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Adaptive Evolution of Recently Duplicated Accessory Gland Protein Genes in Desert Drosophila

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ABSTRACT

The relationship between animal mating system variation and patterns of protein polymorphism and divergence is poorly understood. Drosophila provides an excellent system for addressing this issue, as there is abundant interspecific mating system variation. For example, compared to *D. melanogaster* subgroup species, *repleta* group species have higher remating rates, delayed sexual maturity, and several other interesting differences. We previously showed that accessory gland protein genes (*Acp*'s) of *Drosophila mojavensis* and *D. arizonae* evolve more rapidly than *Acp*'s in the *D. melanogaster* subgroup and that adaptive *Acp* protein evolution is likely more common in *D. mojavensis*/*D. arizonae* than in *D. melanogaster*/*D. simulans*. These findings are consistent with the idea that greater postcopulatory selection results in more adaptive evolution of seminal fluid proteins in the *repleta* group flies. Here we report another interesting evolutionary difference between the *repleta* group and the *D. melanogaster* subgroup *Acp*'s. *Acp* gene duplications are present in *D. melanogaster*, but their high sequence divergence indicates that the fixation rate of duplicated *Acp*'s has been low in this lineage. Here we report that *D. mojavensis* and *D. arizonae* genomes contain several very young duplicated *Acp*'s and that these *Acp*'s have experienced very rapid, adaptive protein divergence. We propose that rapid remating of female desert Drosophila generates selection for continuous diversification of the male *Acp* complement to improve male fertilization potential. Thus, mating system variation may be associated with adaptive protein divergence as well as with duplication of *Acp*'s in Drosophila.

POSTCOPULATORY conflict between males, in the form of sperm competition, can be an important component of male fitness in polyandrous species (Birkhead and Møller 1998). Numerous strategies have evolved to increase sperm competitive ability, often mediated by components of the seminal fluid (Birkhead and Møller 1998; Chapman 2001; Fry and Wilkinson 2004). Females also have an interest in paternity and can play an important role in deciding the outcome of sperm competition (Eberhard 1996; Birkhead and Pizzari 2002; Bernasconi *et al.* 2004). Thus, postcopulatory sexual selection may drive male adaptations to increase sperm competitive ability and female counter-adaptations to bias paternity, maintaining a state of antagonistic coevolution between the sexes (Rice 1996, 1998). Consistent with this hypothesis, proteins that mediate fertilization are known to evolve rapidly in many species (Vacquier 1998; Swanson and Vacquier 2002). Accordingly, postcopulatory interactions and the molecules behind them have drawn considerable attention for their potential role in generating reproductive isolation between populations (Parker and Partridge 1998; Rice 1998; Pitnick *et al.* 1999; Arnqvist *et al.* 2000; Gavrilets 2000; Knowles and Markow 2001).

In Drosophila, empirical studies suggest that there is abundant genetic variation affecting traits related to male–male and male–female postcopulatory interactions (Clark *et al.* 1995; Rice 1996; Clark and Begun 1998). Male accessory gland proteins (*Acp*'s) of the *Drosophila melanogaster* subgroup have received most of the attention as potential molecular agents of male–male and male–female postcopulatory interactions in Drosophila. There are an estimated 70–106 *Acp*'s in *D. melanogaster* (Mueller *et al.* 2005) that are transferred to females during mating as secreted seminal fluid peptides. *Acp*'s have been shown to stimulate ovulation and increase egg-laying rates (Kalb *et al.* 1993; Herndon and Wolfner 1995; Heifetz *et al.* 2000), bind sperm and affect sperm storage (Neubaum and Wolfner 1999; Tram and Wolfner 1999), affect the outcome of sperm competition (Harshman and Prout 1994; Chapman *et al.* 2000), decrease female receptivity (Chen *et al.* 1988; Aigaki *et al.* 1991; Chapman *et al.* 2003; Liu and Kubli 2003), and decrease female life span (Chapman *et al.* 1993, 1995; Lung *et al.* 2002). Furthermore, *Acp*'s evolve rapidly in the *D. melanogaster* subgroup (Begun *et al.* 2000; Swanson *et al.* 2001; Kern *et al.* 2004), in at least some cases as a result of directional

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selection (Tsaur and Wu 1997; Aguadé 1998, 1999; Tsaur *et al.* 1998; Begun *et al.* 2000; Kern *et al.* 2004).

