This is in contrast to *Acp*'s, which are more likely to regulate postcopulatory male-male and male-female interactions (Wolfner 1997, 2002; Chapman 2001; Birkhead and Pizzari 2002). Our contrasting population genetic data for *Acp*'s *vs.* testis-enriched genes support the idea that proteins controlling postcopulatory, prefertilization phenotypes

are more likely to be under directional selection compared to proteins controlling sperm phenotypes *per se*. However, we predict that proteins controlling sperm phenotypes directly involved in male-male or male-female interactions will show evolutionary patterns similar to those observed at *Acp*'s. Our results suggest, not surprisingly, that the functional categorization of genes as male reproduction related or male biased (*e.g.*, Zhang *et al.* 2004) obscures a great deal of heterogeneity regarding mechanisms of evolution. More nuanced treatments of male reproduction-related genes with respect to expression and other aspects of biological annotation will likely add great additional insight into the factors explaining variance of protein evolution for such genes (*e.g.*, Good and Nachman 2005).

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