J Mol Evol (2010) 71:356–363
DOI 10.1007/s00239-010-9390-7

Loss of Octarepeats in Two Processed Prion Pseudogenes in the Red Squirrel, *Sciurus vulgaris*

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Received: 12 July 2010 / Accepted: 8 September 2010 / Published online: 28 September 2010
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Abstract The *N*-terminal region of the mammalian prion protein (PrP) contains an 'octapeptide' repeat which is involved in copper binding. This eight- or nine-residue peptide is repeated four to seven times, depending on the species, and polymorphisms in repeat number do occur. Alleles with three repeats are very rare in humans and goats, and deduced PrP sequences with two repeats have only been reported in two lemur species and in the red squirrel, *Sciurus vulgaris*. We here describe that the red squirrel two-repeat PrP sequence actually represents a retroposed pseudogene, and that an additional and older processed pseudogene with three repeats also occurs in this species as well as in ground squirrels. We argue that repeat numbers may tend to contract rather than expand in prion retropseudogenes, and that functional prion genes with two repeats may not be viable.

Keywords Prion protein · PrP · Octarepeat · Protein evolution · Pseudogene

Teun van Rheede—Deceased May 21, 2003.

Electronic supplementary material The online version of this article (doi:10.1007/s00239-010-9390-7) contains supplementary material, which is available to authorized users.

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Introduction

The prion protein (PrP) is a plasma membrane glycoprotein of essentially unknown function (Steele et al. 2007), which has been implicated in disparate processes, such as copper binding, anti- and pro-apoptotic activities, redox homeostasis, neuron formation and myelin maintenance (Aguzzi et al. 2008; Bremer et al. 2010). Misfolded isoforms of PrP mediate the intra- and inter-species transmission of the mammalian prion diseases (transmissible spongiform encephalopathies, TSEs) (Kovacs and Budka 2009). Copper binding to PrP occurs extracellularly at the *N*-terminal domain, which in most mammals contains five or six repeats of a peptide of eight or nine residues with the consensus sequence P(Q/H)GGG(G/-)WGQ (Wopfner et al. 1999; van Rheede et al. 2003; Acutis et al. 2007). This region can bind up to one $Cu2^{+}$ ion per repeat (Viles et al. 2008; Guerrieri et al. 2009), but there is still no generally accepted role for the binding of copper to PrP (Davies and Brown 2008). The repeat region modulates prion replication and pathogenicity (Flechsig et al. 2000), is involved in the protective effect against oxidative stress (Mitteregger et al. 2007; Malaise et al. 2008), and in PrP aggregation upon copper binding (Yu et al. 2008).

Mammals homozygous for four or seven repeats also occur (van Rheede et al. 2003; Kim et al. 2008; Rongyan et al. 2008; Martin et al. 2009). In humans, which normally have five repeats, a few families are known in which alleles with seven to even fourteen repeats are associated with atypical cases of hereditary prion disorders (Creutzfeldt-Jakob and Gerstmann-Sträussler-Scheinker; Kovacs and Budka 2009). Heterozygosity for four repeats occurs at a frequency of $\sim 1\%$ and is not associated with prion disease susceptibility (Jeong et al. 2004; Bishop et al. 2009), but

might be related to familial Alzheimer's and Parkinson's disease (Perry et al. 1995; Wang et al. 2009). Heterozygosity for three repeats has been reported in two patients with a rapidly progressing dementia (Beck et al. 2001) and Creutzfeldt-Jakob disease (Capellari et al. 2002), respectively. On the other hand, goats heterozygous for a three-repeat allele appeared more resistant to prion infectivity (Goldmann et al. 1998). There are only two reports of PrP deletion mutants with two repeats. Heterozygosity for a 2- and a 5-repeat allele was reported in two specimens of different species of lemurs which are known to be particularly susceptible for TSE (Gilch et al. 2000). A similar apparent heterozygosity has been reported in a red squirrel, *Sciurus vulgaris* (van Rheede et al. 2003). Interestingly, five patients from rural Kentucky with probable or definite Creutzfeldt-Jakob disease had a history of eating squirrel brains (Berger et al. 1997), and consuming squirrel brains has been proposed as a serious risk (Sekercioglu 2004). One might wonder whether this relates to the presence of a two-repeat PrP in squirrels.

