

Negative Purifying Selection Drives Prion and Doppel Protein Evolution

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Abstract The prion protein (PrP) when misfolded into the pathogenic conformer PrP^{Sc} is the major causative agent of several lethal transmissible spongiform encephalopathies in mammals. Studies of evolutionary pressure on the corresponding gene using different datasets have yielded conflicting results. In addition, putative PrP or PrP interacting partners with strong similarity to PrP such as the doppel protein have not been examined to determine if the same evolutionary mechanisms apply to prion paralogs or if there are coselected sites that might indicate how and where the proteins interact. We examined several taxonomic groups that contain model organisms of prion diseases focusing on

primates, bovids, and an expanded dataset of rodents for selection pressure on the prion gene (*PRNP*) and doppel gene (*PRND*) individually and for coevolving sites within. Overall, the results clearly indicate that both proteins are under strong selective constraints with relaxed selection on amino acid residues connecting α -helices 1 and 2.

Keywords Prion · Doppel · *PRNP* · *PRND* · Purifying selection · Interacting sites

Introduction

The prion protein (PrP) is the causal factor in a range of transmissible spongiform encephalopathies (TSEs) including Creutzfeldt–Jakob Disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease (CWD) in wild cervids. Common to all prion diseases regardless of affected species

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is the misfolding of the PrP into a proteinase and heat resistant conformer PrP^{Sc}. Accumulation of this misfolded conformer leads to severe neurodegeneration by an unknown mechanism (Kraus et al. 2013).

The prion gene (*PRNP*) belongs to a gene family with several known members widespread in vertebrates including the prion gene itself and two conserved genes derived from duplications of the prion gene, *doppel* (*PRND*) and *shadoo* (*SPRN*) (Premzl and Gamulin 2007). Although exhibiting different but partially overlapping expression profiles, only *PRNP* and *SPRN* are expressed in the central nervous system (CNS) in healthy animals. Mis-expression of *PRND* is neurotoxic in mouse neuroblastoma cell lines (Silverman et al. 2000). *Shadoo* appears to be down-regulated during prion diseases suggesting potential interaction with PrP (Westaway et al. 2011). Similarly, it has been suggested that PrP and Dpl interact as agonists with PrP preventing Dpl neurotoxicity (Sakaguchi 2008). Specific deletions of *PRNP* result in its inability to prevent Dpl neurotoxicity (Sakaguchi 2008). Further evidence for an interaction is that PrP and Dpl coprecipitate from detergent resistant membrane domains of some cell types from rats (Caputo et al. 2010).

Evolutionary analyses of *PRNP* have demonstrated conflicting results, with the prion gene being under balancing (Mead et al. 2003), purifying (Seabury et al. 2004), and positive selection (Premzl and Gamulin 2009) depending on the dataset. A recent study including representatives of multiple taxa suggested that positive selection was acting on PrP in various domains and intradomains (Premzl and Gamulin 2009). However, the selection analysis to date has been done on either, relatively small datasets, species specific or order specific data sets and only on the *PRNP* gene itself and not its paralogs.

To investigate the interspecific evolution of *PRNP* and *PRND*, we examined lagomorph, eulipotyph, primate, and bovid sequences from GenBank along with 19 *PRNP* and 21 *PRND* sequences from 10 rodent genera and 21 rodent species produced in this study. The bovids, primates, and rodents in particular include animals susceptible to prion diseases, model organisms in prion research or both. Different models were applied to investigate selective pressure on *PRNP* and *PRND* individually and to identify coevolving sites that might indicate interaction sites in the proteins relative to their structure.