Previous work has demonstrated that *Acp*'s evolve more rapidly than most Drosophila genes (Begun *et al.* 2000; Swanson *et al.* 2001; Wagstaff and Begun 2005a) and that they evolve especially rapidly in desert Drosophila of the *repleta* group (Wagstaff and Begun 2005b). Elevated rates of *Acp* evolution in desert Drosophila may be due to differences between their mating system and that of flies from the *D. melanogaster* subgroup (Markow 1996, 2002). For example, desert Drosophila males take at least twice as long as *D. melanogaster* males to reach reproductive maturity (Pitnick *et al.* 1995). Male age at reproductive maturity is positively correlated with sperm size and the size of the female sperm-storage organ in Drosophila species (Pitnick *et al.* 1995, 1999). Moreover, sperm size and sperm-storage organ size are coevolving rapidly in *D. mojavensis*, with geographically distinct populations expressing different phenotypes for these correlated traits (Pitnick *et al.* 2003). Another difference relative to the *D. melanogaster* subgroup mating system is female remating, which occurs much more rapidly and often in desert Drosophila (Markow 2002). Higher remating rates in desert Drosophila could potentially increase selection on phenotypes related to postcopulatory male–male or male–female interactions (Markow 2002; Singh *et al.* 2002).

Additional differences between *repleta* group and *D. melanogaster* subgroup flies are evident in the short-term physiological response of females following copulation. Transfer of seminal fluid triggers an insemination reaction within the female reproductive tract of desert Drosophila (Patterson and Stone 1952) but is diminutive in *D. melanogaster* (Wheeler 1947; Markow and Ankney 1988). This insemination reaction, which is superficially similar to inflammation, results in a mass in the female reproductive tract. Remating does not occur during the several hours that it persists (Patterson 1947; Knowles and Markow 2001). The intensity of the insemination reaction is highly variable, with interspecific matings (*e.g.*, *D. arizonae* and *D. mojavensis*) triggering an exaggerated and harder mass, which persists significantly longer than within-species insemination reactions (Patterson 1947). Interestingly, exaggerated insemination reactions are observed in some crosses between geographically distinct populations of *D. mojavensis*, suggesting that interpopulation postcopulatory incompatibilities may evolve quickly (Knowles and Markow 2001). Finally, ejaculate components of many *repleta* group species, including *D. mojavensis*, are incorporated into female somatic tissues, a phenomenon not known to occur in the *D. melanogaster* subgroup (Markow and Ankney 1984; Pitnick *et al.* 1997).

Our earlier results suggested that, although general patterns of protein variation in *Acp*'s from desert Drosophila and *D. melanogaster* subgroup flies are similar, there are important quantitative differences between the groups. For example, we found faster rates of protein evolution and stronger evidence for directional selection in *repleta* group *Acp*'s relative to *D. melanogaster* subgroup *Acp*'s (Wagstaff and Begun 2005b). Analyses of annotated *D. melanogaster Acp*'s show that, although several *Acp* duplicates exist, they tend to be relatively highly diverged at the nucleotide level (Holloway and Begun 2004; Mueller *et al.* 2005). Here we report the discovery of several recent *Acp* duplications in *D. arizonae*/*D. mojavensis*. Our analyses suggest that several of these recent duplications have diverged under directional selection, a phenomenon not observed in *D. melanogaster* (Holloway and Begun 2004). These data provide additional support for different evolutionary processes acting on *Acp*'s in these lineages, perhaps as a result of mating system divergence.