If the two-repeat allele is more common in lemur and squirrel populations, one would expect the occurrence of two-repeat homozygotes, unless these are lethal. Lethality could be envisaged, considering the role of the intact repeat region in copper binding, and the possibly increased susceptibility for TSE. If viable homozygotes were found, this would challenge the functional and structural importance of the repeat region. This paper describes the search for squirrels homozygous for the two-repeat allele of the prion protein gene, *PRNP*. However, it turned out that the two-repeat *PRNP* sequence in squirrel actually represents a retroposed pseudogene rather than an allele of the functional gene. In addition, during this study a second prion retropseudogene, with three repeats, was found in squirrel.

Materials and Methods

Sampling of Squirrel Material and DNA Isolation

Samples of tissue (liver or ear tips) or genomic DNA were obtained from Eurasian red squirrels (*S. vulgaris*) from various localities in the Netherlands, from Germany (Dresden), Spain (Madrid), Italy (Valfurva), Wales (Anglesey) and Russia (Primorsky Krai, Eastern Siberia). Liver was obtained from a Japanese squirrel (*Sciurus lis*) and genomic DNA from a grey squirrel (*Sciurus carolinensis*). Tissue samples were either stored at −20°C or preserved in 95% ethanol. Genomic DNA was extracted using the Promega Wizard® Genomic DNA purification System, following the manufacturer's protocol.

Amplification and Sequencing of Squirrel Prion Genes

Prion genes were amplified using three different primer combinations, named 'long', 'short' and 'long1' (Table S1). The 'long' primer set (Prp_for1 and Prp_rev1) was identical to the primer set used to amplify mammalian *PRNP* sequences by van Rheede et al. (2003), in which red squirrel yielded two PCR products of 642 and 720 bp, for the 2- and 5-repeat allele, respectively. The 'short' primer set was a combination of the original forward primer (Prp_for1) and a primer located just after the repeat region in squirrel *PRNP* (PrpS2_rev) (van Rheede et al. 2003). The 'short' combination resulted in PCR fragments of 226 and 304 bp in red squirrels. The primer set 'long1' (PrpSq_for and PrpSq_rev) was specifically developed for amplifying the squirrel *PRNP* gene, and resulted in fragments of 540 and 618 bp.

All PCR were performed with the Expand High Fidelity PCR system (Roche) and contained 50–100 ng genomic DNA or ~10 ng cDNA, 200 mM dNTP, 50–100 pmol of each primer, 1.5–2 mM MgCl2, 1 M betain (optional) and 1.75 U Expand High Fidelity Enzyme mix in a final volume of 50 µl. The following PCR program was used: 2 min at 94°C; 35 cycles of 15 s at 94°C, 1 min at 48–62°C (mostly 58°C was used) and 1 min at 72°C and a final step of 10 min at 72°C.

The PCR products were purified from 1% agarose gels using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Gel-extracted PCR products were sequenced directly with one of the PCR primers, mostly the forward primer, to determine the number of repeats. PCR products of *S. lis* and a *S. carolinensis* were sequenced with both PCR primers to determine the complete amplified sequence. Sequence reactions were performed using Big Dye fluorescent technology, and run on an ABI 3730 96-capillary sequencer (Applied Biosystems).

New sequences determined in this study have been deposited in the EMBL Nucleotide Database under the accession numbers FN678793-FN678795.

Flanking PCR

Multiple attempts were made to amplify the unknown 5′ and 3′ flanking sequences of the 2- and 5-repeat red squirrel *PRNP* sequences, applying the 'Flanking PCR' method as described by Sørensen et al. (1993) and van Rheede et al. (2003). The biotinylated primers PrpSqbio_rev and PrpSqbio_for (Table S1) were used in the first round PCR for amplifying the 5′ and 3′ flanking sequences, respectively. The PrpSqbio_rev primer was positioned 3′ to the repeat region, so that it could be determined whether the 2- or 5-repeat flanking sequence had been amplified. With

these primers we only succeeded in determining the 5′ flanking sequence of the two-repeat gene.