Materials and Methods

Laboratory Procedures

Twenty-one rodent samples from Europe and Southeast Asia were used for this study. The Southeast Asian samples were collected as part of a larger project involving authors S. Morand and J. Michaux (CERoPath project, “Community

Ecology of Rodents and their Pathogens in a changing environment,” <http://www.ceropath.org>). *Apodemus* samples were collected by the Conservation Genetics Unit of the University of Liège. The *Myodes glareolus* sample KS 10/1240 was trapped in October 2009 in Lower Saxony/Germany. DNA extractions for all the rodent tissue or blood samples were performed using the DNeasy Blood & Tissue DNA extraction kit (QIAGEN) following the manufacturer’s protocol. Polymerase chain reaction for the *PRNP* and *PRND* genes was performed in 25 µl reactions containing 0.5 U of MyTaq HS polymerase mix (Bioline), 200 nM primers, and 110 ng of DNA template using the following forward (F) and reverse (R) primer sets PrP_F1 (5′-GTTC(C/T)TCATT TTGCAGATCA-3′), Dpl_F1 (5′-CTTCCCTTGCAGATT CACC-3′), Dpl_R1 (5′-TCACCTCTGTGGCTGCCAGC-3′), Dpl_F2 (5′-CGCCCAGGAGCCTT(C/T)ATCAARC-3′), Dpl_R2 (5′-CACAAAT(G/A)AACCAAA(C/T)GAAAC C(C/T)AGCAG-3′), Dpl_F3 (5′-CCATGAAGAACCG (G/T)(C/G)TGG-3′), and Dpl_R3 (5′-CTC(C/T)GANGCC AA(C/T)GTGAC-3′). Thermocycling conditions were 95 °C denaturation for 3 min followed by 35 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 25 s, with a final extension of 72 °C for 2 min. Positive PCR amplification products were purified using the QIAquick PCR purification kit (QIAGEN), and sequenced using BigDye chemistry on a 3730 DNA Analyzer (Applied Biosystems). The existence of heterozygotes was examined by inspecting the traces for multiple peaks at a single position. For the species *Bandicota savilei*, and *Rattus argentiventer* several (up to three) individual rodents from different populations were available to be sequenced and identical sequences obtained and, though not conclusive for all species, suggested that intraspecific polymorphism did not affect the results.

Sequence Alignment, Phylogenetic, and Selection Analyses

PRNP and *PRND* GenBank accession codes were as follows: *Mus cervicolor* (KF466919, KF466939), *Mus cookii* (KF466920, KF466940), *Mus caroli* (KF466921, KF466941), *Mus fragilicauda* (KF466956, KF466957), *Rattus losea* (KF466924, KF466954) *Rattus argentiventer* (KF466955, KF466925), *Rattus nitidus* (KF466938, KF466952), *Apodemus sylvaticus* (KF466922), *Apodemus fulvipectus* (KF466923, KF466953), *Apodemus mystacinus* (KF466942, KF466935), *Leopoldamys sabanus* (KF466928, KF466943), *Leopoldamys edwardsi* (KF466944, KF466929), *Bandicota indica* (KF466930, KF466950), *Bandicota savilei* (KF466951, KF466931), *Saxatilomys paulinae* (KF466937, KF466958), *Chiropodomys gliroides* (KF466945, KF466932), *Berylmys berdmorei* (KF466936, KF466946), *Maxomys surifer* (KF466947, KF466933), *Myodes glareolus* (KF466934).

Nucleotide sequences were aligned in TranslatorX (Abascal et al. 2010) using MAFFT 7 (Katoh and Standley 2013) and inspected for coding frame-disrupting substitutions. Alignments can be found in the supplemental materials. The phylogenetic relationships among orthologs across species were estimated in a maximum likelihood (ML) framework in the POSIX-Threads built of RAxML 8.0.2 (Stamatakis 2006). Nucleotide sequences were examined with the GTR + Γ_4 (Lanave et al. 1984; Yang 1993) substitution model. Ten maximum likelihood (ML) searches were run starting from a maximum parsimony stepwise-addition tree. Internode branch robustness was evaluated through 500 parametric bootstrap pseudoreplicates (Felsenstein 1981).

The signature of natural selection on both gene sequences was examined using three probabilistic methods implemented in HyPhy 2.1.2 (Pond et al. 2005) and the Datamonkey webserver (<http://www.datamonkey.org>; (Delpont et al. 2010); mixed effects model evolution (MEME)—an extension of the fixed effects likelihood method (Kosakovsky Pond and Frost 2005) allowing ω (= dN/dS) to vary along the phylogeny branches (Murrell et al. 2012), Fast Unconstrained Bayesian Approximation (FUBAR)—a new empirical Bayes method for estimating codon-wise trends of negative or positive selection (Murrell et al. 2013), and branch-site random effects likelihood (BSREL)—a “branch-site” method for detecting the branches on which a proportion of codons evolve with $\omega > 1$ (Kosakovsky Pond et al. 2011). MEME is capable of detecting episodic positive selection, especially when these instances are located on a small portion of the tree branches, meaning that it can detect positive selection in the overwhelming presence of negative selection (Murrell et al. 2012). FUBAR is more robust to model mis-specification and also orders of magnitude faster in terms of algorithmic implementation (Murrell et al. 2013). Furthermore, we employed the mechanistic empirical model (MEC) (Doron-Faigenboim and Pupko 2007) that accounts for the different amino acid replacement probabilities based on the JTT empirical substitution matrix (Jones et al. 1992), while estimating the codon rate matrix, thus allowing for positions undergoing radical amino acid exchanges to acquire higher dN rates than those with less radical exchanges. The codon-wise ω estimates were mapped onto predicted protein tertiary structures deposited in PDB (<http://www.pdb.org>; human prion protein (PrP), PDB ID: 1QLZ; human Doppel protein (Dpl), PDB ID: 1LG4) using the Selecton-3D web server (<http://selecton.tau.ac.il>).

We examined the possibility of natural selection driving the non-independent evolution of codons and amino acid sites at the intra- and inter-molecular level. We adopted a protein primary structure spatial approach by searching for amino acid residues that show evidence of concerted evolution in CAPS as detailed in (Fares and McNally 2006).

First, we looked for residues that appear to be linked on the protein structure or potentially functionally as a result of natural selection (Fares and Travers 2006) using the PDB tertiary structures. After searching for coevolving sites within proteins, we contrasted the two proteins. Significance was assessed via random resampling of the correlation coefficients for residue pairs sampled from the alignment (100,000 pseudoreplicates, $\alpha = 0.001$), and the amino acid sequence distances among taxa was Poisson-corrected. Groups of coevolving residues were set at a maximum of 3–5 % of the total alignment length. Protein structures were plotted in Jmol 13.0 (<http://www.jmol.org>) and network relationships among coevolved residues were mapped in Cytoscape 3.0 (<http://www.cytoscape.org>).

Results

Prion Protein Selection

Sequencing of 19 novel rodent *PRNP* sequences yielded a phylogeny generally consistent with species phylogeny as has been previously described for prion sequences (van Rheede et al. 2003). Exceptions were the hedgehog (*Erinaceus europaeus*) and guinea pig (*Cavia porcellus*) positions (Fig. 1). It is generally unsurprising that conflicts arise between a gene tree of a sequence under selection does not precisely recapitulate a species tree as would be expected of a neutral marker. A scan for positive diversifying selection using MEME revealed five codons (codons 22, 68, 130, 197, and 220) to belong significantly ($P < 0.05$) to this regime, but once the significance cut-off was reduced to 0.01, codon 197 was dismissed (Fig. 1, Supplementary Table 1). Codon 22 had two inferred nonsynonymous substitutions along the branch leading to the greater kudu (*Tragelaphus strepsiceros*). Codon 68 had two nonsynonymous substitutions on the internal branch connecting the European rabbit (*Oryctolagus cuniculus*) with the other Glires. Codon 128 showed similar evidence (1–2.7 nonsynonymous substitutions) on the branches leading to the Malayan colugo (*Galeopterus variegatus*), the rabbit, the pangolin (*Manis* sp.), the Assam macaque (*Macaca assamensis*), and the dog (*Canis familiaris*). Codon 220 harbored two nonsynonymous substitutions on the branch of the house mouse (*Mus musculus* M180071). The FUBAR method did not detect any codons under pervasive diversifying selection exceeding a $PP(dN > dS) > 0.9$, but at $PP = 0.81$ codons 125 and 127 emerged, although with an expected number of false positives of 0.38 (CI 95 % 0–2). When we searched for episodic positive selection along the branches of the *PRNP* gene tree, we found small, isolated bursts of diversifying selection acting on a limited number of codons on the internal branch before the Rodentia-Scandentia clade ($p = 0.000055$, Holm's

Fig. 1 Maximum likelihood phylogenetic tree for *PRNP*. Branches with significant ($p < 0.05$) evidence of episodic positive selection as indicated by the branch-site REL model are marked with *star symbols*. Sequences obtained from GenBank are shown with accession numbers. All rodent sequences generated in this study are shown without accession numbers. Accession numbers for sequences generated in this study can also be found in the “Materials and Methods” section. The area of the *circles* at the nodes indicates the magnitude of internode branch support. The *scale bar* denotes 0.02 nucleotide substitutions per site

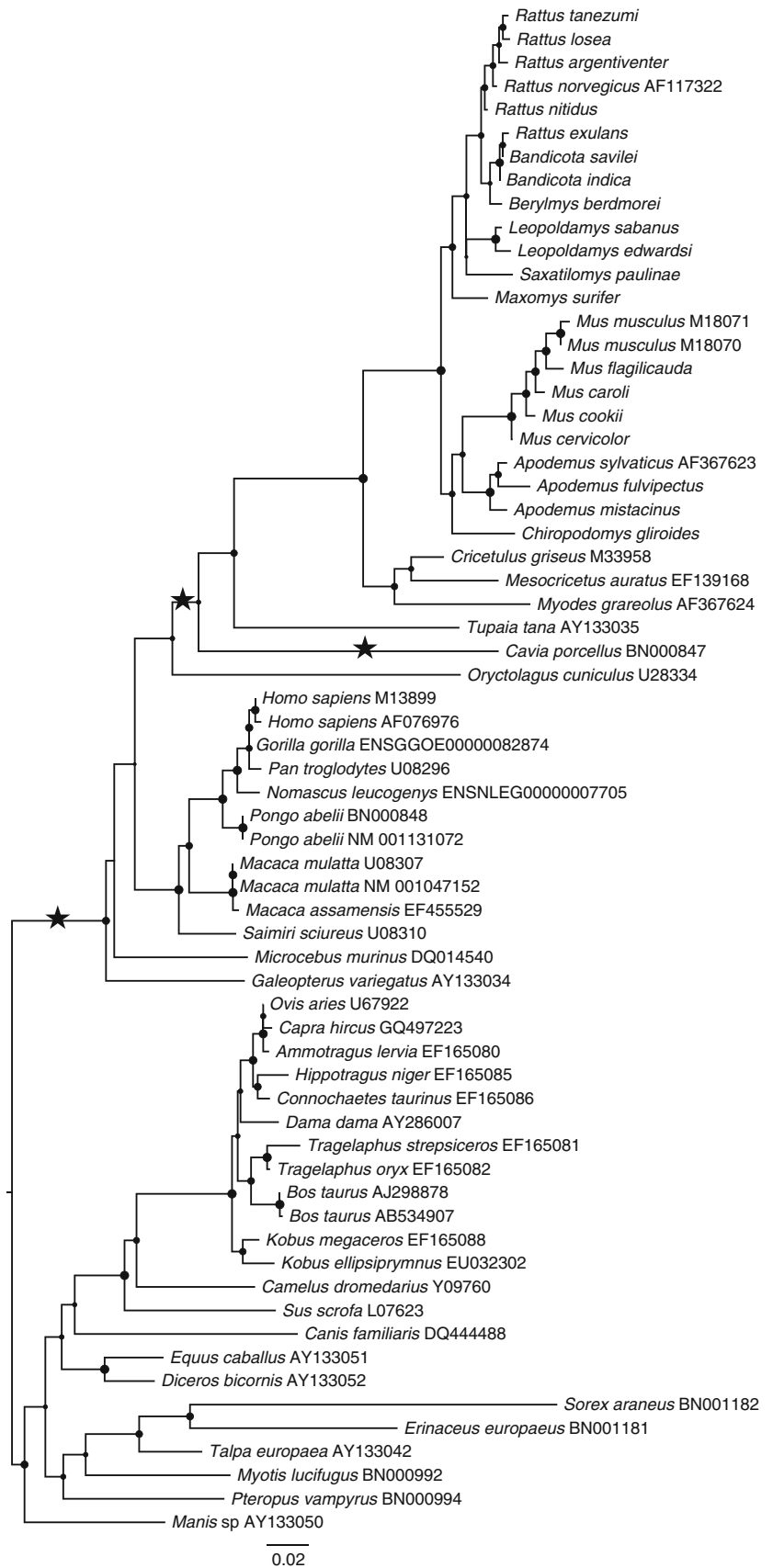
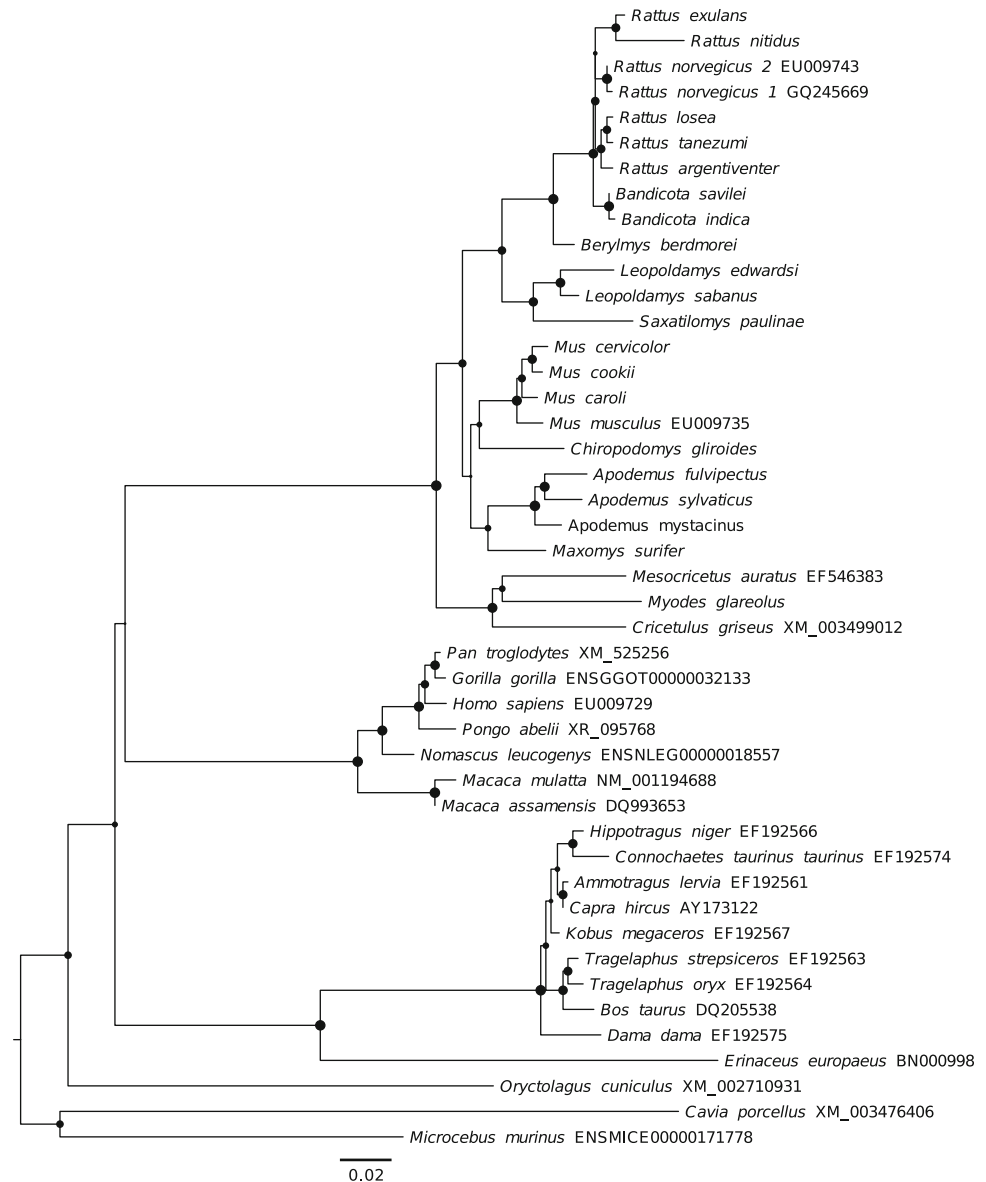


Fig. 3 Maximum likelihood phylogenetic tree for *PRND*. Sequences obtained from GenBank are shown with accession numbers. All rodent sequences generated in this study are shown without accession numbers. Accession numbers for sequences generated in this study can also be found in the “Materials and Methods” section. The area of the circles at the nodes indicates the magnitude of internode branch support. The scale bar denotes 0.02 nucleotide substitutions per site



Discussion

No evidence of balancing selection or widespread positive selection could be identified in our study for either *PRNP* or *PRND*. This is consistent with an earlier interspecific study (Krakauer et al. 1998), but at odds with a recent one (Premzl and Gamulin 2009). This latter study examined seven *PRNP* sequences from representatives of the Euarchonta focusing on primates (five species) as well as twelve sequences from Laurasiatheria (3 Cetartiodactyla, 2 Perissodactyla, 1 Carnivora, 1 Pholidota, 2 Chiroptera, and 3 Eulipotyphla species) and found evidence for positive selection on specific branches and codon sites. Some of those amino acid sites are potentially important for PrP function: His-100-Asn replacement is implicated in cross-primate transmission (Schatzl et al. 1995), and

two changes spatially related to the binding site of protein X (Kaneko et al. 1997; Perrier et al. 2002). We could not confirm these specific results for *PRNP* using the original data set of (Premzl and Gamulin 2009) or by expanding our dataset to 48 taxa and by utilizing more powerful selection detection computational methods, other than a burst of positive selection on the branch leading to the Perissodactyla clade (BSREL, $p = 0.04$) given the Premzl and Gamulin (2009) sequences alone. Once we added our sequences this result was no longer significant (BSREL, $p = 0.147$). Our contrasting findings with respect to Premzl and Gamulin (2009), who previously reported the action of positive selection on specific codons in *PRNP*, are very likely due to the taxonomic and—subsequently—sequence variability between their 19-taxon and our 48-taxon datasets.

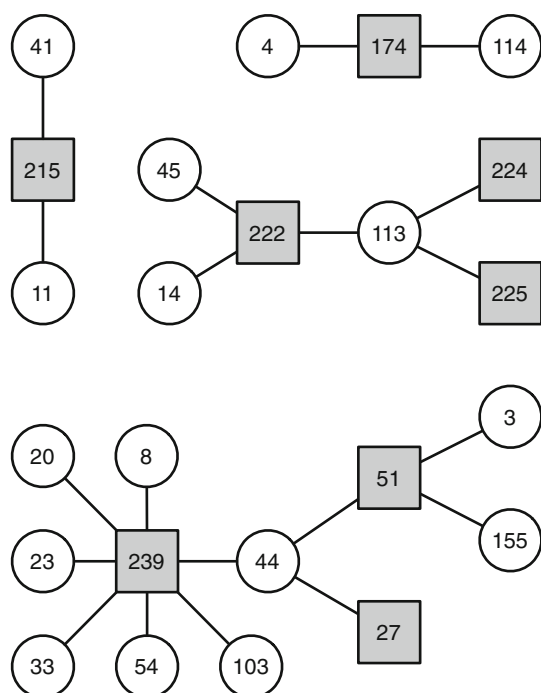


Fig. 4 PrP–Dpl coevolution analysis. Numbers in *boxes* indicate codons in *gray* (PrP) and *white* (Dpl). Four coevolution groups were identified. Network edges denote inferred interprotein functionally interacting amino acid residues

While an earlier study (Mead et al. 2003) reported intense balancing selection against homozygous polymorphisms in humans and concluded on a recurring pattern of balancing selection in *PRNP* in the latest 500,000 years of human evolution, subsequent studies criticized that claim on the basis of the introduction of a bias in the ascertainment of single nucleotide polymorphisms (SNPs) by selecting and scoring SNPs with frequency $>5\%$ (Kreitman and Di Rienzo 2004; Soldevila et al. 2006). Resequencing efforts of the *PRNP* exon 2 and population genetic estimations (Soldevila et al. 2006) were unable to support the balancing selection claim and found evidence suggesting the potentially heterotachous action of positive selection. The presence of some intermediate-frequency polymorphisms remains interesting and warrants a wider resequencing study and examination of the effect of the polymorphisms on protein structure and function. Another study (Seabury et al. 2004) evaluated genetic variation in the exon 3 of *PRNP* in 36 breeds of domestic cattle and other bovine species and found negative selection against nonsynonymous changes and an excess of rare silent polymorphisms upstream the N-terminal cleavage site coding sequence.

Our results are most consistent with that of Seabury et al. (2004). Although they had a limited interspecific focus using cross-species comparisons in a pairwise fashion examining cattle and bison at the population level with

various bovine species as outgroups, their analysis supported purifying selection. Our analysis suggests this and extends it to non-domestic animals as a general principle. Purifying selection was not limited to the Bovidae but extended to all mammal groups studied here, including all primates and the expanded rodent taxa as well. The principle can be generalized to *PRNP* paralogs such as *PRND*, which demonstrated a very similar pattern of selection dominated by negative selection in all included taxa. A portion of the constraints may be a result of coevolutionary pressure as several putative interacting sites were detected between PrP and Dpl. Although no interactions were found among residues that correlate with disease and interactions did not strongly correlate with predicted structural motifs, some evidence for interaction between the H2 and H3 α -helices of PrP and Dpl were identified. Further experimentation will be necessary to determine the significance of these sites in interaction between the protein products of these two genes.

From a biological standpoint, purifying negative selection could be interpreted as a consequence of the pathogenic effects of misfolding of the prion protein. Selection against changes that could favor the pathogenic conformations would be advantageous. Existing polymorphisms in prion genes can lead to a higher probability of seeding conversion to PrP^{Sc} (Christen et al. 2013). It has also been suggested that variants conferring susceptibility to TSEs are in generally conserved regions and that such variation is generally derived (Martin et al. 2009). This also extends to the species barrier to prion diseases whereby most species are resistant to infectious prions of other species. The species barrier may be determined by variation in few amino acids in disordered regions of the prion protein (Richmond et al. 2014). Overall there is strong conservation of the PrP at the sequence and structural levels among diverse species (Richmond et al. 2014; Wopfner et al. 1999). In this context, our result of general negative purifying selection could indicate that variants that confer higher probability of seeding misfolding are quickly removed from the population. However, relaxation of selection at specific domains such as at the amino acids connecting α -helices 1 and 2 could suggest that substitutions in these regions do not destabilize the prion protein in such a way as to provoke it into adopting a pathogenic conformation.

Surprisingly, *PRND* is under very similar constraints although it is not directly associated with neurological disease. This may reflect either linkage disequilibrium effects as *PRND* and *PRNP* are closely linked or may be due to constraints imposed by the putative interaction between the two proteins identified as coevolving sites in this study. The similar selection regimes on *PRNP* and *PRND* in all taxa examined suggest that purifying selection

on the prion genes and its homologs may be a general feature for this gene family in all species. This is somewhat surprising as the species tested are not equally susceptible to prion diseases with most species, particularly primates and rodents, being generally resistant to disease but several cervid species quite disease prone including to the emergence of CWD in wild cervids (Saunders et al. 2012). Nonetheless, there was no evidence for relaxed selection in cervids suggesting that their relative susceptibility to prion diseases is related to other factors such as ease of transmission or within species polymorphism (Hunter 2007) as opposed to the general selective regime on the gene.

Rodents have been hypothesized to be potential reservoirs of TSEs (Heisey et al. 2010). Therefore, we specifically extended the *PRNP* and *PRND* data sets for diverse rodent species. Consistent with other taxa, rodent *PRNP* and *PRND* were under a strict regime of negative selection. This is consistent with the difficulty of establishing rodent-adapted scrapie models which involves intracerebral inoculation. Therefore, we conclude that the prion gene and its homolog doppel have been evolving under purifying selection among mammalian taxa.

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