MATERIALS AND METHODS

Stocks and DNA sequencing: All fly stocks were acquired from the Drosophila Species Stock Center (Tucson, AZ). They included seven *D. arizonae* lines (15081-1271.00, 15081-1271.04, 15081-1271.05, 15081-1271.08, 15081-1271.12, 15081-1271.13, 15081-1271.14 from various locations in mainland Mexico) and seven *D. mojavensis* lines. Of the seven *D. mojavensis* stocks, four were *D. mojavensis baja* (15081-1351.03, 15081-1351.09, 15081-1351.12, 15081-1351.14 from various locations in Baja, Mexico), and three were *D. mojavensis mojavensis* (15081-1352.00, 15081-1352.01, 15081-1352.02 from various locations in southern California).

Most duplicate *Acp*'s described here were accidentally amplified during our earlier survey (Wagstaff and Begun 2005b) as secondary PCR products from primers designed from *D. mojavensis* accessory gland ESTs. Sequence data from each putative duplicate *Acp* were used to design duplicate-specific PCR primers for amplifying additional copies. However, the very short length of some *Acp*'s under investigation made it difficult to isolate duplicates from all of the fly lines in this survey. Expand high-fidelity polymerase (Roche Molecular Biochemicals) was used for PCR amplification. Single alleles for sequencing were isolated by TOPO vector (Invitrogen, San Diego) cloning of PCR products. PCR-amplified colony-PCR products and their associated sequences were obtained using M13 reverse and T7 vector primers. All sequencing was done on an Applied Biosystems 377 automated sequencer (ABI).

Organization of duplicated *Acp*'s: Patterns of sequence divergence (see below) in most cases provided unambiguous evidence that the *Acp*'s in question are duplications rather than highly diverged alleles. However, we used molecular and further computational analysis to investigate the genomic organization of putative duplicate *Acp*'s. Under the premise that recent duplications are often tandemly arranged, we designed PCR primers to amplify genomic DNA across the putative duplicates. We used LA-Taq long PCR polymerase (TaKaRa, Shiga, Japan) with an extension time of 10 min and cycling parameters according to the manufacturer's instructions. Successfully amplified fragments were end sequenced to confirm that the amplified product corresponded to the expected genomic sequence under the tandem duplication hypothesis. When the draft version of the whole-genome shotgun (WGS) *D. mojavensis* genome became available, we used BLAT (BLAST-like alignment tool, UCSC Genome Browser) analysis (Kent 2002) to confirm our observations and localize additional duplicate *Acp*'s.

TABLE 1

Sample and distribution of duplicate genes

	Sample		Documented in the same fly line?			
Duplicate gene	*ari*	*moj*	*a* + *b*	*a* + *c*	*b* + *c*	*a* + *b* + *c*
Acp5a	7	7				
Acp5b	3	1	3 *ari*, 1 *moj*	1 *moj*	No	No
Acp5c	0	1				
Acp16a	7	6				
Acp16b	7	4	7 *ari*, 3 *moj*	2 *moj*	1 *moj*	No
Acp16c	0	3				
Acp21a	6	7	No	—	—	—
Acp21b	1	0				
Acp27a	7	7	5 *moj*	—	—	—
Acp27b	0	5				

ari, *D. arizonae*; *moj*, *D. mojavensis*.

Molecular population genetic statistics and hypothesis tests of adaptive protein evolution: Alleles and duplicate gene sequences were aligned and edited using the DNASTAR software package (Lasergene, Madison, WI). The DnaSP program of Rozas and Rozas (1999) was used to measure levels of polymorphism and divergence for duplicate genes represented by multiple alleles. Group averages (*i.e.*, duplicates *vs.* other *Acp*'s) were calculated by taking averages weighted according to sequence length.

Nucleotide distances were used to infer the topologies of duplicate family genealogies. Maximum-likelihood estimation of branch-specific d_N and d_S values used the free-ratio model (model 1) of the PAML computer program (Yang 1997). Outgroups were determined by pairwise distance estimates and corroborated by PAML branch length output. For genes sampled for multiple alleles, one random allele was chosen for PAML analyses. Alignments were generated using the DNASTAR software package (Lasergene) and manually adjusted where appropriate. Indel variation for codon positions that were gapped in >50% of the aligned sequences were omitted from the analyses. PAML tests for branch heterogeneity compared likelihood estimates from the free-ratio model to estimates from the one-ratio model (model 0). We then tested entire gene trees for significant evidence of $d_N/d_S > 1$ by comparing the one-ratio model to a one-ratio model with $d_N/d_S = 1$ (fix_ omega = 1; omega = 1). To test whether the d_N value of a given branch significantly exceeds the d_S value (*Acp27* only), we used the two-ratio model (model 2) and set all background branches to have the same d_N/d_S value. The branch of interest either was allowed to be free or was fixed at $d_N/d_S = 1$. For likelihood-ratio tests, twice the log-likelihood difference was compared to a χ^2 distribution with 1 d.f. [or (number of branches − 1) for the branch heterogeneity tests] to determine significance levels.

RESULTS

Evidence of gene duplication: In the course of our molecular population genetic analysis of 18 *Acp*'s in *D. arizonae* and *D. mojavensis* (Wagstaff and Begun 2005b), sequence data from four genes revealed alleles that were unusually highly diverged from the majority of alleles sampled. These genes were clearly related to the target genes, but had levels of divergence that in most cases could be plausibly interpreted only as evidence of gene duplication. Table 1 provides a summary of alleles sampled and the number of fly lines that have been verified by PCR to carry particular putative duplicate gene copies.

Under the assumption that recent duplicate *Acp*'s likely originated through unequal crossing over and therefore were organized tandemly, we designed PCR primers to amplify intergenic sequence between putative paralogs. We successfully amplified intergenic sequences (data not shown) for *Acp16a-b*, *Acp21a-b*, and *Acp27a-b*, thereby confirming their duplicate status. BLAT analysis of the *D. mojavensis* assembly (UCSC Genome Browser) supports the proposition that the other highly diverged "alleles" isolated in our previous work (Wagstaff and Begun 2005b) are actually tandem duplicates. Table 2 shows the scaffold assembly positions of these paralogs. Thus, there is PCR evidence and/or support from genome assemblies for the duplicate status of the *Acp*'s discussed here.

Polymorphism and interspecific divergence of duplicate *Acp*'s: Polymorphism and interspecific orthologous divergence of several duplicate *Acp*'s (*Acp5a*, *Acp16a*, *Acp16b*, *Acp21a*, and *Acp27a*) was previously reported in Wagstaff and Begun (2005b). We include these data in Table 3 along with the newly reported duplicates. Table 3 also shows estimates of average polymorphism and divergence for duplicate *vs.* single-copy *Acp*'s in *D. mojavensis* and *D. arizonae*. Although synonymous and nonsynonymous heterozygosity shows considerable variation among duplicate genes, the small number of sites surveyed per gene precludes any speculation about heterogeneous forces. Overall, synonymous heterozygosity and divergence are slightly lower in duplicated *D. arizonae* and *D. mojavensis Acp*'s compared to single-copy *Acp*'s from these species. In contrast, nonsynonymous heterozygosity and divergence are higher for duplicated *Acp*'s than for single-copy *Acp*'s in *D. arizonae* and *D. mojavensis*, with

TABLE 2

Duplicate *Acp* genomic organization inferred from *D. mojavensis* assembly

Gene	Scaffold	Strand	CDS start	CDS stop	Intergenic sequence
Acp5a	6540	+	3,094,356	3,094,529	a–b: 22,390
Acp5b	6540	−	3,071,966	3,071,802	b–c: 16,862
Acp5c	6540	−	3,054,940	3,054,758	
Acp16a	—	—	—	—	—
Acp16b	6680	+	18,985,243	18,985,461	2,761
Acp16c	6680	+	18,988,222	18,988,437	
Acp21a	6540	+	33,094,203	33,094,553	—
Acp21b	—	—	—	—	
Acp27a	6496	−	3,965,482	3,965,189	—
Acp27b	—	—	—	—	

CDS, coding sequence.

nonsynonymous divergence marginally significantly higher for duplicate *Acp*'s (Wagstaff and Begun 2005b, Mann–Whitney one-tailed *U*-test, $P = 0.046$). The average d_N/d_S ratio for duplicated *Acp*'s is 2.123 (Table 3), which is significantly higher than the ratio for single-copy *Acp*'s (0.761) from these species (Wagstaff and Begun 2005b, Mann–Whitney one-tailed *U*-test, $P = 0.00679$; one value was omitted from each group because $d_S = 0$) and higher than the average ratio for *Acp*'s in *D. melanogaster vs. D. simulans* comparisons (0.47) (Swanson *et al.* 2001).

Contingency table comparison of synonymous to nonsynonymous polymorphisms (Table 4) provides further evidence that different evolutionary forces are acting on duplicate *vs.* other *Acp*'s in *D. mojavensis* ($P = 0.012$). The heterogeneity can be interpreted as either a deficit of synonymous or an excess of nonsynonymous polymorphisms. Comparison to non-*Acp D. mojavensis* polymorphisms from our previous survey on the same fly lines (Wagstaff and Begun 2005b) suggests that the heterogeneity is primarily attributable to an excess of nonsynonymous polymorphisms, although a smaller

TABLE 3

Polymorphism and orthologous divergence of duplicate *Acp*'s

			No. of sites						
Gene	No. of alleles	Sample	Synonymous	Nonsynonymous	θ_{syn}	θ_{rep}	d_S	d_N	d_N/d_S
Acp5a[a]	7	*ari*	27	72	0.0151	0.0057	0.111	0.110	0.990
Acp5a[a]	7	*moj*	27	72	0.0000	0.0170	—	—	—
Acp5b	3	*ari*	27	69	0.0000	0.0000	0.000	0.112	$d_N > d_S$
Acp16a[a]	7	*ari*	38	103	0.0000	0.0159	0.060	0.132	2.205
Acp16a[a]	6	*moj*	38	103	0.0000	0.0299	—	—	—
Acp16b[a]	7	*ari*	49	155	0.0251	0.0184	0.062	0.05	0.808
Acp16b[a]	4	*moj*	49	155	0.0336	0.0070	—	—	—
Acp16c	3	*moj*	45	156	0.0000	0.0086	—	—	—
Acp21a[a]	6	*ari*	48	132	0.0092	0.0066	0.055	0.227	4.121
Acp21a[a]	7	*moj*	48	132	0.0086	0.0278	—	—	—
Acp27a[a]	7	*ari*	68	214	0.0000	0.0019	0.006	0.013	2.138
Acp27a[a]	7	*moj*	68	214	0.0120	0.0076	—	—	—
Acp27b	5	*moj*	71	208	0.0068	0.0115	—	—	—
All duplicates		*ari*	257	745	0.0080	0.0083	0.044	0.094	2.123
		moj	346	1040	0.0097	0.0139	—	—	—
Other *Acp*'s[a]		*ari*	712	2336	0.0149	0.0058	0.068	0.052	0.761
		moj	712	2336	0.0166	0.0075	—	—	—

ari, *D. arizonae*; *moj*, *D. mojavensis*; θ_{syn}, synonymous heterozygosity; θ_{rep}, nonsynonymous heterozygosity.
[a] Data are from Wagstaff and Begun (2005b).

TABLE 4

Contingency analysis of duplicate *vs.* single-copy *Acp* polymorphism

	Synonymous	Nonsynonymous	
	D. mojavensis		
Duplicates	7	32	$G = 6.350$
Other *Acp*'s	29	42	$P = 0.012$
	D. arizonae		
Duplicates	5	17	$G = 3.250$
Other *Acp*'s	26	33	$P = 0.071$

deficit of synonymous polymorphisms also contributes to the pattern. A comparable contingency table from *D. arizonae* shows a similar trend in the same direction, although it is not significantly heterogeneous ($P = 0.071$).

Paralogous d_N/d_S ratios: Paralogous d_N/d_S estimates are >1 for all pairwise comparisons and are extraordinarily high in some cases (Table 5). For example, d_N/d_S is >4 in at least one pairwise comparison for three of the four *Acp* families. For the *Acp16* family, the highest value is for *D. arizonae Acp16a-b* (1.934). These extremely high d_N/d_S estimates seem particularly noteworthy, given that only 3 of 14 putative single-copy *Acp*'s investigated in *D. arizonae*/*D. mojavensis* (Wagstaff and Begun 2005b) have a $d_N/d_S > 1$.

Analysis of duplicate *Acp* gene trees: Maximum-likelihood analysis of the *Acp5* duplicate gene family reveals very high rates of protein evolution along most gene-tree branches, with average $d_N/d_S = 2.982$ (Figure 1B). The complete gene tree has a d_N/d_S ratio that is significantly >1 (Figure 1B; $P < 0.01$) and provides no evidence of branch heterogeneity. We report individual branch d_N/d_S estimates as a guideline for future investigations (Figure 1A). However, the lack of evidence for branch heterogeneity precludes individual branch hypothesis testing. Similarly, the *Acp16* gene tree shows high nonsynonymous divergence along most branches (Figure 2A), significant evidence of adaptive evolution with an average d_N/d_S of 1.923 (Figure 2B; $P < 0.05$), and no statistical evidence for branch heterogeneity (Figure 2B). Note that, although our PCR and sequencing efforts identified only *D. mojavensis* alleles for both *Acp5c* and *Acp16c*, paralogous synonymous and nonsynonymous divergence associated with both duplicates (Table 5) greatly exceeded average levels of orthologous divergence (Table 3). Thus, our failure to identify *D. arizonae* alleles might be explained by interspecific divergence that was too great to amplify *D. arizonae* alleles using primers designed from *D. mojavensis* DNA sequences or by loss of duplications in *D. arizonae*. As the genome assembly becomes more complete, a strategy of long PCR, cloning, and sequencing should reveal the full complement of *Acp5* and *Acp16* duplicates in *D. arizonae* and *D. mojavensis*.

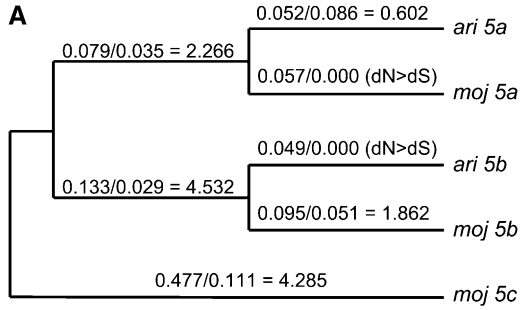
Divergence estimates and tests of branch heterogeneity for *Acp21* and *Acp27* are shown in Table 6. Both gene families generally show little synonymous divergence and very high levels of nonsynonymous divergence. However, in many cases, an informative estimate of d_N/d_S cannot be reported because synonymous divergence is too close to zero. The *Acp21* gene family shows dramatically higher levels of nonsynonymous *vs.* synonymous divergence, with d_N/d_S ratios significantly >1 for the complete gene tree ($P < 0.001$), and all three

TABLE 5

Paralogous divergence of duplicate *Acp*'s

	No. of alleles		No. of sites				
Gene pair	First gene	Second gene	Synonymous	Nonsynonymous	d_S	d_N	d_N/d_S
			Acp5				
ari (a:b)	7	3	27	69	0.199	0.272	1.370
moj (a:b)	7	1	24	63	0.043	0.205	4.799
moj (a:c)	7	1	25	65	0.124	0.474	3.817
moj (b:c)	1	1	25	68	0.157	0.434	2.757
			Acp16				
ari (a:b)	7	7	40	116	0.229	0.442	1.934
moj (a:b)	6	4	40	113	0.247	0.461	1.867
moj (a:c)	6	3	40	116	0.196	0.314	1.599
moj (b:c)	4	3	46	149	0.313	0.378	1.209
			Acp21				
ari (a:b)	6	1	49	137	0.014	0.134	9.734
			Acp27				
moj (a:b)	7	5	65	196	0.021	0.103	4.899

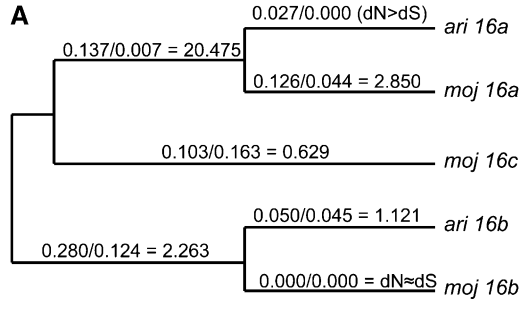
ari, *D. arizonae*; *moj*, *D. mojavensis*.



B

	dN/dS	2Δℓ	df	Prob.
Branch Heterogeneity	—	4.46	6	NS
Tree ω > 1	2.982	8.57	1	$P < 0.01$

FIGURE 1.—Phylogeny of *Acp5* duplicate genes. (A) Evolution along each branch is shown as d_N/d_S ratios. (B) Maximum-likelihood analyses of the complete gene tree. There is no significant evidence of branch heterogeneity; however, the complete *Acp5* gene tree has $d_N/d_S > 1$ ($P < 0.01$). ω, d_N/d_S ratio; $2\Delta\ell$, likelihood-ratio test; NS, not significant.



B

	dN/dS	2Δℓ	df	Prob.
Branch Heterogeneity	—	5.58	6	NS
Tree ω > 1	1.923	5.36	1	$P < 0.05$

FIGURE 2.—Phylogeny of *Acp16* duplicate genes. (A) d_N/d_S values are shown for each branch. (B) Maximum-likelihood analyses of the complete gene tree. There is no significant evidence of branch heterogeneity; however, the complete *Acp16* gene tree has $d_N/d_S > 1$ ($P < 0.05$). ω, d_N/d_S ratio; $2\Delta\ell$, likelihood-ratio test; NS, not significant.

branches are individually estimated at $d_N > d_S$. Because there is no significant evidence of *Acp21* branch heterogeneity (Table 6), we do not report individual branch likelihood-ratio tests. Even so, note that the *D. mojavensis Acp21a* branch has estimated d_N at severalfold higher than d_N along the *D. arizonae Acp21a* and *Acp21b* branches.

Unlike *Acp21*, *Acp27* does show significant evidence of branch heterogeneity ($P < 0.05$) that is clearly attributable to most of the nonsynonymous divergence occurring along the *D. mojavensis Acp27b* branch. Both this branch individually and the complete gene tree have d_N/d_S significantly >1 ($P < 0.01$; Table 6). Furthermore, all 17 nonsynonymous differences between *D. mojavensis Acp27a* and *Acp27b* fall on the *Acp27b* branch when *D. arizonae Acp27a* is used as the outgroup. Note that levels of divergence for *D. arizonae Acp21b* and *D. mojavensis Acp27b* are low enough to be consistent with postspeciation duplication events. Further sequence analysis should confirm whether or not this is the case.

DISCUSSION

Sequence analysis of *Acp* genes from the *D. melanogaster* subgroup has demonstrated that seminal fluid proteins evolve rapidly relative to other classes of genes (BEGUN *et al.* 2000; SWANSON *et al.* 2001; KERN *et al.* 2004). This rapid evolution is often interpreted as evidence of natural selection, which is thought to play an important role in sperm competition and male–female postcopulatory interactions (RICE 1996; SWANSON and VACQUIER 2002). We have previously shown that single-copy *Acp* genes in *D. arizonae* and *D. mojavensis* evolve more rapidly than the *D. melanogaster* subgroup *Acp*'s (WAGSTAFF and BEGUN 2005b), an observation that is consistent with

TABLE 6

Divergence and branch heterogeneity of *Acp21* and *Acp27* duplicate families

Gene family	d_N	d_S	ω	$2\Delta l^a$	Significance
Acp21 heterogeneity	—	—	—	0.41	NS
Acp21 complete tree	—	—	$d_N > d_S$	18.52	$P < 0.001$
ari a branch	0.090	0.000	$d_N > d_S$	—	—
ari b branch	0.060	0.000	$d_N > d_S$	—	—
moj a branch	0.180	0.014	12.857	—	—
Acp27 heterogeneity	—	—	—	6.40	$P < 0.05$
Acp27 complete tree	—	—	8.348	8.24	$P < 0.01$
ari a branch	0.014	0.000	$d_N > d_S$	0.97	NS
moj a branch	0.000	0.013	0.000	—	—
moj b branch	0.110	0.000	$d_N > d_S$	12.12	$P < 0.01$

w, d_N/d_S ratio ($d_N > d_S$ is shown when $d_S = 0$); NS, not significant.

[a] Likelihood-ratio tests *vs.* the null model ($d_N/d_S = 1$ or d_N/d_S is constant for branch heterogeneity tests).

expectations based on their dramatically different mating systems (Markow 1996, 2002). Here we have documented that desert Drosophila *Acp*'s differ from the *D. melanogaster* subgroup *Acp*'s in other important ways. In contrast to the *D. melanogaster* subgroup *Acp*'s (Saudan *et al.* 2002; Holloway and Begun 2004; Mueller *et al.* 2005), *Acp* duplications in *D. mojavensis/D. arizonae* are very recent (Table 5), including two (*D. arizonae Acp21b* and *D. mojavensis Acp27b*) that potentially originated after the *D. mojavensis/D. arizonae* speciation event.

The four *D. arizonae/D. mojavensis Acp* gene families investigated here evolve more rapidly than putative single-copy *Acp*'s, with evidence of adaptive evolution in all four families. These results are consistent with observations suggesting that gene duplication can facilitate adaptive protein evolution (Ohno 1970; Ohta 1994; Li 1995). Interspecific d_N/d_S ratios for all duplicate *Acp*'s varied from 0.808 to 4.121, significantly exceeding the distribution of d_N/d_S ratios for putative single-copy *Acp*'s. Moreover, paralogous d_N/d_S ratios were even higher, demonstrating a broad time frame for adaptive evolution since most duplication events clearly predate *D. arizonae/D. mojavensis* speciation. Our maximum-likelihood analyses show that 16 of 20 duplicate gene tree branches have d_N/d_S ratios >1. The complete gene tree for each duplicate gene family significantly exceeds $d_N/d_S = 1$.

Duplicated *Acp*'s also show higher levels of nonsynonymous polymorphism compared to single-copy *Acp*'s. The high rate of adaptive protein evolution at these loci and the evidence for significant geographical variation in postcopulatory *D. mojavensis* phenotypes (Knowles and Markow 2001; Pitnick *et al.* 2003; Reed and Markow 2004) suggest that some nonsynonymous polymorphism in *Acp* gene families might be due to divergent selection between geographically isolated populations. Alternatively, the high level of nonsynonymous polymorphism could be due to selected amino acid polymorphisms during their sojourn through the population. The lower synonymous heterozyogsity in duplicated *vs.* single-copy *Acp*'s is consistent with this scenario. Additional population genetics and functional data comparing intra- and interpopulation dynamics between conspecific desert Drosophila populations are needed to resolve this question.

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