RNA Isolation and RT-PCR

Total RNA was isolated from red squirrel whole brain tissue using GibcoBRL Trizol® reagent according to the manufacturer's protocol. Isolated RNA was dissolved in DEPC-treated water and stored at −20°C until use. Before cDNA synthesis any remaining DNA was removed by treating the RNA with 5 units of RNase-free DNase per µg of RNA for 15 min at 37°C followed by 10 min at 70°C. The reverse transcriptase reaction was performed using the first-strand cDNA synthesis kit for RT-PCR (AMV; Roche Applied Science) according to the manufacturer's instructions, with 1 µg of DNA-free total RNA and oligo-p(dT)15 primers. PCR was done on 2 µl of the synthesized cDNA using the squirrel specific primer combination 'long1' and PCR conditions as described above. Possibly remaining contamination with genomic DNA was assessed by performing first-strand cDNA synthesis with and without reverse transcriptase.

Sequence Retrieval, Alignment and Phylogenetic Analyses

The NCBI and Ensembl databases (as on 10 Jan 2010) were searched for additional sciurid prion sequences. In the Genbank database one *PRNP* gene of the ground squirrel *Spermophilus tridecemlineatus* (acc. nr. BN000993.2) was found, and from the EST database two *PRNP* ESTs of the ground squirrel *Sp. tridecemlineatus* and four of the related *Spermophilus lateralis* were retrieved. These sequences all have four repeats and apparently correspond with the normal *PRNP* gene of the two species. BLAST/BLAT searches with the red squirrel 2- , 3- and 5-repeat *PRNP* sequences and with the *Sp. tridecemlineatus PRNP* gene and EST sequences against sciurid genomic sequences revealed two *PRNP*-like sequences in the *Sp. tridecemlineatus* genome (acc. nr. AAQQ01175405), one corresponding with the four-repeat EST and the other with a three-repeat pseudogene (acc. nr. AAQQ01193410).

The eight available sciurid *PRNP* nucleotide sequences were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and manually adjusted for optimal alignment with GeneDoc (v 2.6.002). After removal of the repeat sequences and sites missing in more than two of the eight sequences, the remaining 479-bp alignment (corresponding with positions 120–153 and 232–667 of the red squirrel 5r *PRNP* sequence) was used for phylogenetic analysis. An unrooted ML tree was obtained with PhyML v3.0 (Guindon and Gascuel 2003), using K80 as the best fitting model of sequence evolution as indicated with jModeltest (v. 0.1.1) (Posada 2008). Statistical nodal support was estimated by 100 non-parametric bootstrap replicates.

Results

We began our search for animals homozygous for the two-repeat *PRNP* allele by amplifying the *PRNP* gene from 27 red squirrels from all over the Netherlands. Amplification was done with two primer sets: the 'short' squirrel specific primer set should amplify fragments of 226 and 304 bp for the 2- and 5-repeat *PRNP* alleles, respectively, and the 'long' primer set fragments of 642 and 720 bp. Surprisingly, all 27 squirrels yielded the same patterns. The short primer pair revealed the two expected bands and a third band of intermediate size (Fig. 1a, lanes 1 and 2), and the long primer pair gave the two bands corresponding to the 2- and 5-repeat bands as originally observed by van Rheede et al. (2003) (Fig. 1a, lanes 3–7). In other mammals only the long primer pair resulted in successful amplification, and yielded a single band, corresponding with five-(human, rhinoceros, elephant) or six-(tenrec) repeats (Fig. 1a, lanes 8–11). The fact that all the squirrels displayed the 2- and 5-repeat bands strongly indicated that these bands represent two different *PRNP* gene loci, rather than alleles.

Sequencing of the PCR products confirmed that the 2R and 5R bands in lanes 1–7 of Fig. 1a corresponded with the 2- and 5-repeat *PRNP* sequences originally reported by van Rheede et al. (2003) (*S. vulgaris* r2 and r5 in Fig. 2). In some of the red squirrels single base heterozygosity was observed in both the r2 and r5 sequences (Fig. 3), which further confirmed that r2 and r5 represent two different *PRNP* gene loci. In addition to the 2- and 5-repeat sequences, the 3R middle band in lanes 1 and 2 of Fig. 1a turned out to represent a *PRNP*-like sequence with three repeats (*S. vulgaris* r3 in Fig. 2). The presence of an in-frame stop codon and a frame-shift mutation in the displayed sequence identifies it as a pseudogene. A database search with this sequence revealed a homologous r3 *PRNP* pseudogene in the ground squirrel *S. tridecemlineatus* (Fig. 2). The presence of several synapomorphous mutations indicates that the 3r pseudogenes in the two species are orthologs.

Apart from the number of repeats, the 2- and 5-repeat *PRNP* coding sequences of the red squirrel differ at only three synonymous and one non-synonymous sites. The corresponding genes must thus be the products of a relatively recent duplication event. To assess whether this duplication is present in Eurasian red squirrels throughout their distribution range, and in related squirrel species, we amplified the *PRNP* gene sequences from red squirrels from

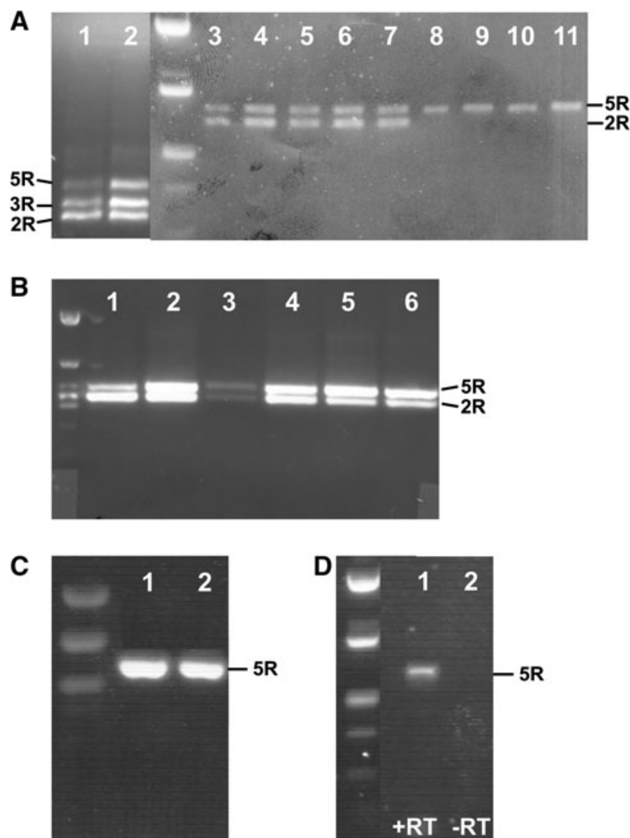

Fig. 1 PCR amplification with *PRNP*-specific primers on samples of genomic DNA (**a**–**c**) and total brain cDNA (**d**) resolved on 1% agarose gels. In *panel* **a**, *lanes 1* and *2* were amplified with the 'short' primer pair, all other lanes with the 'long' primer pair; in *panels* **b**, **c** and **d** the 'long1' primer pair was applied (see 'Materials and Methods'). All PCR products were sequenced to determine the number of repeats. 2R, 3R and 5R correspond with the presence of 2, 3 and 5 (or 6 in tenrec) repeats. **a** *Lanes 1–7*, red squirrels (*S. vulgaris*) from different localities in the Netherlands; *lane 8*, human (*Homo sapiens*); *lane 9*, black rhino (*Diceros bicornis*); *lane 10*, Indian elephant *(Elephas maximus); lane 11*, tenrec (*Tenrec ecaudatus*). **b** *Lanes 1–6*, red squirrels (*S. vulgaris*) from Germany, Spain, Wales, Italy, Eastern Siberia and the Netherlands, respectively. **c** *Lane 1*, grey squirrel (*S. carolinensis*); *lane 2*, