Molecular evolution of the carboxy terminal region of the zona pellucida 3 glycoprotein in murine rodents

Christine A Swann^{1,3}, Steven J B Cooper^{2,3} and William G Breed^{1,3}

¹School of Medical Sciences, Faculty of Health Sciences, University of Adelaide, North Terrace, Adelaide, 5005 South Australia, ²Evolutionary Biology Unit, South Australian Museum, Adelaide, South Australia and ³Centre for Evolutionary Biology and Biodiversity, University of Adelaide, Adelaide, South Australia

Correspondence should be addressed to W G Breed; Email: bill.breed@adelaide.edu.au

Abstract

In mammals, before fertilization can occur, sperm have to bind to, and penetrate, the zona pellucida (ZP). In the laboratory mouse, which has been used as a model system for fertilization studies, sperm–ZP binding has been found to be mediated by a region at the carboxy terminal, encoded by exon 7 of the *Zp3* gene. This region shows considerable interspecific sequence diversity with some evidence of adaptive evolution in mammals, suggesting that it may contribute to species-specific sperm–ZP binding. However, in a previous study of sequence diversity of ZP3 of three species of Australian murine rodents, we found an identical protein sequence of the region encoded by exon 7. Here, we expand this earlier study to determine the sequence diversity of this region in 68 out of the 130 species of Australasian murine rodents. Maximum likelihood analyses, using representatives of both New Guinean and Australian taxa, provide evidence of positive selection at three codons adjacent to, or within, the putative combining-site for sperm of ZP3, but this was not evident when the analysis was restricted to the Australian taxa. The latter group showed low levels of both intra- and inter-generic sequence divergences in the region encoded by exon 7 of *Zp3*, with little evidence that this region contributes to species specificity of sperm–ZP binding. These findings suggest that the selective forces acting on the *Zp3* exon 7 region during the evolution of the Australasian murine rodents have been variable, and that positive selection has only occurred in a few lineages.

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Introduction

The mammalian zona pellucida (ZP) is an extracellular glycoprotein matrix that surrounds the ovulated oocyte through which the spermatozoon has to pass before it can bind to, and fuse with, the cell membrane of the oocyte (Yanagimachi 1994). The ZP is largely composed of three or four glycoproteins (ZP1–4), and a model based on studies of the ZP of the laboratory mouse (*Mus musculus*, ‘the mouse’), has proposed that it is composed of filaments of alternating units of ZP2 and ZP3 cross-linked together by ZP1 (for reviews, see Green 1997, Wassarman 1999). A fourth ZP gene (*Zp4*) has been found to be expressed in the oocytes of the laboratory rat (*Rattus norvegicus*, ‘the rat’), but there is no evidence that a functional *Zp4* gene exists in the mouse (Conner *et al.* 2005). In the mouse, ZP3 (mZP3) is the smallest of the three glycoproteins and appears to be important, not only as a structural component of the matrix (Rankin *et al.* 1996), but also as the primary binding site for the spermatozoon and for the induction of the acrosome reaction (Bleil & Wassarman 1983).

Based on results from exon swapping and site-directed mutagenesis experiments (Kinloch *et al.* 1995, Chen *et al.* 1998), it has been suggested that the region encoded by exon 7, at the carboxyl terminus of the ZP3 glycoprotein, is the primary binding site for the spermatozoon (Rosiere & Wassarman 1992, Kinloch *et al.* 1995). Within this region is a short stretch of amino acids (mZP3_{328–343}, the so-called ‘combining-site for sperm’) containing a series of serine residues (Ser-329, Ser-331 to Ser-334), of which Ser-332 and Ser-334 are thought to provide O-linked glycosylation sites for primary sperm–ZP binding (Chen *et al.* 1998). By comparing this region of ZP3 of the mouse with that of a disparate group of other eutherian mammalian species, such as hamster, human, and the bonnet monkey, it was found that this region has a high level of sequence divergence when compared with the rest of the ZP3 glycoprotein (Wassarman & Litscher 1995). This finding has led to the proposal that changes in this region may result in ‘species specificity’ of sperm–ZP binding (Kinloch *et al.* 1995, Wassarman 1995,

1999, 2002, Wassarman & Litscher 1995, Wassarman *et al.* 1999, 2001, 2004a, Williams *et al.* 2003, 2006). In addition, there is evidence that this region has undergone adaptive evolution with a few of the sites being under positive selection (Swanson *et al.* 2001). Recently, when comparing the sequence of exon 7 within several species of the genera *Mus* (Jansa *et al.* 2003) and *Peromyscus* (Turner & Hoekstra 2006), a similar conclusion was reached, although it has also been suggested that the methodology underlying such conclusions may be prone to result in false positives (Berlin & Smith 2005).

In a previous study on the ZP3 glycoprotein of three endemic species of Australian murine rodents, we found that they shared an identical sequence within the region encoded by exon 7, albeit that this sequence differed from that of the mouse and rat (Swann *et al.* 2002). This study suggested that there was a lack of sequence divergence in and around the putative combining-site for sperm of ZP3, and thus contrasts with the conclusions from the studies on *Mus* (Jansa *et al.* 2003) and *Peromyscus* (Turner & Hoekstra 2006). However, a subsequent reanalysis of the data on the

Mus species, after removal of *Rattus* as the outgroup, suggested that no positive selection had occurred (Turner & Hoekstra 2006).

Here, we expand our previous study by determining the nucleotide and predicted amino acid sequence of the exons 6 and 7 regions of ZP3 in a broad range of non-*Rattus* Australasian (New Guinean and Australian 'old endemic') murine rodents. Previous molecular phylogenetic analyses suggest that this group of rodents is monophyletic and it does not appear to have any close relatives outside Australasia (Lee *et al.* 1981, Watts & Baverstock 1995, Strahan 2002). They are only distantly related to *Mus*, although are clearly part of the radiation of the Old World rats and mice (subfamily Murinae) within the family Muridae. Amongst the Australasian species at least six groupings, or divisions as defined by Musser & Carleton (2005), are recognized. They are the *Pseudomys* division which is almost entirely restricted to Australia; the *Uromys*, *Xeromys*, and *Hydromys* divisions each of which has species that occur in both Australia and New Guinea; the *Pogonomys* division that includes a disparate group of genera that are almost entirely restricted to New Guinea; and the *Lorentzimys*

Figure 1 Ancestral reconstruction of the region encoded by exon 7 of ZP3 from the New Guinean and Australasian murines. Proposed phylogeny of the New Guinean and Australasian murines, based on microcomplement fixation of albumin data (Watts & Baverstock 1995). Amino acid substitutions (single letter code) for selected residue positions for the exon 7 coding regions have been plotted against lineages, with ZP3 from *Mus musculus* used as reference. Black dots indicate those nodes where amino acid substitutions have taken place. Note the many unresolved branches, and not all genera are represented. The divisions devised by Musser & Carleton (2005) are noted in red. Under this classification, *Crossomys* and *Leggadina* (denoted with an asterisk) have been placed in the *Hydromys* and *Pseudomys* divisions respectively.

division that contains just one species restricted to New Guinea. The first four of these divisions are sometimes placed in a separate subfamily, the Hydromyinae (Watts *et al.* 1992).

The most comprehensive phylogeny of the New Guinean and Australian murines is one derived from microcomplement fixation of albumin data (see Fig. 1 from Watts & Baverstock 1995). Recently, a phylogeny of

Australian members of the Pseudomys, Uromys, and Xeromys divisions, based on the nucleotide sequence of the control region of the mitochondrial gene, has been published (see Fig. 2 from Ford 2006). In the present study, these two phylogenies have been used to test for positive selection during the evolution of the *Zp3* gene in these species using maximum likelihood analyses. In addition, extensive amino acid sequence comparisons

Figure 2 Ancestral reconstruction of the region encoded by exon 7 of ZP3 from the Australian murines. Proposed phylogeny of the Australian murine rodents, based on nucleotide sequence data from the mitochondrial control region (Ford 2006). Amino acid substitutions (single letter code) for selected residue positions for the exon 7 coding regions have been plotted against lineages, with ZP3 from *Mus musculus* used as reference only. Black dots indicate those nodes where amino acid substitutions have taken place.

have been carried out to further investigate whether this region may contribute to species specificity of sperm–ZP binding in these murines.

Materials and Methods

Nomenclature

The nomenclature of ZP genes and proteins is confusing since two different systems are in use. Simplification of this nomenclature has been proposed by Harris *et al.* (1994) and Spargo & Hope (2002). Recently, Conner *et al.* (2005) recommended that the numerical system (ZP1–ZP4) based on the size of the ZP glycoproteins when separated by one-dimensional electrophoresis, be adopted, stating that this system has been accepted by the nomenclature committee of the National Center for Biotechnology Information. The recommendation by Conner *et al.* has been followed in the present study.

Samples and extraction of DNA

A total of 68 species of New Guinean and Australian murine rodents were included in the study, representing 27 of a total of 35 genera from each of the six divisions as proposed by Musser & Carleton (2005). DNA was extracted using the PureGene DNA Isolating Kit (Gentra, Minneapolis, MN, USA) from frozen liver tissue provided by the Australian Biological Tissue Collection (ABTC) located at the South Australian Museum. Species names, common names, divisions, ABTC numbers, and GenBank accession numbers for all samples are given in Supplementary Table 1 which can be viewed online at www.reproduction-online.org/supplemental/.

PCR amplification

Primers were designed using conserved regions of exons 6 and 7 of *Zp3* cDNA from the *Spinifex* Hopping mouse (*Notomys alexis*; GenBank accession number AY078054; Swann *et al.* 2002). Forward primer sequence was ACCTGCCATCTCAAAGTCGC and reverse primer sequence was TGCGGTTTCGAGAGGTTAGC. PCR amplifications were carried out in 25 µl reaction volumes containing 1 × PCR buffer (Applied Biosystems, Foster, CA, USA), 4 mM MgCl₂, 0.8 mM dNTPs, 5 pM each primer, 2.5 µl of 1:50 diluted DNA, and 0.6 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR amplifications were carried out on a Hybaid Omni Thermocycler (Hybaid, Middlesex, UK) at 95 °C for 9 min; 34 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, and ending with a final elongation cycle at 72 °C for 6 min. PCR products were then purified using the UltraClean PCR Clean Up Kit (MoBio Laboratories, Solana Beach, CA, USA) following the manufacturer's instructions.

PCR sequencing

PCR sequencing was performed using the BigDye Sequencing Ready Reaction Kit version 3 (ABI, Perkin Elmer) in 20 µl reactions with the PCR primers as sequencing primers. The amount of DNA template used was 5 µl per reaction. Reaction products were purified using isopropanol precipitation and sequenced on an ABI 3700 Sequencing Analyser using procedures specified by the manufacturer.

Sequence analysis

Forward and reverse sequences of each species were compared and manually edited using GeneDoc software (Nicholas *et al.* 1997) and multiple sequences were then visually aligned. Where the nucleotide sequence between species was identical, repeated extractions of the same species and PCR were performed in order to verify the validity of the sequence.

For comparative purposes, the following *Zp3* cDNA sequences were extracted from GenBank: the laboratory mouse (*Mus musculus*: accession numbers NM_011775) and the laboratory rat (*Rattus norvegicus*: accession number NM_053762).

Analyses to test for evidence of positive selection

We first tested for evidence of positive selection by estimating the nonsynonymous (replacement) substitutions per site (d_N) and synonymous (silent) substitutions per site (d_S) within the exons 6 and 7 regions of *Zp3*. If d_N is less than d_S , that is the ratio of d_N/d_S (ω) < 1, the gene is considered to be evolving under stabilizing or purifying selection, whereas when $d_N = d_S$ ($\omega = 1$) the gene is evolving under neutral evolution. A higher d_N than d_S substitution rate (i.e., $\omega > 1$) is indicative of positive selection, resulting from nonsynonymous substitutions providing a fitness advantage and being fixed within the population at a higher rate than silent mutations (Yang 1998).

We estimated d_N and d_S using the Yang & Nielsen (2000) approximation method which allows for transition and base/codon frequency biases. Pairwise comparisons were conducted using the yn00 program of the Phylogenetic Analysis by Maximum Likelihood (PAML) software package (version 3.15; Yang 1997) and mean estimations were calculated over all species, within and between the divisions categorized by Musser & Carleton (2005). The ω ratio of d_N/d_S was not calculated from the d_N and d_S estimations using this method as the high percentage of $d_S = 0.000$ produced undefined d_N/d_S ratios (given as 99 in the PAML software), and would, therefore, introduce bias into the mean d_N/d_S calculations.

As the above method averaged the d_N and d_S over all codons, it is not a robust method for detecting evidence

of positive selection, particularly when it only occurs at a few codon positions, with the majority of sites under stabilizing selection. We, therefore, conducted a maximum likelihood analysis for detecting positive selection using the approach developed by Nielsen & Yang (1998). This method uses likelihood ratio tests (LRTs) to compare a null model that does not allow for $\omega > 1$ and an alternative model which does. We applied three LRTs to the data, using 'site models', rather than 'branch-site' models, as the latter is considered sensitive to small sequences, while the former is not (Yang personal communication). The first pair of models compared was the nearly neutral model (M1a) and the positive selection model (M2a; Wong *et al.* 2004). The M1a model assumes two codon site classes in proportions p_0 and $p_1 = 1 - p_0$ with $0 < \omega < 1$ and $\omega_1 = 1$. The M2a model adds a proportion (p_2) of sites with $\omega_2 > 1$ estimated from the data. The next pair of models compared was the null M7 model with the alternative model of M8. The M7 model assumes a beta distribution for ω (within the interval $0 < \omega < 1$) and the M8 model adds an extra class of sites under positive selection ($\omega_s > 1$). The third LRT compared a null model of M8 (M8a) with ω_s constrained to 1 ($\omega_s = 1$) with the M8 model with $\omega_s > 1$ (Swanson *et al.* 2003). For each LRT, the null model is nested within the alternative (positive selection) model so that a test statistic $-2\Delta \ln L$ (where $\Delta \ln L$ is the difference in log-likelihood values of the two models) follows a χ^2 distribution, with the degrees of freedom (df) equal to the difference in the number of parameters between the null and the alternative models. We, therefore, used two df for the M1a/M2a and M7/M8 LRTs and one df for the M8a/M8 LRT. The correct distribution of the latter test statistic is unknown and, hence, we chose a more conservative one df, rather than using a χ^2 distribution with a 50:50 mix of a χ^2_1 and χ^2 with point mass of 0, a suggested alternative (Wong *et al.* 2004). All tests are considered to be conservative (Yang *et al.* 2005). If the LRT is significant ($P < 0.05$) then positive selection can be inferred, and the Bayes Empirical Bayes method (Yang *et al.* 2005) was then used to calculate the posterior probability (PP) that each codon site is from a particular class of ω . Sites with $\omega > 1$ and high posterior probabilities (PP > 95%) are considered to be under positive selection.

The implementation of the above site models requires a phylogenetic tree of the species studied. Unfortunately, no published tree, based on nucleotide sequences, currently exists that contains all the taxa in the present study. We, therefore, carried out the analyses using a subset of the taxa and two phylogenies of Australasian murine rodents. The most comprehensive tree of Australasian murine rodents currently available was based on microcomplement fixation of albumin (Watts & Baverstock 1995; see Fig. 1). This tree contains representative species from most of the New Guinean and Australian old endemic genera. Secondly, we used a

recently published phylogenetic tree based on nucleotide data from the mitochondrial control region for 40 Australian species (Ford 2006; see Fig. 2). Sequences used in the analysis are highlighted in Fig. 3. *Mus* and *Rattus* data were excluded from the PAML analysis to allow an assessment of whether positive selection occurs specifically within Australasian murine lineages.

Results

After exclusion of the primer sequences, PCR amplification and sequencing generated fragments of the *Zp3* gene between 273 and 305 nucleotides in length. The partial sequences of exons 6 and 7 (171 nucleotides in length) were identified by their similarity to the corresponding cDNA sequence of the mouse and rat *Zp3* gene and by the presence of conserved intron/exon splicing signals. Intron 6 varied in length from 102 nucleotides in two *Pseudomys* species (*P. shortridgei* and *P. desertor*) to 134 nucleotides in *Pseudomys occidentalis*.

The coding sequence of exon 6/7 provided a predicted amino acid sequence of 57 residues in length, corresponding to mZP3 glycoprotein Pro-289 to Ser-345 (Fig. 3). Exon 6 of mZp3 encodes 31 amino acids, of which the last 21 residues have been determined; and exon 7 of mZp3 encodes 46 amino acids, within which the putative combining-site for sperm occurs, of which the first 36 residues have been determined in the present study.

Protein sequence comparisons

The results show that, when compared with the mouse (Boja *et al.* 2003), all five cysteine residues that are potential sites for disulfide bonding (Cys-301, Cys-320, Cys-322, Cys-323, and Cys-328) and all three potential N-linked glycosylation sites (Asn-304, Asn-327, and Asn-330) have been conserved in all species investigated.

Region encoded by exon 6 of *Zp3*

All but 9 out of the 68 species investigated have an identical amino acid sequence to that of the mouse and rat (Fig. 3). The exceptions were: in the *Uromys* division, five *Melomys* species and *Solomys salebrosus*, where there was a histidine in position 298; in the *Pogonomys* division, *P. macrourus*, with a tyrosine in position 303; and in the *Pseudomys* division, *Notomys cervinus*, with a serine residue in position 291 and *Zyzomys woodwardi* with a valine in position 290.

Region encoded by exon 7 of *Zp3*

The five serine residues (Ser-329, Ser-331 to Ser-334) identified in the mouse ZP3 (mZP3) as providing the O-linked glycosylation sites (serine/threonine residues) that could be important in sperm-ZP binding are conserved in all but seven species from the *Pseudomys* division (see below).

	mZP3	289	300	310	320	330	340	345
		*	*	*	*	*	*	*
<i>Mus musculus</i>	:	PANQIPDKLNKACSFNKTSQS	WLPVEGDADICDCSHGNC	SNSSSSQFQIHGPROWS				
<i>Rattus norvegicus</i>	:		N.....	E.ET.E.A..				
<u>Lorentzimys Division</u> (1 genera, 1 species)								
<i>Lorentzimys nouhuysi</i>	:		W.....	P.R.				
<u>Pogonomys Division</u> (13 genera, 25 species)								
<i>Anisomys imitator</i>	:		S.....	N.....	W.....	P.R.		
<i>Chiruromys vates</i>	:		S.....	D.....	W.R....	AP.R.		
<i>Coccymys ruemmleri</i>	:		S.....	D.....	W.....	P.R.		
<i>Hyomys goliath</i>	:		S.....	N.....	W.....	P.R.		
<i>Macruromys major</i>	:		S.....	D.....	W.....	LP.R.		
<i>Mallomys aroaensis</i>	:		S.....	D.....	W.....	P.R.		
<i>Mallomys rothschildi</i>	:		S.....	D.....	W.....	P.R.		
<i>Mammelomys lanosus</i>	:		S.....	A.....	WL.....	P.R.		
<i>Mammelomys rattoides</i>	:		S.....	A.....	WL.....	P.R.		
<i>Pogonomelomys mayeri</i>	:		S.....	D.....	W.....	LP.R.		
<i>Pogonomys macrourus</i>	:		Y.....	S.....	RE.....	W.....	P.R.	
<u>Hydromys Division</u> (5 genera, 11 species)								
<i>Crossomys moncktoni</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Hydromys chrysogaster</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Parahydromys asper</i>	:		S.....	D.....	WS.....	SP.R.		
<u>Xeromys Division</u> (3 genera, 8 species)								
<i>Leptomys elegans</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Pseudohydromys ellermani</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Xeromys myoides</i>	:		S.....	D.....	WS.....	SP.R.		
<u>Uromys Division</u> (5 genera, 46 species)								
<i>Melomys burtoni</i>	:		H.....	S.....	D.....	WS.....	AP.R.	
<i>Melomys capensis</i>	:		H.....	S.....	D.....	WS.....	AP.R.	
<i>Melomys cervinipes</i>	:		H.....	S.....	D.....	WSR....	AP.R.	
<i>Melomys rubicola</i>	:		H.....	S.....	D.....	WS.....	SP.R.	
<i>Melomys rufescens</i>	:		H.....	S.....	RD.....	WS.....	SP.R.	
<i>Paramelomys levipes</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Paramelomys platyops</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Paramelomys rubex</i>	:		S.....	D.....	WS.....	SS.R.		
<i>Solomys salebrosus</i>	:		H.....	S.....	RD.....	WS.....	SP.R.	
<i>Uromys anak</i>	:		S.....	S.....	ID.....	WS.....	SP.R.	
<i>Uromys caudimaculatus</i>	:		S.....	ID.....	WS.....	SP.R.		
<u>Pseudomys Division</u> (8 genera, 39 species)								
<i>Conilurus penicillatus</i>	:		S.....	ID.....	TWS.....	SP.R.		
<i>Leggadina forresti</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Leggadina lakedownensis</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Leporillus conditor</i>	:		S.....	ID.....	WS.....	SP.R.		
<i>Mesembriomys gouldii</i>	:		S.....	TD.....	WS.....	SP.R.		
<i>Mesembriomys macrurus</i>	:		S.....	TD.....	WS.....	SP.R.		
<i>Mastacomys fuscus</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Notomys alexis</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Notomys aquilo</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Notomys cervinus</i>	:	S.....	W.....	D.....	WS.....	SP.R.		
<i>Notomys fuscus</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Notomys mitchellii</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys albocinereus</i>	:		W.....	D.....	L.....	WS.....	SP.R.	
<i>Pseudomys apodemoides</i>	:		W.....	D.....	L.....	WS.....	SP.R.	
<i>Pseudomys australis</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys bolami</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys calabyi</i>	:		W.....	D.....	PWS.....	SP.R.		
<i>Pseudomys chapmani</i>	:		W.....	D.....	PWS.....	SP.R.		
<i>Pseudomys delicatulus</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys desertor</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys fieldi</i>	:		W.....	N.....	WS.....	SP.R.		
<i>Pseudomys fumeus</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys gracilicaudatus</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys hermannsburgensis</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys higginsii</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys johnsoni</i>	:		W.....	D.....	PWS.....	SP.R.		
<i>Pseudomys laborifex</i>	:		W.....	D.....	PWS.....	SP.R.		
<i>Pseudomys nanus</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys novaehollandiae</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys occidentalis</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys oralis</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys patrius</i>	:		W.....	D.....	PWS.....	SP.R.		
<i>Pseudomys pilligaensis</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Pseudomys shortridgei</i>	:		W.....	D.....	WS.....	SP.R.		
<i>Zyzomys argurus</i>	:		S.....	D.....	WS.....	SP.RY		
<i>Zyzomys maini</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Zyzomys palatilis</i>	:		S.....	D.....	WS.....	SP.R.		
<i>Zyzomys pedunculatus</i>	:		S.....	N.....	D.....	WS.....	SP.R.	
<i>Zyzomys woodwardi</i>	:	V.....	S.....	D.....	WS.....	SP.RF		

Lorentzimys division. *Lorentzimys nouhuysi* is the only species in this division. Three amino acid changes have occurred between the ZP3 sequence from *L. nouhuysi* and the mouse. *L. nouhuysi* has a tryptophan in position 335 (mZP3: Gln-335), a proline in position 342 (mZP3: Arg-342), and an arginine in position 344 (mZP3: Trp-344). None of the amino acid differences that exist between the mouse and the rat are present in the exon 7 region of ZP3 of *L. nouhuysi* (Fig. 3).

Pogonomys division. The Pogonomys division is composed of 23 extant species (13 genera) of which we have determined the predicted amino acid sequence for 11 species from 9 of the genera. These species differ from the mouse in having Trp-335, Pro-342, and Arg-344. In addition, all species have a serine in position 311 (mZP3: Leu-311). The two species of *Mammelomys* have an identical amino acid sequence to each other and two species of *Mallomys* have an identical amino acid sequence to that of *Coccymys ruemmleri*, albeit different from *Mammelomys*. *Anisomys imitator* and *Hyomys goliath* also share an identical amino acid sequence. Within the ten amino acid residues stretch from 335 to 344, 54% (6 out of 11) of species in this division share an identical sequence with each other as well as to that of *L. nouhuysi*, although they differ at these sites from the mouse.

Hydromys and Xeromys divisions. Of the 11 extant species in the Hydromys division and 8 species in the Xeromys division, we determined the predicted amino acid sequence for three species from three genera in both of the two divisions. In all six species, we found an identical sequence. They all share Ser-311, Trp-335, Pro-342, and Arg-344 with species in the Pogonomys division. In addition, all six species have an aspartic acid in position 325 (mZP3: His-325) and serine residues in positions 336 and 341 (mZP3: Phe-336 and Pro-341).

Uromys division. The Uromys division consists of 46 extant species from five genera and we have determined the predicted amino acid sequence from 11 of these species from four genera. All species in this division have, in common with the Hydromys and Xeromys division species, Ser-311, Asp-325, Trp-335, Ser-336, Pro-342 (with one exception being *Melomys burtoni*), and Arg-344. In addition, in common with species in the Hydromys and Xeromys divisions, all but three *Melomys* species have a serine residue in position 341. Of the 23 extant *Melomys* species, we have determined the amino acid sequence in three Australian species (*M. burtoni*, *M.*

capensis, and *M. cervinipes*), one species from New Guinea (*M. rufescens*), and the fifth, *M. rubicola*, from Bramble Cay, a Torres Strait island. The three Australian *Melomys* species all have an alanine residue in position 341, which is not present in the other two species of this genus where a serine occurs.

The genus *Paramelomys* consists of nine species, all endemic to New Guinea, of which we have determined the amino acid sequence for three species. *Paramelomys rubex* has a serine in position 342, with all other species having a proline in this position. *Solomys salebrosus*, which is one out of five extant *Solomys* species endemic to the Solomon Islands, has an identical amino acid sequence to *Melomys rufescens* with the two species sharing an arginine in position 324, albeit that the majority of the Australasian murines have a serine in this position. Of the eight extant *Uromys* species, we have determined the amino acid sequence from two, one of which, *Uromys anak* occurs only in New Guinea and the other, *U. caudimaculatus*, occurs in both north-east Australia and New Guinea. *U. anak*, uniquely, has a serine in position 316, while other species, including the mouse and rat, have a glutamic acid in this position. Both *Uromys* species share the common residues with the other members of this division in addition to having an isoleucine residue at position 324, rather than a serine. Within the ten amino acid stretch, from residue 335 to 344, 64% of species from the Uromys division (7 out of 11) shared an identical amino acid sequence.

Pseudomys division. The Pseudomys division comprises 39 extant species, at present allocated to eight genera and we have determined the amino acid sequence from all these species. All species have retained the common Trp-335, Pro-342, and Arg-344, which are also shared with species in the other divisions, but not with mouse and rat. In addition, all species have the two additional serine residues at positions 336 and 341 in common with all six species from the Xeromys and Hydromys divisions, and most species from the Uromys division. Furthermore, all but *Pseudomys fieldi* have an aspartic acid in position 325, in common with all species from Xeromys, Hydromys, and Uromys divisions. All species investigated in the large genus of *Pseudomys*, together with five species of *Notomys* and *Mastacomys fuscus*, have a tryptophan in position 311. All other species in this division (from the genera of *Conilurus*, *Leggadina*, *Leporillus*, *Mesembriomys*, and *Zyzomys*), together with species from the Lorentzimys,

Figure 3 Alignment of the predicted amino acid sequence for the region encoded by the partial exons 6 and 7 of *Zp3* for New Guinean and Australasian endemic rodent species respectively. Species are grouped under the divisions defined by Musser & Carleton (2005). In parenthesis, next to each division, are the number of genera per division, and the number of extant species (according to Musser & Carleton (2005)). Amino acid residues are numbered according to the corresponding mouse ZP3. The exon 6/exon 7 boundary is indicated by the space between residues 309 and 310. The putative combining-site for sperm, identified by Wassarman & Litscher (1995), is highlighted in grey. Each amino acid is represented by its single letter code: A: (Ala) alanine, C: (Cys) cysteine, D: (Asp) aspartic acid, E: (Glu) glutamic acid, F: (Phe) phenylalanine, G: (Gly) glycine, H: (His) histidine, I: (Ile) isoleucine, K: (Lys) lysine, L: (Leu) leucine, M: (Met) methionine, N: (Asn) asparagine, P: (Pro) proline, Q: (Gln) glutamine, R: (Arg) arginine, S: (Ser) serine, T: (Thr) threonine, V: (Val) valine, W: (Trp) tryptophan, Y: (Tyr) tyrosine.

Pogonomys, Hydromys, Xeromys, and Uromys divisions, have a serine in position 311.

At residue position 324, *Conilurus penicillatus* and *Leporillus conditor* have an isoleucine in common with two *Uromys* species, whereas the two *Mesembriomys* species have a threonine at this position (Fig. 3). The majority of species also retained the five serine residues (Ser-329, Ser-331 to Ser-334) found in the mouse and rat amino acid sequence. Of the 22 species of *Pseudomys* investigated two sibling taxa, *P. albocinereus* and *P. apodemoides*, have a leucine in position 331 and five species collectively known as pebble-mound *Pseudomys* (*P. calabyi*, *P. chapmani*, *P. johnsoni*, *P. laborifex*, and *P. patrius*) have a proline in position 334. In addition, *Conilurus penicillatus* has a threonine in this position. Hence, the five potential O-linked glycosylation sites (serine/threonine residues) conserved in the *Mus*, *Rattus*, *Lorentzimys*, *Pogonomys*, *Hydromys*, *Xeromys*, and *Uromys* divisions have been conserved in all but six of the *Pseudomys* division species (Fig. 1).

The five *Notomys* species all share an identical amino acid sequence within the exon 7 coding region, in common with the single *Mastacomys* species and 64% of species from the *Pseudomys* genus. Within the ten amino acid stretch from residue 335 to 344, all 39 species share the same identical sequence.

Rates of nonsynonymous and synonymous substitutions per site

Exon 6

Over all six divisions, the mean d_s for exon 6 was 0.041 and d_N was 0.006 (Table 1). Within each division, d_s was always higher than d_N , suggesting that exon 6 is evolving under selective constraints (Table 1). Between divisions, the highest mean pairwise comparison of d_N was 0.014 between the *Pogonomys* and the *Uromys* divisions. Mean pairwise estimations of d_s ranged from 0.016 between the *Lorentzimys* and the *Uromys* divisions to 0.057 between the *Pogonomys* and the *Hydromys* divisions (Table 1).

Exon 7

Over all divisions, the mean pairwise estimate of d_N for exon 7 was 0.031 (Table 1). The mean pairwise estimate of d_s was 0.037, a rate similar to that observed for the coding region of exon 6. Within all divisions, mean pairwise d_N estimates were lower than d_s estimates, as expected for a sequence under purifying selection (Table 1). However, comparisons between divisions showed a number of cases, where d_N was higher than d_s (e.g., *Xeromys* versus *Lorentzimys* and *Pogonomys*; *Uromys* versus *Lorentzimys*; *Pseudomys* versus *Lorentzimys*; and *Pogonomys*), suggesting some evidence for

Table 1 Mean estimated pairwise comparisons of nonsynonymous substitutions per nonsynonymous site (d_N , in bold) and synonymous substitutions per synonymous site (d_s) within and between each of the New Guinean and Australasian murine divisions (according to Musser & Carleton, 2005) for exon 6 (A) and exon 7 (B) coding region of *Zp3*.

		Between					
	Within	Lorentzimys	Pogonomys	Hydromys	Xeromys	Uromys	Pseudomys
(A) Exon 6: Overall, mean d_N =0.006, d_s =0.041							
Lorentzimys	d_N						
	d_s						
Pogonomys	d_N	0.004	0.002				
	d_s	0.042	0.022				
Hydromys	d_N	0.000	0.000	0.002			
	d_s	0.076	0.038	0.057			
Xeromys	d_N	0.000	0.000	0.002	0.000		
	d_s	0.038	0.038	0.053	0.051		
Uromys	d_N	0.012	0.012	0.014	0.012	0.012	
	d_s	0.034	0.016	0.037	0.049	0.044	
Pseudomys	d_N	0.002	0.001	0.003	0.001	0.001	0.013
	d_s	0.041	0.023	0.043	0.053	0.046	0.036
(B) Exon 7: Overall, mean d_N =0.031, d_s =0.037							
Lorentzimys	d_N						
	d_s						
Pogonomys	d_N	0.029	0.037				
	d_s	0.049	0.062				
Hydromys	d_N	0.000	0.055	0.042			
	d_s	0.041	0.055	0.050			
Xeromys	d_N	0.000	0.055	0.042	0.000		
	d_s	0.000	0.033	0.028	0.020		
Uromys	d_N	0.025	0.065	0.051	0.014	0.014	
	d_s	0.043	0.037	0.055	0.043	0.025	
Pseudomys	d_N	0.017	0.060	0.058	0.016	0.015	0.029
	d_s	0.030	0.049	0.041	0.036	0.015	0.041

positive selection acting on the exon 7 region for these lineages. This is particularly evident when mean pairwise comparisons of d_N are made between the New Guinean divisions of *Lorentzimys* and *Pogonomys* and the other four divisions.

LRTs of positive selection

To further assess the evidence for positive selection using a statistical approach, we conducted LRTs on two sets of data: *Lorentzimys* and *Pogonomys* divisions with some representative species from the other divisions of *Hydromys*, *Xeromys*, *Uromys*, and *Pseudomys*, using the [Watts & Baverstock \(1995\)](#) phylogeny ([Fig. 1](#)); and Australian species only, using the [Ford \(2006\)](#) mitochondrial control region phylogeny ([Fig. 2](#)). Results are shown in [Table 2](#).

LRTs, using representative species from all six divisions (using the [Watts & Baverstock 1995](#) phylogeny), provided strong evidence of positive selection ([Table 2A](#)). In all three tests, the null model of no positive selection was rejected ($P < 0.01$) in favor of the alternative model, i.e., the model of positive selection fits the data significantly better than the null model. Models M2a and M8 identified 12% of codons as being under strong positive selection ($\omega_s = 3.57$). Of these seven codon sites, two amino acid sites, mZP3-324 and mZP3-325, gave a PP > 95% for all models, with a third site identified only under the M8 model (mZP3-341)

with a PP > 95%. All three sites are within the exon 7 coding region, although only the first two are close to, but not within, the sequence of residues identified by [Wassarman & Litscher \(1995\)](#) as the combining-site for sperm. Position 324 contains a serine residue in the majority of species, but this residue was replaced with an arginine in one species in the *Pogonomys* division, and with either an arginine (two species) or an isoleucine (two species) within the *Uromys* division. This common serine residue was substituted with either an isoleucine residue (two species) or a threonine (two *Mesembriomys* species) within the large *Pseudomys* division. The second site identified as being under positive selection, position 325, changed from an aspartic acid in the majority of species to either an asparagine, alanine, or glutamic acid within the *Pogonomys* division, but within other divisions, only one species did not have an aspartic acid in this position. Residue mZP3-341 contains a proline in the majority of *Lorentzimys* and *Pogonomys* division species, with substitutions to an alanine or a leucine occurring. Within the other four divisions, the majority of species, including the New Guinea *Melomys* species, have a serine in this position with three Australian *Melomys* species having an alanine. Each of the above three sites shows parallel site changes, where the same amino acid has independently evolved in different rodent lineages ([Fig. 3](#)).

The LRTs using a phylogeny containing 40 species from three divisions of mainly Australian species

Table 2 Likelihood ratio tests of models of codon substitution using PAML (version 3.15).

$2\Delta\ell$									
Mod- el	ℓ	M1a-M2a	M7-M8	M8a-M8	df	$P(\chi^2)$	ω for each branch	Parameter estimates	Positively selected sites (PP>95%, PP> 99%*)
(A) New Guinea and Australasian species (using combined phylogenetic tree of Watts & Baverstock (1995) derived from microcomplement fixation of albumin)									
M1a	-487.39						0.22	$p_0=0.79, p_1=0.21, \omega_0=0.011, \omega_1=1$	None
M2a	-482.69	9.4			2	0.009	0.49	$p_0=0.88, p_2=0.12, \omega_0=0.06, \omega_1=1, \omega_2=3.57$	324S, 325H
M7	-487.45						0.21	$p=0.005, q=0.018$	None
M8	-482.71		9.48		2	0.008	0.49	$p=6.43, q=99, p_0=0.88, p_s=0.12, \omega_s=3.57$	324S*, 325H*, 341P
M8a	-487.39			9.36	1	0.002	0.22	$p=1.15, q=99, p_0=0.79, p_s=0.21, \omega_s=1$	None
(B) Australasian species only (using phylogenetic tree, based on data derived from the mitochondrial control region Ford (2006))									
M1a	-458.67						0.22	$p_0=0.85, p_1=0.15, \omega_0 \& 262 \neq 0.076, \omega_1=1$	None
M2a	-458.66	0.02			2	0.99	0.22	$p_0=0.87, p_2=0.13, \omega_0=0.08, \omega_1=1, \omega_2=1.13$	None
M7	-458.83						0.21	$p=0.18, q=0.66$	None
M8	-458.66		0.34		2	0.84	0.22	$p=8.20, q=99$	None
M8a	-458.67			0.02	1	0.88	0.22	$p_0=0.87, p_s=0.13, \omega_s=1.13$ $p=8.30, q=99$ $p_0=0.85, p_s=0.15, \omega_s=1$	None

Notes: In the above tables, ℓ , log likelihood ratio: $2\Delta\ell$ ($-2\Delta\ln L$)=twice the difference between log likelihood ratios; df, degrees of freedom; $P(\chi^2)=P$ values of LRT under χ^2 distribution, significant values (<0.05); $\omega = d_N/d_S$, for each branch, calculated under each model; p , parameters; PP, posterior probability, calculated under Bayes Empirical Bayes method.

(Xeromys, Uromys, and Pseudomys divisions; Ford 2006), failed to show support for positive selection occurring within this sequence. All three LRTs consistently failed to reject the null model in favor of the positive selection model ($P > 0.05$). The average ω value across the tree was either 0.21 or 0.22 and, in models allowing for a proportion of sites to have $\omega > 1$, the parameter estimate for ω was 1.13, which is close to the ratio expected under a neutral model of evolution. In addition, only one residue (mZP3-334) was identified as being under possible positive selection, but the Bayesian PP was $< 70\%$ (Table 2B).

Discussion

In the mouse, there is good evidence that a region encoded by exon 7 of the *Zp3* gene of the extracellular ZP complex acts as the primary binding site for spermatozoa (Kinloch *et al.* 1992, 1995, Rosiere & Wassarman 1992, Liu *et al.* 1995, Wassarman *et al.* 1997, Chen *et al.* 1998, Williams *et al.* 2003, 2006) and cross-species comparisons, using a disparate group of mammals from several orders, have shown that this region of the *Zp3* gene exhibits a high degree of sequence divergence and evidence of adaptive evolution (Swanson *et al.* 2003). This has led, in part, to the suggestion that in mammals, this sequence divergence may contribute to species specificity of sperm–ZP binding (Kinloch *et al.* 1995, Wassarman 1995, 1999, 2002, Wassarman & Litscher 1995, Wassarman *et al.* 1999, 2001, 2004a, Williams *et al.* 2003, 2006) and that some of the sites either in, or close to, this region are under positive selection (Swanson *et al.* 2001, Swanson & Vacquier 2002, Jansa *et al.* 2003, Turner & Hoekstra 2006).

In the present study, we have used the old endemic rodents of New Guinea and Australia to determine the extent of the divergence of exons 6 and 7 of *Zp3*. We have found evidence for positive selection within exon 7, but not exon 6, of the *Zp3* gene for species within this clade. The amino acid sites where positive selection was found to have occurred (324, 325, and 341) are close to, but not identical with, those found to be under positive selection in the studies using species of *Mus* by Jansa *et al.* (2003) and *Peromyscus* by Turner & Hoekstra (2006). Two of the sites (324 and 325) are located just outside, and the third site (341) within the combining-site for sperm as defined by Wassarman & Litscher (1995). Each of these sites also showed evidence of parallel or convergent evolution of a number of amino acid changes, a finding that can be interpreted as further evidence for adaptive evolution (Briscoe 2001).

Evidence for positive selection was also suggested when the average d_N and d_S values were compared, with mean d_N values greater than d_S for pairwise comparisons between species in the two New Guinean divisions, Lorentzimys and Pogonomys, and those in the other divisions. However, when a maximum likelihood

analysis was performed on data from species in the Hydromys, Xeromys, Uromys, and Pseudomys divisions, no evidence of positive selection was found for either exons 6 or 7. This finding is, perhaps, not too surprising in the light of the fact that there are only a few amino acid differences in the sequence of ZP3 for these species. For example, we found an identical amino acid sequence for the region encoded by exon 6 as well as exon 7 of *Zp3* in all six species in the Hydromys and Xeromys divisions, three species in the Uromys division, and in four of the species in the Pseudomys division. Average d_N was lower than d_S in all these groups.

The above findings suggest that positive selection of exon 7 may have only taken place in the lineages leading to extant taxa within the Pogonomys and Lorentzimys divisions and has probably not occurred during the evolution of the exon 7 region of *Zp3* in the other lineages. A failure to detect evidence for positive selection among the Australian taxa could possibly be due to a lack of power of the analyses when closely related species are compared and/or due to low sequence variation (Anisimova *et al.* 2001). However, mean pairwise d_S values between these divisions (0.03) were only marginally less than that of the overall mean (0.037) of d_S between all the divisions, including the New Guinea lineages. In contrast to our findings, the recent study of the *Zp3* gene of *Peromyscus* species by Turner & Hoekstra (2006) showed considerable amino acid sequence variation in the region encoded by exon 7 both between, and even within, the species they studied. Amino acid variation was also found to vary across lineages with some showing multiple substitutions, whilst other lineages had comparable synonymous divergence levels with little, or no, amino acid variation (Turner & Hoekstra 2006). The latter result is similar to our finding of lineage-specific differences using the Australasian murine rodents.

Although there is ongoing debate, as to whether the spermatozoon binds either to the oligosaccharides on the ZP3 (Wassarman *et al.* 2004a, 2004b, Williams *et al.* 2006) or to the supramolecular structure of the ZP matrix (Dean 2003, Rankin *et al.* 2003, Hoodbhoy & Dean 2004, Clark & Dell 2006) in the mouse, there is strong evidence that the region encoded by exon 7 of mZp3 is involved in sperm–ZP binding. Changes to the primary structure adjacent to the combining-site for sperm may alter ionic interactions and/or glycosylation patterns, and hence affect sperm-binding capacity of this molecule. It is possible that, as a result of divergence in the amino acid sequence, the sperm–ZP binding region becomes refractory to the binding of sperm from other species regardless of how this takes place. To prevent cross-species fertilizations, sequence divergence in this region might occur between closely related species that are present in sympatry in parts of their ranges. However, our data

have shown an identical amino acid sequence in the region encoded by exon 7, including the residues 335–345, in three pairs of such species in the Australian *Pseudomys* division: those of *Notomys alexis* and *N. mitchelli*, *Pseudomys hermannsburgensis* and *P. bolami*, and the two *Leggadina* species. This finding suggests that, at least within this group of Australian old endemic rodents, positive selection for divergence of exon 7 of *Zp3* has not taken place in spite of the occurrence of sympatry between these pairs of species.

Swanson *et al.* (2003) have suggested that, where divergence of reproductive proteins has taken place, this may have been driven by either sperm conflict and/or sperm competition. In comparative studies of eutherian mammals, relative testis size has been shown to relate to the intensity of potential intermale sperm competition with species that have a polyandrous mating system, and hence exhibiting intense sperm competition, having larger relative testes mass than those where this is not the case (Harcourt *et al.* 1981, Kenagy & Trombulak 1986). The Australian endemic rodents have a very large range of relative testes sizes across species. For instance, within the *Pseudomys* division, most *Notomys* have relative testes masses of only 0.1–0.2%, and in *P. shortridgei* and *P. novaehollandiae*, it is 0.35–0.45% of body mass. By contrast, in other *Pseudomys* species, for example, *P. australis*, *P. desertor*, *P. fumeus*, *P. nanus*, and *Mastacomys fuscus*, relative testis mass is between 1.4 and 3.6% of body weight (Breed & Taylor 2000). In the present study, a comparison of exons 6 and 7 coding regions of these species does not show any association between testis size and variation in the combining-site for sperm of the ZP3 glycoprotein. These comparative observations support the recent conclusion from *Peromyscus* (Turner & Hoekstra 2006) that there is not an association between adaptive evolution of *Zp3* and the potential for intermale sperm competition.

In conclusion, our data provide independent support from the old endemic Australasian murine rodents that positive selection occasionally takes place in the exon 7 region of the *Zp3* gene. That this region of *Zp3* has undergone adaptive evolution adds further support to the conclusions based on data from *Mus* and *Peromyscus*. However, within the Australasian murines, the occurrence of positive selection appears to be restricted to just a few lineages. Most of these species share an identical amino acid sequence between closely related species with no evidence of positive selection having taken place. This low rate of sequence divergence within the lineages of the Australasian murine rodents suggests that it is unlikely that divergence in this region has contributed to species specificity of sperm–ZP binding, if it exists, in these animals.

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