Molecular Ecology (2008) **17**, 2616–2628 doi: 10.1111/j.1365-294X.2008.03780.x

Reproductive protein evolution within and between species: maintenance of divergent ZP3 alleles in *Peromyscus*

LESLIE M. TURNER*† and HOPI E. HOEKSTRA†
*Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093, USA, †Department of Organismic and Evolutionary Biology and The Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138, USA

Abstract

In a variety of animal taxa, proteins involved in reproduction evolve more rapidly than non-reproductive proteins. Most studies of reproductive protein evolution, however, focus on divergence between species, and little is known about differentiation among populations within a species. Here we investigate the molecular population genetics of the protein ZP3 within two *Peromyscus* species. ZP3 is an egg coat protein involved in primary binding of egg and sperm and is essential for fertilization. We find that amino acid polymorphism in the sperm-combining region of ZP3 is high relative to silent polymorphism in both species of *Peromyscus*. In addition, while there is geographical structure at a mitochondrial gene (*Cytb*), a nuclear gene (*Lcat*) and eight microsatellite loci, we find no evidence for geographical structure at *Zp3* in *Peromyscus truei*. These patterns are consistent with the maintenance of ZP3 alleles by balancing selection, possibly due to sexual conflict or pathogen resistance. However, we do not find evidence that reinforcement promotes ZP3 diversification; allelic variation in *P. truei* is similar among populations, including populations allopatric and sympatric with sibling species. In fact, most alleles are present in all populations sampled across *P. truei's* range. While additional data are needed to identify the precise evolutionary forces responsible for sequence variation in ZP3, our results suggest that in *Peromyscus*, selection to maintain divergent alleles within species contributes to the pattern of rapid amino acid substitution observed among species.

Keywords: balancing selection, fertilization, reinforcement, reproductive isolation, sexual conflict, zona pellucida

Received 17 August 2007; revision received 24 February 2008; accepted 20 March 2008

Introduction

One of the most striking genetic patterns seen in animals is that proteins involved in reproduction often evolve much more rapidly than do nonreproductive proteins (Singh & Kulathinal 2000; Swanson & Vacquier 2002b; Clark *et al*. 2006). In mammals, evidence for rapid reproductive protein evolution comes from comparing widely divergent species (Queralt *et al*. 1995; Swanson *et al*. 2001, 2003; Torgerson *et al*. 2002; Glassey & Civetta 2004) or comparing diverse species within orders (Retief *et al*. 1993; Wyckoff *et al*. 2000; Kingan *et al*. 2003; Dorus *et al*. 2004; Clark & Swanson 2005; Podlaha *et al*. 2005). Only a few studies have focused on comparisons of closely related species (Jansa *et al*. 2003; Turner & Hoekstra 2006) or on populations within a single species.

In fact, intraspecific studies of reproductive protein variation have been limited to invertebrates. In these studies, patterns of genetic variation differ among proteins and among taxa. In some cases (e.g. sea urchin bindin, Metz & Palumbi 1996; *Drosophila* accessory gland proteins, Begun *et al*. 2000; Wagstaff & Begun 2005), amino acid variation is extensive, while in others (e.g. abalone lysin, Lee *et al*. 1995), variation is limited or absent. In some species, population-genetic data support a role for natural selection in driving the evolution of fertilization proteins, with directional selection operating in some instances and balancing selection in others. For example, there is evidence for recent positive selection on seminal proteins (accessory gland proteins, Acps) in several species of *Drosophila* (Begun *et al*. 2000; Wagstaff

Correspondence: Leslie M. Turner, Max Planck Institute for Evolutionary Biology, Department of Evolutionary Genetics, August Thienemann Str. 2, 24306 Plön, Germany. Fax: +49 45 022763 281; E-mail: turner@evolbio.mpg.de

& Begun 2005) and on female reproductive-tract proteins in *D. melanogaster* (Panhuis & Swanson 2006).

We know much less, however, about the patterns of reproductive protein variation within mammalian species or about the evolutionary forces affecting these patterns (reviewed in Turner & Hoekstra in press). At present, surveys of intraspecific variation are limited to two sperm proteins, zonadhesin and PKDREJ, in humans. In both cases, sliding window analyses of nucleotide variation suggest some protein regions may be subject to balancing selection, whereas other regions may be subject to directional selection (Gasper & Swanson 2006; Hamm *et al*. 2007).

Proteins directly involved in gamete interaction are likely targets of natural and sexual selection. In mammals, the initial binding of sperm to the zona pellucida, or egg coat, is thought to be the critical step of sperm-egg recognition. The sperm protein(s) involved in this interaction have not yet been identified, but the egg protein ZP3 (zona pellucida 3) is essential in sperm binding (Wassarman *et al*. 2001). *In vitro* binding assays indicate that interaction with sperm occurs in a small region of ZP3 known as the 'sperm-combining' region (Kinloch *et al*. 1995). In the laboratory mouse, amino acid substitutions at critical sites in the sperm-combining region (Chen *et al*. 1998) or replacement of the region with hamster sequence (Williams *et al*. 2006) reduces the strength of sperm binding. ZP3 was long thought to be the single primary sperm-receptor protein, but a recent model of egg–sperm interaction, based on evidence from transgenic studies, suggests that binding specificity is based on the three-dimensional structure of the zona pellucida, a matrix composed of ZP3 plus two additional proteins, ZP1 and ZP2 (Rankin *et al*. 2003; Dean 2004).

Comparative sequence analysis of mammalian ZP3 shows evidence for positive selection on amino acid changes. Moreover, a cluster of positively selected amino acid sites was identified in the sperm-combining region (Swanson *et al*. 2001). In previous work, we analysed patterns of genetic variation of ZP3 in 15 species of deer mice (genus *Peromyscus*, Turner & Hoekstra 2006). We found evidence that ZP3 experienced positive selection during diversification of the genus. Further, despite small sample sizes, we observed substantial amino acid variation *within* some species.

Here, we expand our previous results by characterizing intraspecific patterns of ZP3 sequence variation in two *Peromyscus* species. First, we sequenced regions of ZP3 (including the sperm-combining region) and a nonreproductive nuclear gene in *Peromyscus truei* (the pinyon mouse) and one population of its sister species, *P. gratus* (Osgood's mouse). To compare patterns of population structure, we also sequenced a mitochondrial DNA (mtDNA) gene and genotyped eight microsatellite markers in a large and geographically diverse sample of *P. truei*. Together, these data provide evidence for natural selection maintaining divergent ZP3 alleles in both species.

Materials and methods

Extraction, amplification and sequencing

Tissues samples (liver, kidney, or tail) from 46 *Peromyscus truei* individuals from 14 localities and 10 *P. gratus* individuals from a single locale were obtained from natural history museums or from our field collections (Table 1, Fig. 1a). We extracted DNA from tissue using a DNeasy kit (QIAGEN).

To make a preliminary assessment of intraspecific *Zp3* variation in *P. truei*, we sequenced a region containing exons 3–8 in 10 individuals from Williams Butte, California (population CA-N, Fig. 1a). The ~4910 bp fragment was amplified using a nested polymerase chain reaction (PCR) with primers Z33FD (5′-GTAACCCYGACCCCACACAAAC-3′) and Z38RA (5′-TCAGGGACAGGCACTAAACCAC-3′) followed by Z33FF (5′-GATGGAGTTATATGGAAGCAGC-3′) and Z38RC (5′-WGGWGTGACACTTCCTGGTGAC-3′). Based on the exons 3–8 sequences (see Results), we chose to sequence a variable region of *Zp3* containing exons 6–7 (358 bp), which includes the region essential for sperm binding (Wassarman *et al*. 2001), for all individuals using previously published primers and conditions (Turner & Hoekstra 2006).

For comparison to variation in *Zp3*, we sequenced the nonreproductive nuclear gene lecithin cholesterol acyltransferase (*Lcat*). *Lcat* is an enzyme involved in glycerophospholipid metabolism (Kuivenhoven *et al*. 1997) located on *Mus* chromosome 8 and thus not likely to be linked to *Zp3* (*Mus* chromosome 5). Like *Zp3*, *Lcat* is an autosomal locus, and thus the effects of demographic events on genetic variation in *Lcat* are expected to be comparable to *Zp3*. We sequenced two regions of *Lcat*: fragment one, containing exons 2–5 (664 bp), was amplified using conserved primers LCAT2FA (5′-ACAGAGGACTTCTTCACCATC-3′) and LCAT5RA (5′-AATAGAGCACATGTAGGCAGC-3′), and fragment two, containing most of *Lcat* exon 6 (487 bp), was amplified as described previously (Turner & Hoekstra 2006) using published primers (Robinson *et al*. 1997).

In addition, to control for demographic effects, we sequenced the mitochondrial gene *Cytb* in all 56 individuals. *Cytb* should be more sensitive to population structure than a nuclear locus because of its lower N_e. J.L. Patton generously provided partial *Cytb* sequences for 20 individuals. For the remaining samples, we amplified a 1055-bp fragment using primers tCytbF1 (5′-CGACCTCCCAACTCCATCCAAC-3′) and tCytbR1 (5′-TGCCTGCCATAGGTATTAGGAC-3′).

To further characterize genetic structure at autosomal loci in *P. truei*, we genotyped individuals at eight microsatellite loci. Microsatellites, like mtDNA, have higher mutation rates than nuclear genes and thus may be more sensitive to population structure. However, unlike mtDNA, microsatellites have biparental inheritance and thus genetic

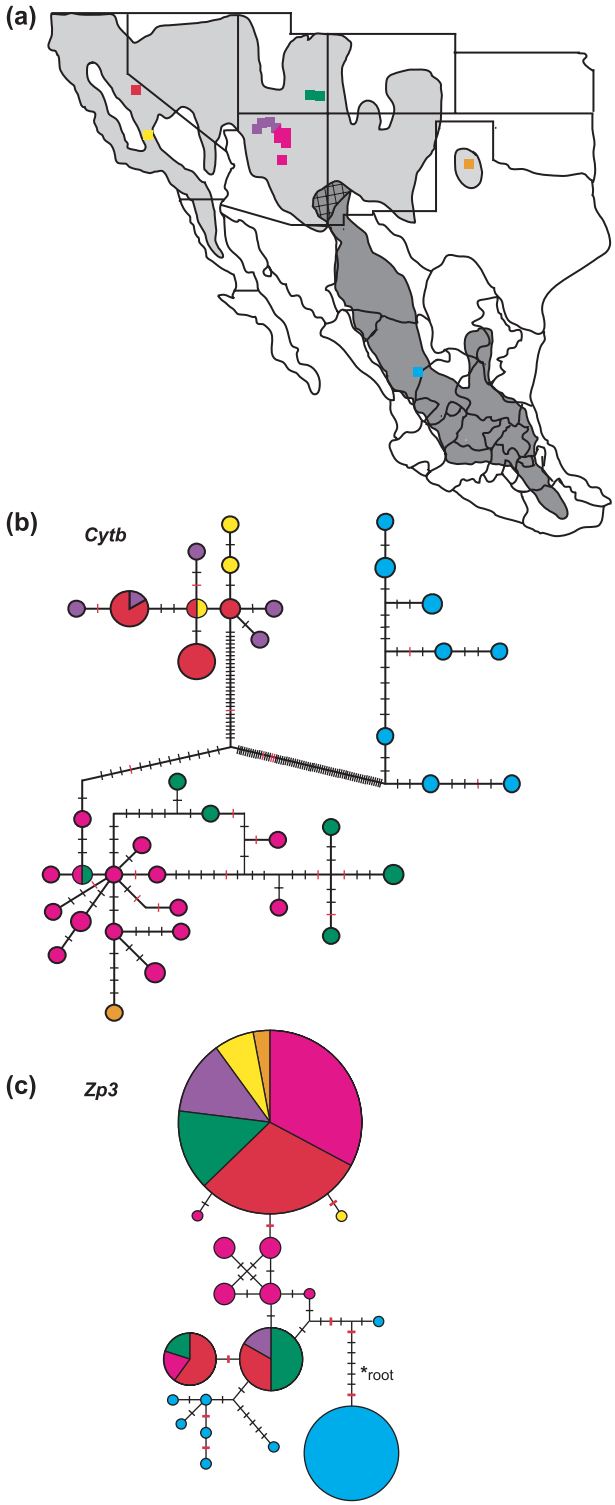

Fig. 1 (a) Ranges of *Peromyscus truei* (light grey) and *P. gratus* (dark grey); overlapping region is cross-hatched (following Durish *et al*. 2004). Sample locations are indicated with squares and colour indicates grouping: red, northern California (CA-N); yellow, southern California (CA-S); purple, Arizona, western *Cytb* clade (AZ-W); pink, Arizona, eastern *Cytb* clade (AZ-E); green, Utah (UT); orange, Texas (TX); turquoise, Durango, Mexico (*P. gratus*). Haplotype networks for (b) *Cytb* and (c) *Zp3*. Each circle represents a unique haplotype; the size of the circle is proportional to the number of alleles, with the exception of the most common *Zp3* allele, which is reduced to one-fourth. Colours indicate geographical origins of sampled alleles (following a). Each tick mark represents a nucleotide difference: black marks indicate synonymous differences and red marks indicate nonsynonymous differences.

structure will be affected by gene flow mediated by both sexes. Microsatellite loci (*PO-9*, *PO3-68*, *PO3-85*, *Pm103*, *Pm108*, *Pm109*, *PLGT62*, *PMBW0514*) were amplified successfully for 89–100% of *P. truei* individuals using published primers (Schmidt 1999; Chirhart *et al*. 2000; Prince *et al*. 2002; Steiner *et al*. 2007).

We purified PCR products using a MinElute Gel Extraction Kit (QIAGEN), a PerfectPrep PCR cleanup 96 kit (Eppendorf) or treatment with exonuclease I and shrimp alkaline phosphatase (Fermentas). PCR products of *Zp3* exons 3–8 were cloned (TOPO-XL, Invitrogen) and plasmid DNA was purified using a Qiaprep Spin Miniprep kit (QIAGEN). We performed cycle sequencing using BigDye 3.1 chemistry (Applied Biosystems) and ran those products on an ABI 3100 genetic analyser (Applied Biosystems). We aligned sequences using SEQUENCHER (version 4.2, Gene Codes). If more than one heterozygous site was detected for nuclear genes, we determined phase by sequencing cloned products (TOPO-TA, Invitrogen) or inferred phase computationally using PHASE (version 2.1.1, Stephens *et al*. 2001; Stephens & Donnelly 2003). We scored microsatellite alleles on an ABI 3130 or 3730 sequencer and assigned genotypes using PEAK SCANNER (version 1.0, Applied Biosystems).

Sequence data have been deposited with the GenBank Data Libraries under Accession nos EU568373–EU568800.

Statistical analysis

To compare levels of nucleotide diversity within and among populations and species, we estimated summary statistics of sequence polymorphism data using ANALYSERHKA (version 7, Haddrill *et al*. 2005). We calculated nucleotide diversity for silent sites, replacement sites and all sites, separately. Diversity measures include π, which is based on the average pairwise difference between sequences (Tajima 1983) and θ_W, which is based on the number of segregating sites (Watterson 1975). We excluded sites with alignment gaps or more than two variants within a species from further analysis. For microsatellite loci, we calculated observed (H_O) and expected (H_E) heterozygosities using GENEALEX (version 6.1, Peakall & Smouse 2006).

To test for deviations from neutral evolution, we applied two statistical tests based on population parameters estimated from polymorphism data; both analyses test for a skew in the frequency distribution of polymorphisms, which indicates deviation from neutrality. Tajima's *D* test

Table 1 Samples of *Peromyscus* included in this study

Subspecies	Sampling location	*n*	Specimen numbers*
P. t. montipinoris	Walker Pass, Kern County, CA	3	LMT 010 – 012
P. t. truei	Williams Butte, Mono County, CA	13	LMT 007 – 009, JNW007, 009, 011 - 012, MVZ 208477 – 208481
P. t. truei	Yavapai County, AZ	1	TK113804
P. t. truei	Ryan, Coconino County, AZ	2	MVZ 197284 – 5
P. t. truei	Kaibab Plateau, Coconino County, AZ	2	MVZ 197293 – 4
P. t. truei	Tanner Tank, Coconino County, AZ	2	MVZ 197295 – 6
P. t. truei	Woodhouse Mesa, Coconino County, AZ	12	MVZ 199469 – 80
P. t. truei	Toroweap Valley, Mohave County, AZ	1	MVZ 199467
P. t. truei	Hack Canyon, Mohave County, AZ	1	MVZ 199468
P. t. truei	Little Colorado River, Coconino County, AZ	1	MVZ 199481
P. t. truei	Onion Creek, Grand County, UT	1	MVZ 199482
P. t. truei	Rock Canyon Corral, Grand County, UT	3	MVZ 199483 – 5
P. t. truei	0.5 mi E Rock Canyon Corral, Grand County, UT	3	MVZ 199486 – 8
P. t. comanche	Armstrong County, TX	1	TK40209
P. gratus gentilis	Durango, Mexico	10	TK48798, TK48826, TK48834, TK48839, TK48854, TK48856, TK48892 – 3, TK48899, TK48911

*LMT, JNW = Hoekstra laboratory; MVZ = Museum of vertebrate zoology (Univ. California, Berkeley); TK = Museum of Texas Tech University.
§Samples from this population were identified as *P. truei* in previous work (see Supplementary methods; Turner & Hoekstra 2006).

measures the difference between π and θ_W; negative values indicate an excess of rare mutations, a pattern consistent with a selective sweep, and positive values indicate an excess of high frequency mutations, consistent with balancing selection (Tajima 1989). Fay and Wu's *H* test measures the difference between π and θ_H, a measure of nucleotide diversity that gives greater weight to high frequency polymorphisms (Fay & Wu 2000). Negative values of *H* indicate an excess of high frequency derived mutations, consistent with hitchhiking. We determined significance by comparing observed values to the results of 10 000 coalescent simulations performed in MS (Hudson 2002) under neutral conditions given the observed θ_W. For nuclear genes, we estimated the population recombination parameter (ρ, Hudson 2001), and ran simulations with this empirical estimate of ρ and four additional ρ values such that ρ/θ_W equalled 0, 1, 10, and 50. The additional values were included to determine the sensitivity of the results of *D* and *H* to variation in recombination rate; they represent a large range of recombination, from $\rho = 0$ up to values that are greater than values in *Drosophila* ($\rho = 50$), which has much higher levels of recombination than mammals. We report ρ values based on simulations run with empirically estimated ρ values; the significant *D* and *H* values reported remained significant when simulations were run with $P = 0$.

We used the McDonald–Kreitman (MK) test to look for evidence of balancing or directional selection on *Zp3* in *P. truei* and *P. gratus*. The MK test is based on the expectation under neutrality that the ratio of replacement to silent polymorphism should equal the ratio of replacement to silent divergence. A statistically significant excess of replacement divergent sites is interpreted as evidence for directional selection while excess replacement polymorphism is expected if a gene is subject to balancing selection (McDonald & Kreitman 1991).

To assess genetic structure among populations for each of the three genes and the microsatellite loci, we used the program STRUCTURE, which clusters individuals based on their genotypes (version 2.2, Pritchard *et al*. 2000). We ran the analyses with values of *K* (number of genotypic clusters) ranging from 1 to the number of populations sampled plus 3 (4 for *P. gratus*, 9 for *P. truei*). For each value of *K*, we ran 10 analyses for 130 000 cycles and discarded the first 30 000 cycles as burn-in. We used the ΔK statistic to determine the appropriate *K* value for each data set (Evanno *et al*. 2005). ΔK is not defined for $K = 1$; thus, if the difference in likelihood values between $K = 1$ and $K = 2$ was < 5%, we used $K = 1$. In addition, we estimated genetic distance between populations (γ_{ST}, Nei 1982) using DNASP (version 4.20.2, Rozas *et al*. 2003) and constructed haplotype networks for each gene using TCS (version 1.21, Clement *et al*. 2000) to visually represent the extent and type (i.e. nonsynonymous vs. synonymous) of variation between alleles. For microsatellites, we tested for linkage disequilibrium among loci using GENEPOP (version 3.4, Raymond & Rousset 1995) and estimated genetic distance between populations (F_{ST}) using ARLEQUIN (version 2.000, Schneider *et al*. 2000).

ZP3 divergence in mammals

To compare rates of evolution among regions of ZP3, we obtained coding sequences for 18 mammalian species from GenBank (see Table S1, Supplementary material for accession numbers). We aligned nucleotide sequences using MUSCLE (version 3.6, Edgar 2004), and adjusted gaps based on an alignment of amino acid sequences. Four species with deletions in exons 6 or 7 were excluded from further analysis (indicated in Table S1). We performed a sliding window analysis of the nonsynonymous divergence rate (d_N, window 22 sites, step size 8 sites) and the ratio of nonsynonymous to synonymous divergence rates (d_N/d_S, window 30 sites, step size 10 sites) using DNASP.

Results

Polymorphism and divergence

To determine which regions of *Zp3* may be most variable in an intraspecific analysis, we sequenced exons 3–8 in 10 *Peromyscus truei* individuals from the CA-N population. We found five polymorphic sites in this large coding region, three of which were nonsynonymous changes. Two of the three variable amino acid sites were in exon 7; the third site was the last aa encoded by exon 5, and thus is directly adjacent to the region sequenced previously (Turner & Hoekstra 2006). Therefore, we focused our sequencing efforts on exons 6–7, as this region likely would capture most of the intraspecific amino acid variation in ZP3.

Measures of genetic diversity for the three genes sequenced are shown in Table 2. Each gene shows a unique pattern of genetic variation. Within species, *Cytb* has the most genetic diversity, as expected based on high mtDNA mutation rate, and levels of variability are similar in *P. truei* and *P. gratus*. When all sites are considered, diversity estimates are similar for *Lcat* in both species and for *Zp3* in *P. truei*, but *Zp3* diversity in the single *P. gratus* population is much higher. Importantly, both *Lcat* and *Cytb* have fixed nucleotide differences between *P. truei* and *P. gratus*, while *Zp3* has neither silent nor replacement fixed differences.

When just replacement sites are considered, levels of polymorphism are much higher in *Zp3* than in *Lcat* and even in *Cytb,* when samples from the highly divergent eastern and western clades of *P. truei* are separated. If diversity were high at both replacement and silent sites, we might infer that *Zp3* has a higher mutation rate than the other genes. Thus, we determined the ratio of replacement to silent diversity to correct for mutation rate when comparing amino acid polymorphism between genes. In *P. gratus*, measures of nucleotide diversity for both replacement and silent sites are higher for *Zp3* than for the nuclear gene *Lcat*; however, ratios of replacement to silent diversity ($\theta_R/\theta_S = 0.302$, $\pi_R/\pi_S = 0.312$; Table 2) are at least an order of

Table 2 DNA polymorphism and divergence

				Silent					Replacement					Total						
Locus	Species	Hap	N	No. of sites	S	θ	π	D_{xy}	Fixed	No. of sites	S	θ	π	D_{xy}	Fixed	No. of sites	S	θ	π	D_{xy}	Fixed	θ_R/θ_S	π_R/π_S
Cytb	*P. truei* (all)	31	46	236	72	0.069	0.101	0.380	53	724	10	0.003	0.003	0.007	3	964	82	0.020	0.028	0.099	56	0.050	0.034
	P. truei (E)	21	25	235	32	0.035	0.024	0.182	31	725	8	0.003	0.002	0.005	2	964	40	0.011	0.007	0.049	33	0.084	0.090
	P. truei (W)	10	21	237	9	0.011	0.010			723	1	4×10^{-4}	2×10^{-4}			964	10	0.003	0.003			0.036	0.026
	P. gratus	8	10	239	20	0.030	0.032			721	2	0.001	8×10^{-4}			964	22	0.008	0.008			0.033	0.024
Lcat	*P. truei*	23	88	418	14	0.007	0.004	0.028	3	495	2	0.001	1×10^{-4}	7×10^{-5}	0	917	16	0.003	0.002	0.013	3	0.121	0.034
	P. gratus	13	14	445	10	0.006	0.007			578	0	0	0			1027	10	0.003	0.003			0	0
Zp3	*P. truei*	10	92	177	5	0.004	0.002	0.034	0	176	3	0.003	0.003	0.017	0	354	8	0.004	0.002	0.025	0	0.754	1.360
	P. gratus	10	20	174	13	0.021	0.026			177	4	0.006	0.008			352	17	0.014	0.017			0.302	0.312

Hap, number of haplotypes. N, number of alleles sampled. S, segregating sites. θ, Watterson's θ (per site). π , pairwise differences (per site). D_{xy}, average number of nucleotide substitutions (per site) between *P. truei* and *P. gratus* or for *P. truei* (W) with *P. truei* (E). Fixed, number of fixed differences, comparisons as for D_{xy}, θ_R, replacement sites; θ_S, silent sites; π_R, replacement sites; π_S, silent sites.

Table 3 Population-genetic tests of neutrality. Significant *P* values (< 0.05) are in bold

Locus	Species	Tajima's *D* (*P*)	Fay & Wu's *H* (*P*)
Cytb	*P. truei* (all)	1.51 (0.952)	0.16 (0.342)
	P. truei (W)	–1.17 (0.112)	0.087 (0.328)
	P. truei (E)	–0.36 (0.390)	–0.93 (0.189)
	P. gratus	0.37 (0.680)	3.56 (0.893)
Lcat	*P. truei*	–1.28 (0.076)	–7.97 **(0.012)**
	P. gratus	0.17 (0.600)	1.45 (0.856)
Zp3	*P. truei*	–0.96 (0.173)	–1.88 (0.066)
	P. gratus	0.85 (0.843)	3.44 (0.976)

magnitude higher than the ratios in nonreproductive genes (*Cytb* – $\theta_R/\theta_S = 0.033$, $\pi_R/\pi_S = 0.024$; *Lcat* – $\theta_R/\theta_S = 0$, $\pi_R/\pi_S = 0$; Table 2). In *P. truei*, ratios of replacement to silent diversity observed for *Zp3* are six to 40 times greater ($\theta_R/\theta_S = 0.754$, $\pi_R/\pi_S = 1.360$) than for the control genes.

In addition, all microsatellite loci genotyped in *P. truei* have substantial variation (Table S2, Supplementary material): the total number of alleles per locus ranged from 12 to 37 (mean 22.2), overall H_O ranged from 0.095 to 0.905 (mean = 0.572) and overall H_E ranged from 0.754 to 0.960 (mean = 0.884). No significant linkage disequilibrium was detected among loci.

Tests of neutrality

To test for deviations from neutral evolution, we applied two statistical tests based on population parameters estimated from polymorphism data (Tajima 1989; Fay & Wu 2000). Neither the Tajima's *D* nor Fay and Wu's *H* statistic was significant in the positive or negative direction for *Cytb* or *Zp3* for either *P. truei* or *P. gratus*, based on comparison of observed values to neutral coalescent simulations (Table 3). For *Lcat*, neither *D* or *H* values were significant in *P. gratus*; however, in *P. truei*, *D* was not significant but *H* was negative (*H* = –7.97, *P* = 0.012). The mixed results of the two tests suggest that evidence for directional selection acting on *Lcat* in *P. truei* is weak at best. Because there are neither high frequency replacement polymorphisms within *P. truei* nor fixed replacement differences between *P. truei* and *P. gratus* in the sequenced region of *Lcat*, any selection has been on a linked site(s).

We also used the McDonald–Kreitman (MK) test to distinguish between two alternative explanations for high levels of amino acid polymorphism in *Zp3* — balancing selection that maintains divergent alleles or relaxed levels of purifying selection. MK tests do not indicate significant excess of replacement polymorphism or of replacement divergence for either *P. truei* or *P. gratus* vs. an outgroup sequence from *P. boylii* (Turner & Hoekstra 2006).

Population structure

We found that the spatial distribution of alleles in *P. truei* varied among loci. There is striking geographical structure in *Cytb* (Figs 1b and 2); the species comprises two highly diverged clades ($D_{xy} = 0.049$, $\gamma_{ST} = 0.807$), representing eastern and western populations. These mitochondrial clades meet in northern Arizona, where individuals representing both *Cytb* groups are found in close proximity (Fig. 1a). The location of the transition between clades does not coincide with any reported boundary between subspecies or other previously described geographical patterns of variation in *P. truei* (Hoffmeister 1951). Similar geographical structure is also apparent in *Lcat* (Fig. 2). STRUCTURE identified two genotypic clusters (*K* = 2), one of which is found predominantly in the eastern clade, and the other predominantly in the western clade. Thus, there is a distinct transition between eastern and western groups ($D_{xy} = 0.004$, $\gamma_{ST} = 0.162$). This geographical structure appears to be genome-wide as evidenced by patterns of microsatellite variation: there is statistically significant genetic structure between eastern and western groups ($F_{ST} = 0.049$), although the geographical transition is less distinct. (Pairwise population F_{ST} estimates are provided in Table S3, Supplementary material). In contrast to the structure observed at all other loci, nucleotide variation in *Zp3* has little or no geographical structure. The two genotypic clusters identified by STRUCTURE analysis are represented in each *P. truei* population sampled and are relatively evenly distributed among the western and eastern clades (Fig. 2). Moreover, genetic distance between the western and eastern groups is an order of magnitude lower than in *Lcat* ($D_{xy} = 0.003$, $\gamma_{ST} = 0.014$).

Geographic distribution of allelic diversity in Zp3

To test for geographical variation in *Zp3* allelic diversity, we compared allele frequencies among populations of *P. truei* distributed across the species' range (Fig. 1a). (We do not describe geographical variation in *Zp3* in *P. gratus* because we sampled only a single population). Levels of allelic variation are remarkably similar across the species' range ($\theta_R = 0.002$–0.004; Table 4). With the exception of the locale where only a single individual was sampled (TX), at least two haplotypes, differing by at least one amino acid site, were represented at each locale. In addition, there is haplotype-sharing between populations across large geographical distances (Fig. 1c). Although no alleles are shared between *P. truei* and *P. gratus*, some alleles of *P. gratus* are more closely related to *P. truei* alleles than to other *P. gratus* alleles (Fig. 1c).

The sampled populations include localities both where *P. truei* is sympatric with other members of the *truei* species group (AZ-W, AZ-E, TX, Fig. 1a) and also localities where *P. truei* is the only species group member present (allopatric

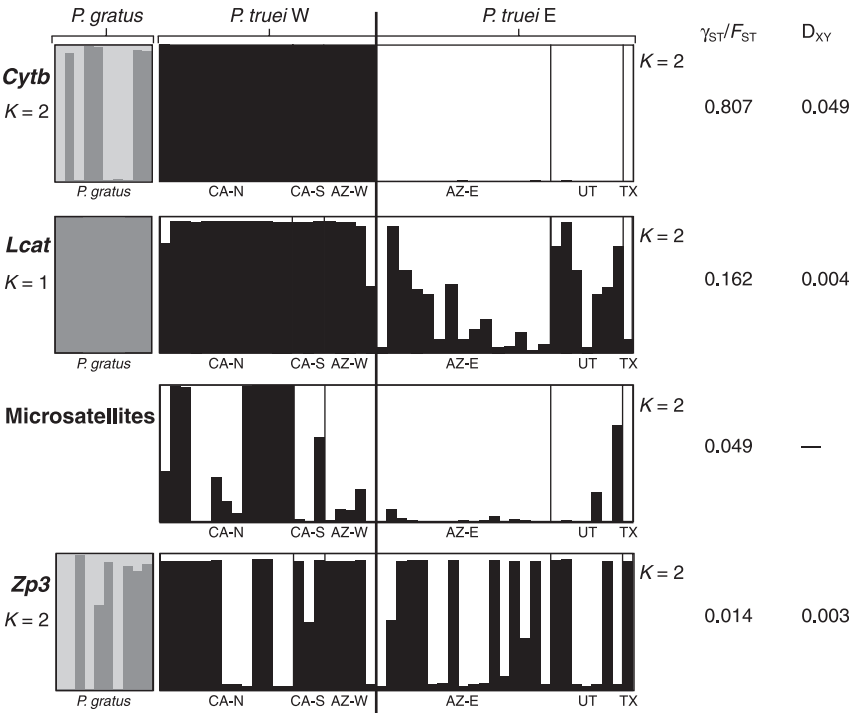

Fig. 2 Genotypic clustering within species for *Cytb*, *Lcat*, microsatellites, and *Zp3* determined using STRUCTURE (Pritchard *et al*. 2000); *K* = estimated number of subpopulations (*P. gratus* estimates are shown on the Left and *P. truei* on the right). Each vertical bar represents a single individual and each shade represents a genotype cluster. Thin black lines separate geographical regions of sampling sites. Estimates of γ_{ST} (for genes), F_{ST} (for microsatellites) and D_{XY} (for genes) between eastern and western mtDNA clades of *P. truei* are given.

Table 4 Diversity in sympatric versus allopatric populations

Locale	*N*	θ	θ_R	aa alleles
Sympatric				
AZ-W	10	0.003	0.002	2
AZ-E	34	0.006	0.003	3
Allopatric				
CA-N	26	0.003	0.003	3
CA-S	6	0.001	0.002	2
UT	12	0.004	0.004	3

N, number of alleles sampled. θ, Watterson's θ (per site). θ_R, replacement sites. aa alleles, number of alleles with unique amino acid sequences.

populations: CA-N, CA-S, UT; Durish *et al*. 2004). Allelic composition and levels of amino acid variation are similar between sympatric and allopatric populations (Table 4), providing no evidence of increased divergence of *Zp3* between *P. truei* and sibling species in sympatric populations.

Discussion

Our study of intraspecific variation of the female fertilization protein ZP3 reveals several intriguing patterns. While we do not find evidence of directional selection in either *Peromyscus truei* or *P. gratus*, we do find more than a 10-fold increase in the ratio of replacement to silent polymorphism in *Zp3* relative to the ratios seen for two nonreproductive genes. In addition, while there is geographical structure at both autosomal and mtDNA loci, we do not detect any structure at *Zp3*. In fact, most *Zp3* alleles are found in all *P. truei* populations. These results are consistent with the maintenance of divergent alleles due to sexual conflict (Gavrilets & Waxman 2002) or to pathogen resistance (Roy & Kirchner 2000). However, populations of *P. truei* sympatric with other members of the *truei* species group do not show reduced levels of polymorphism, derived substitutions, or unique haplotypes compared to allopatric populations; thus, reinforcement does not seem to promote ZP3 diversification. This work represents one of the few studies in mammals that examine intraspecific variability and the evolutionary forces responsible for the striking patterns of reproductive protein diversification at the interspecific level.

No evidence of strong directional selection in Zp3

There are several population-level scenarios that could explain the diversification of reproductive proteins among species. In abalone, for example, rapid diversification of the sperm protein lysin due to strong directional selection is observed among species, and there is almost no variation within species (Lee *et al*. 1995). By contrast, in *Peromyscus* we found relatively high levels of ZP3 variation within both *P. truei* and *P. gratus*. In addition, most of the variation in ZP3 occurs within populations rather than between populations. When we conducted population-genetic tests of neutrality in *Zp3*, we did not find evidence for

directional selection or recent selective sweeps, although these tests are weak if selection occurred in the distant past (Simonsen *et al*. 1995; Przeworski 2002) or from standing genetic variation (Hermisson & Pennings 2005). Thus, it is unlikely that strong and recent directional selection has acted on *Zp3* within these *Peromyscus* species.

Diversifying selection acting on ZP3

In some other species, intraspecific variation in reproductive proteins is high and has been attributed to diversifying selection. In urchins, for example, both reinforcement (Geyer & Palumbi 2003) and complex frequency-dependent selection (Levitan & Ferrell 2006) are thought to drive the rapid evolution of a fertilization protein. In *Peromyscus*, several lines of evidence suggest that diversifying selection maintains amino acid polymorphisms in ZP3.

First, in both *P. truei* and *P. gratus*, we observe high levels of *Zp*3 diversity. In *P. gratus*, in which only a single population was sampled, we find five distinct haplotypes (each differing by at least one amino acid) among just 20 sampled alleles. In *P. truei*, average pairwise differentiation (π) at replacement sites among *Zp*3 alleles is 30 times higher than *Lcat (*and has three times the number of segregating sites when corrected for sequence length), a pattern not mirrored in silent sites. In fact, the ratio of replacement to silent polymorphism is more than 10-fold higher for *Zp3* than for either *Cytb* or *Lcat*. Moreover, we find high ratios of replacement to silent diversity for both θ and π, and π_R/π_S is greater than θ_R/θ_S for both *P. truei* and *P. gratus*, indicating that the replacement polymorphisms are not likely to be newly arisen slightly deleterious mutations.

Such high levels of amino acid polymorphism can result from balancing selection that maintains divergent alleles or from relaxed levels of purifying selection. The MK test, which is commonly used to distinguish between these alternatives, is not significant for *Zp3* for either *P. truei* or *P. gratus*. However, the MK test is conservative and lacks power to detect selection maintaining divergent alleles within species when both divergence and polymorphism are elevated at replacement sites relative to silent sites. Further, the importance of ZP3's role in fertilization leads us to believe that relaxed purifying selection is an unlikely explanation for the high amino acid diversity we observe. ZP3 has sperm-binding activity in all mammals that have been tested, including hamster, human, pig and laboratory mouse (Fig. 3a; reviewed in McLeskey *et al*. 1997). Levels of amino acid variation in ZP3 are generally low, consistent with purifying selection, with the exception of the sperm-combining region (Fig. 3c, d). Perhaps most importantly, in the laboratory mouse, amino acid mutations in the sperm-combining region (encompassed in the region sequenced here) inactivate ZP3 as a sperm receptor (Chen *et al.* 1998), thus having obvious effects on fitness. Therefore, it is unlikely that selection would be relaxed in a region of such functional importance.

Second, there is evidence that the sperm-combining region of ZP3 has been a common target of positive selection in mammals. A sliding window analysis of nonsynonymous divergence (d_N) in *Zp3* in 14 species shows that the rate of amino acid evolution is high and the ratio of nonsynonymous relative to synonymous variation (d_N/d_S) is highest in the sperm-combining region (Fig. 3c). Positively selected sites have been identified in the sperm-combining region of ZP3 within *Peromyscus* (Turner & Hoekstra 2006), within other rodents (Jansa *et al*. 2003; Swann *et al*. 2007), and in a phylogenetically diverse sample of mammals (Swanson *et al*. 2001). In fact, two of the variable amino acid sites in *P. truei* are positively selected in Australasian murine rodents, and the amino acid substitution at one of these sites is convergent between these distantly related taxa. This observation also supports the hypothesis that amino acid variation in *P. truei* and *P. gratus* is adaptive.

Finally, the geographical distribution of *Zp3* variation in *P. truei* suggests a role for selection. Whereas all other genetic markers we surveyed showed genetic structure between eastern and western populations, by contrast, *Zp3* shows extensive allele-sharing among populations and among these divergent geographical clades. If proportions assigned to the two genetic clusters in *Zp3* differed substantially between the eastern and western groups, we might infer that the alleles of the two *Zp3* clusters evolved in isolated populations and that variation within clades resulted from recent neutral introgression after secondary contact. The relatively uniform distribution of genetic clusters between clades (Fig. 2) and across great geographical distances (Fig. 1c) indicates that either amino acid variation predates the divergence of the eastern and western clades or that alleles spread rapidly in both directions due to selection following secondary contact. In either case, selection is necessary to explain the spatial distribution of *Zp3* alleles.

The detection of balancing selection within populations, especially within mammals that generally have small population sizes (N_e) compared to other species like marine invertebrates or *Drosophila*, is challenging (Garrigan & Hedrick 2003; Hughes 2007). Even with strong evidence for selection based on patterns of nucleotide variation, additional data showing functional differences between alleles is an important next step (Turner & Hoekstra in press). For example, evidence that variation in polymorphic sites of ZP3 affects female fertility in captive crosses or *in vitro* fertilization assays would support the hypothesis that polymorphism contributes to fitness differences in nature. Further, ZP3 is a glycoprotein and it has been shown that oligosaccharides attached in the sperm-combining region are essential for sperm binding in the laboratory mouse (Chen *et al*. 1998). We have documented variation in the

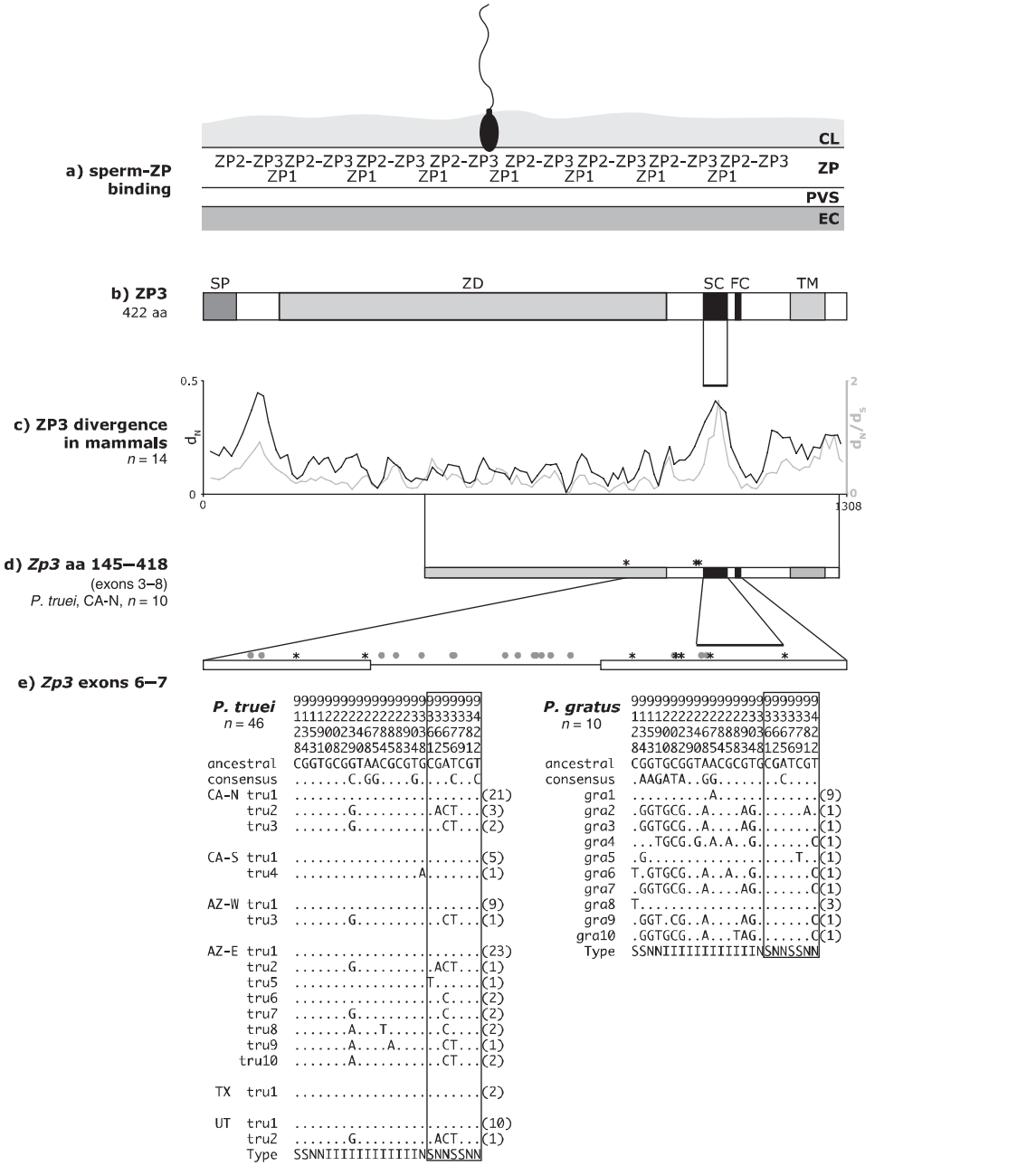

Fig. 3 (a) Schematic representation of sperm binding to the egg zona pellucida. ZP2-ZP3 dimers are shown, cross-linked by ZP1. Egg layers are indicated to the right: CL, cumulus layer; ZP, zona pellucida; PVS, perivitelline space; EC, egg cytoplasm. (b) ZP3 protein structure with predicted functional domains drawn to scale, based on alignment with *Mus* (Akatsuka *et al*. 1998; Jovine *et al*. 2004). Abbreviations for domains are: SP, signal peptide; ZD, zona domain; SC, sperm-combining region; FC, furin cleavage site; and TM, transmembrane domain. (c) A sliding window analysis of nonsynonymous divergence (d_N, in black) and the ratio of nonsynonymous to synonymous divergence (d_N/d_S, in grey) for an alignment of *Zp3* coding sequences from 14 mammals. (d_N – 22 sites, step size 8 sites, d_N/d_S – 30 sites, step size 10 sites). Black bar highlights the sperm-combining region. (d) Protein structure for aa 145–418 of ZP3 with variable amino acid sites [in 10 *P. truei* individuals from Williams Butte, CA (CA-N site, Fig. 1a)] indicated with asterisks. (e) Genomic structure for exons 6–7 of *Zp3*. Boxes indicate exons, black bar highlights SC, symbols show the spatial distribution of synonymous (grey circles) and nonsynonymous (black asterisks) polymorphisms in both *Peromyscus truei* and *P. gratus* samples. Specific polymorphic sites in *Zp3* exons 6–7 for *P. truei* and *P. gratus* are shown below. Nucleotide site numbers refer to inferred position in the genomic sequence based on alignment of exons 6–7 with *Peromyscus polionotus* genomic sequence (Turner & Hoekstra 2006). All unique alleles found within each sampling location are shown (location names follow Fig. 1a) and the number of alleles sampled with each haplotype is given in parentheses. Consensus sequences are provided. Ancestral sequences were inferred using parsimony through comparison to an outgroup sequence (*P. boylii*). Below each position, we indicate the site class (S, synonymous; N, nonsynonymous; I, intron). Black box indicates sites in or near the SC.

amino acid sequence of ZP3 but additional variation at the carbohydrate level is possible and fertilization assays may reveal differences in sperm-binding activity even among alleles that are identical in amino acid sequence.

Allelic diversity in Zp3 *among populations of* P. truei

Reinforcement to prevent or reduce hybridization in areas of sympatry between closely related species can also contribute to the rapid evolution of reproductive proteins. Consistent with this hypothesis, in urchins of the genus *Echinometra*, there is more allelic variation in areas of sympatry than in allopatry (Geyer & Palumbi 2003). For reinforcement to occur, diverging populations must hybridize in nature and experience incomplete postzygotic isolation (Coyne & Orr 2004). Hybridization of *P. truei* with sibling species has not been reported in nature (Dice 1968); however, laboratory crosses provide evidence of some postzygotic isolation between *P. truei* and sympatric populations of *P. difficilis* (sometimes referred to as *P. nasutus*). Crosses follow Haldane's rule: that is, hybrid female offspring are fertile and hybrid male offspring are partially or completely sterile (Haldane 1922; Dice 1968). Thus, although there may be opportunity for reinforcement in natural populations of *P. truei*, the geographical distribution of allelic variation in *Zp3* in *P. truei* is inconsistent with reinforcement. We find similar levels of nucleotide and haplotype diversity among populations (Table 4). It should be noted, however, that the lack of a signature of reinforcement in *Zp3* is not evidence against reinforcement contributing to the evolution of reproductive isolation between *P. truei* and its sibling species. Reinforcing selection may have acted on another male–female reproductive protein interaction or may have acted on premating isolation (such as assortative mating), so that there may be no opportunity for selection to cause increased protein divergence at the gametic level (Lorch & Servedio 2005).

Conclusions

In both *Peromyscus truei* and *P. gratus*, we observe high levels of replacement polymorphism, a pattern that can result from selection acting to maintain divergent alleles. This type of selection is consistent with theories involving sexual conflict (Frank 2000; Gavrilets & Waxman 2002; Haygood 2004) as well as defence against pathogens (Roy & Kirchner 2000), two hypotheses that have been proposed to explain rapid rates of reproductive protein evolution (Swanson & Vacquier 2002a). However, additional data measuring differences between alleles in function or fitness are needed to rule out the formal possibility of relaxed purifying selection, identify the specific amino acid sites that are targets of selection, and determine the precise selective agent promoting ZP3 diversification.

Understanding the molecular interactions between ZP3 and sperm protein(s) may also help determine whether the observed patterns of polymorphism reflect fitness differences among genotypes in nature. There has been recent progress in functionally characterizing two promising candidate proteins that interact with ZP3: zonadhesin (Bi *et al*. 2003) and PKDREJ (Sutton *et al*. 2006). Further, evolutionary analysis of both proteins within and between species of primates has identified promising regions of the proteins that may interact and co-evolve with ZP3. Interestingly, there is evidence that balancing selection promotes amino acid variation in both of these putative ZP3-binding loci (Gasper & Swanson 2006; Hamm *et al*. 2007).

Results of our intraspecific analysis provide insight into the pattern of repeated amino acid substitution in ZP3 first identified in an interspecific analysis (Turner & Hoekstra 2006). The lack of evidence for directional selection in both *P. truei* and *P. gratus* suggests that the interspecific pattern is not likely to be the result of repeated selective sweeps, causing rapid turnover in alleles. Instead, selection may favour two or more alleles simultaneously; consequently, newly arising alleles may increase in frequency but not replace older alleles. An extreme example of this type of selection is self-incompatibility alleles in several families of plants; positive selection promotes amino acid divergence, yet high levels of allelic variation are maintained through frequency-dependent selection resulting in shared polymorphism between species of different genera (Vekemans & Slatkin 1994; Ishimizu *et al*. 1998; Takebayashi *et al*. 2003). While we did not identify any alleles shared between *P. truei* and *P. gratus,* some alleles from *P. gratus* are more closely related to *P. truei* alleles than to other *P. gratus* alleles. Similar lack of monophyly at *Zp3* was found for several other *Peromyscus* species (Turner & Hoekstra 2006). Additional data about the extent and distribution of amino acid polymorphism across species of *Peromyscus* are necessary to evaluate whether there are trans-specific polymorphisms and whether selection maintaining diversity in ZP3 is widespread.

The lack of geographically structured variation at ZP3 in *P. truei,* suggests that this protein likely does not contribute to any current barriers to gene flow between populations. However, measuring sequence variation in additional sibling species and assessing functional differences between alleles will be an exciting next step in evaluating whether ZP3 may have contributed to the evolution or maintenance of isolating barriers between species.

Acknowledgements

We thank members of the Hoekstra Laboratory, P. Andolfatto, B. Payseur, J. Jensen, J. Kohn and V. Vacquier for helpful discussion. Additional comments from J. Coyne and two anonymous reviewers greatly improved the manuscript. J. Patton generously provided

help in the field, advice, and sequence data. J. Weber assisted with fieldwork. The Museum of Texas Tech University and the Museum of Vertebrate Zoology (University of California, Berkeley) kindly provided tissue samples. This research was supported by a Howard Hughes Medical Institute Predoctoral Fellowship and a University of California Reserve System Mildred Mathias Award (to L.M.T.), National Science Foundation Doctoral Dissertation Improvement Grant DEB 0608030 (to L.M.T. and H.E.H.), and National Science Foundation grant DEB 0344710 (to H.E.H.).

References

Akatsuka K, Yoshida-Komiya H, Tulsiani DRP *et al*. (1998) Rat zona pellucida glycoproteins: molecular cloning and characterization of the three major components. *Molecular Reproduction and Development*, **51**, 454–467.

Begun DJ, Whitley P, Todd BL, Waldrip-Dail HM, Clark AG (2000) Molecular population genetics of male accessory gland proteins in *Drosophila*. *Genetics*, **156**, 1879–1888.

Bi M, Hickox JR, Winfrey VP, Olson GE, Hardy DM (2003) Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome. *Biochemical Journal*, **375**, 477–488.

Chen J, Litscher ES, Wassarman PM (1998) Inactivation of the mouse sperm receptor, mZP3, by site-directed mutagenesis of individual serine residues located at the combining site for sperm. *Proceedings of the National Academy of Sciences, USA*, **95**, 6193–6197.

Chirhart SE, Honeycutt RL, Greenbaum IF (2000) Microsatellite markers for the deer mouse *Peromyscus maniculatus*. *Molecular Ecology*, **9**, 1669–1671.

Clark NL, Swanson WJ (2005) Pervasive adaptive evolution in primate seminal proteins. *Public Library of Science, Genetics*, **1**, 335–342.

Clark NL, Aagaard JE, Swanson WJ (2006) Evolution of reproductive proteins from animals and plants. *Reproduction*, **131**, 11–22.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Dean J (2004) Reassessing the molecular biology of sperm-egg recognition with mouse genetics. *Bioessays*, **26**, 29–38.

Dice LR (1968) Speciation. In: *Biology of Peromyscus (Rodentia)* (ed. King JA), pp. 75–97. American Society of Mammalogists.

Dorus S, Evans PD, Wyckoff GJ, Choi SS, Lahn BT (2004) Rate of molecular evolution of the seminal protein gene *SEMG2* correlates with levels of female promiscuity. *Nature Genetics*, **36**, 1326–1329.

Durish ND, Halcomb KE, Kilpatrick CW, Bradley RD (2004) Molecular systematics of the *Peromyscus truei* species group. *Journal of Mammalogy*, **85**, 1160–1169.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Fay JC, Wu CI (2000) Hitchhiking under positive Darwinian selection. *Genetics*, **155**, 1405–1413.

Frank SA (2000) Sperm competition and female avoidance of polyspermy mediated by sperm-egg biochemistry. *Evolutionary Ecology Research*, **2**, 613–625.

Garrigan D, Hedrick PW (2003) Perspective: Detecting adaptive molecular polymorphism: Lessons from the MHC. *Evolution*, **57**, 1707–1722.

Gasper J, Swanson WJ (2006) Molecular population genetics of the gene encoding the human fertilization protein zonadhesin reveals rapid adaptive evolution. *American Journal of Human Genetics*, **79**, 820–830.

Gavrilets S, Waxman D (2002) Sympatric speciation by sexual conflict. *Proceedings of the National Academy of Sciences, USA*, **99**, 10533–10538.

Geyer LB, Palumbi SR (2003) Reproductive character displacement and the genetics of gamete recognition in tropical sea urchins. *Evolution*, **57**, 1049–1060.

Glassey B, Civetta A (2004) Positive selection at reproductive ADAM genes with potential intercellular binding activity. *Molecular Biology and Evolution*, **21**, 851–859.

Haddrill PR, Thornton KR, Charlesworth B, Andolfatto P (2005) Multilocus patterns of nucleotide variability and the demographic and selection history of *Drosophila melanogaster* populations. *Genome Research*, **15**, 790–799.

Haldane JBS (1922) Sex ratio and unisexual sterility in hybrid animals. *Journal of Genetics*, **12**, 101–109.

Hamm D, Mautz BS, Wolfner MF, Aquadro CF, Swanson WJ (2007) Evidence of amino acid diversity–enhancing selection within humans and among primates at the candidate sperm-receptor gene *PKDREJ*. *American Journal of Human Genetics*, **81**, 44–52.

Haygood R (2004) Sexual conflict and protein polymorphism. *Evolution*, **58**, 1414–1423.

Hermisson J, Pennings PS (2005) Soft sweeps: molecular population genetics of adaptation from standing genetic variation. *Genetics*, **169**, 2335–2352.

Hoffmeister DF (1951) A taxonomic and evolutionary study of the pinon mouse. *Illinois Biological Monographs*, **21**, 1–104.

Hudson RR (2001) Two-locus sampling distributions and their application. *Genetics*, **159**, 1805–1817.

Hudson RR (2002) Generating samples under a Wright–Fisher neutral model of genetic variation. *Bioinformatics*, **18**, 337–338.

Hughes AL (2007) Looking for Darwin in all the wrong places: The misguided quest for positive selection at the nucleotide sequence level. *Heredity*, **99**, 364–373.

Ishimizu T, Endo T, Yamaguchi-Kabata Y *et al*. (1998) Identification of regions in which positive selection may operate in S-RNase of Rosaceae: implication for S-allele-specific recognition sites in S-RNase. *Federation of European Biochemical Societies Letters*, **440**, 337–342.

Jansa SA, Lundrigan BL, Tucker PK (2003) Tests for positive selection on immune and reproductive genes in closely related species of the murine genus *Mus*. *Journal of Molecular Evolution*, **56**, 294–307.

Jovine L, Qi HY, Williams Z, Litscher ES, Wassarman PM (2004) A duplicated motif controls assembly of zona pellucida domain proteins. *Proceedings of the National Academy of Sciences, USA*, **101**, 5922–5927.

Kingan SB, Tatar M, Rand DM (2003) Reduced polymorphism in the chimpanzee semen coagulating protein, semenogelin I. *Journal of Molecular Evolution*, **57**, 159–169.

Kinloch RA, Sakai Y, Wassarman PM (1995) Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis. *Proceedings of the National Academy of Sciences, USA*, **92**, 263–267.

Kuivenhoven JA, Pritchard H, Hill J *et al*. (1997) The molecular

pathology of lecithin cholesterol acyltransferase (LCAT) deficiency syndromes. *Journal of Lipid Research*, **38**, 191–205.

Lee YH, Ota T, Vacquier VD (1995) Positive selection is a general phenomenon in the evolution of abalone sperm lysin. *Molecular Biology and Evolution*, **12**, 231–238.

Levitan DR, Ferrell DL (2006) Selection on gamete recognition proteins depends on sex, density, and genotype frequency. *Science*, **312**, 267–269.

Lorch PD, Servedio MR (2005) Postmating–prezygotic isolation is not an important source of selection for reinforcement within and between species in *Drosophila pseudoobscura* and *D. persimilis*. *Evolution*, **59**, 1039–1045.

McDonald JH, Kreitman M (1991) Adaptive protein evolution at the *Adh* locus in *Drosophila*. *Nature (London)*, **351**, 652–654.

McLeskey SB, Dowds C, Carballada R, White RR, Sailing P (1997) Molecules involved in mammalian sperm–egg interaction. *International Review of Cytology*, **177**, 57–113.

Metz EC, Palumbi SR (1996) Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. *Molecular Biology and Evolution*, **13**, 397–406.

Nei M (1982) Evolution of human races at the gene level. In: *Human Genetics Part A: The Unfolding Genome* (eds Tamir B, Cohen T, Goodman RM), pp. 167–181. Alan R. Liss, New York.

Panhuis TM, Swanson WJ (2006) Molecular evolution and population genetic analysis of candidate female reproductive genes in *Drosophila*. *Genetics*, **173**, 2039–2047.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Podlaha O, Webb DM, Tucker PK, Zhang J (2005) Positive selection for indel substitutions in the rodent sperm protein CATSPER1. *Molecular Biology and Evolution*, **22**, 1845–1852.

Prince KL, Glenn TC, Dewey MJ (2002) Cross-species amplification among peromyscines of new microsatellite DNA loci from the oldfield mouse (*Peromyscus polionotus subgriseus*). *Molecular Ecology Notes*, **2**, 133–136.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Przeworski M (2002) The signature of positive selection at randomly chosen loci. *Genetics*, **160**, 1179–1189.

Queralt R, Adroer R, Oliva R *et al*. (1995) Evolution of *protamine P1* genes in mammals. *Journal of Molecular Evolution*, **40**, 601–607.

Rankin TL, Coleman JS, Epifano O *et al*. (2003) Fertility and taxon-specific sperm binding persist after replacement of mouse sperm receptors with human homologs. *Developmental Cell*, **5**, 33–43.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Retief JD, Winkfein RJ, Dixon GH *et al*. (1993) Evolution of *protamine P1* genes in primates. *Journal of Molecular Evolution*, **37**, 426–434.

Robinson M, Catzeflis F, Briolay J, Mouchiroud D (1997) Molecular phylogeny of rodents, with special emphasis on murids: evidence from nuclear gene *Lcat*. *Molecular Phylogenetics and Evolution*, **8**, 423–434.

Roy BA, Kirchner JW (2000) Evolutionary dynamics of pathogen resistance and tolerance. *Evolution*, **54**, 51–63.

Rozas J, Sanchez-De I, Barrio JC, Messeguer X, Rozas R (2003) DNASP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.

Schmidt CA (1999) Variation and congruence of microsatellite markers for *Peromyscus leucopus*. *Journal of Mammalogy*, **80**, 522–529.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland.

Simonsen KL, Churchill GA, Aquadro CF (1995) Properties of statistical tests of neutrality for DNA polymorphism data. *Genetics*, **141**, 413–429.

Singh RS, Kulathinal RJ (2000) Sex gene pool evolution and speciation: a new paradigm. *Genes and Genetic Systems*, **75**, 119–130.

Steiner CC, Weber JN, Hoekstra HE (2007) Adaptive variation in beach mice produced by two interacting pigmentation genes. *Public Library of Science, Biology*, **5**, 1880–1889.

Stephens M, Donnelly P (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *American Journal of Human Genetics*, **73**, 1162–1169.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics*, **68**, 978–989.

Sutton KA, Jungnickel MK, Ward CJ, Harris PC, Florman HM (2006) Functional characterization of PKDREJ, a male germ cell-restricted polycystin. *Journal of Cellular Physiology*, **209**, 493–500.

Swann CA, Cooper SJB, Breed WG (2007) Molecular evolution of the carboxy terminal region of the zona pellucida 3 glycoprotein in murine rodents. *Reproduction*, **133**, 697–708.

Swanson WJ, Nielsen R, Yang QF (2003) Pervasive adaptive evolution in mammalian fertilization proteins. *Molecular Biology and Evolution*, **20**, 18–20.

Swanson WJ, Vacquier VD (2002a) Reproductive protein evolution. *Annual Review of Ecology and Systematics*, **33**, 161–179.

Swanson WJ, Vacquier VD (2002b) The rapid evolution of reproductive proteins. *Nature Reviews Genetics*, **3**, 137–144.

Swanson WJ, Yang Z, Wolfner MF, Aquadro CF (2001) Positive Darwinian selection drives the evolution of several female reproductive proteins in mammals. *Proceedings of the National Academy of Sciences, USA*, **98**, 2509–2514.

Tajima F (1983) Evolutionary relationship of DNA sequences in finite populations. *Genetics*, **105**, 437–460.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–596.

Takebayashi N, Brewer PB, Newbigin E, Uyenoyama MK (2003) Patterns of variation within self-incompatibility loci. *Molecular Biology and Evolution*, **20**, 1778–1794.

Torgerson DG, Kulathinal RJ, Singh RS (2002) Mammalian sperm proteins are rapidly evolving: evidence of positive selection in functionally diverse genes. *Molecular Biology and Evolution*, **19**, 1973–1980.

Turner LM, Hoekstra HE (2006) Adaptive evolution of fertilization proteins within a genus: variation in ZP2 and ZP3 in deer mice (*Peromyscus*). *Molecular Biology and Evolution*, **23**, 1656–1669.

Turner LM, Hoekstra HE (in press) The evolution of reproductive proteins: Causes and consequences. *International Journal of Development Biology*.

Vekemans X, Slatkin M (1994) Gene and allelic genealogies at a gametophytic self-incompatibility locus. *Genetics*, **137**, 1157–1165.

Wagstaff BJ, Begun DJ (2005) Molecular population genetics of accessory gland protein genes and testis-expressed genes in *Drosophila mojavensis* and *D. arizonae*. *Genetics*, **171**, 1083–1101.

Wassarman PM, Jovine L, Litscher ES (2001) A profile of fertilization in mammals. *Nature Cell Biology*, **3**, E59–E64.

Watterson GA (1975) Number of segregating sites in genetic models without recombination. *Theoretical Population Biology*, **7**, 256–276.

Williams Z, Litscher ES, Jovine L, Wassarman PM (2006) Polypeptide encoded by mouse ZP3 exon-7 is necessary and sufficient for binding of mouse sperm *in vitro*. *Journal of Cellular Physiology*, **207**, 30–39.

Wyckoff GJ, Wang W, Wu CI (2000) Rapid evolution of male reproductive genes in the descent of man. *Nature*, **403**, 304–309.

This work represents part of Leslie Turner's PhD dissertation research on the evolution of male and female reproductive proteins and their potential role in reproductive isolation in the genus *Peromyscus*. She is continuing to pursue research on the genetic basis of speciation in rodents as a postdoctoral fellow. Hopi Hoekstra's research focuses on mammalian evolutionary genetics. Currently, her laboratory is interested in the genetics of adaptation (morphology, behaviour, reproduction and development) and speciation in wild populations of rodents.

Supplementary material

The following supplementary material is available for this article:

Supplementary methods: Change in classification of some samples previously identified as *Peromyscus truei* (Turner & Hoekstra 2006) to *P. gratus*.

Figure S1 Polymorphic sites in c*ytb* and *Lcat* for *Peromyscus truei* and *P. gratus*.

Table S1 *Zp3* Accession numbers

Table S2 Genetic diversity at microsatellite loci

Table S3 Genetic differentiation between *Peromyscus truei* populations

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-294X.2008.03780.x
(This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.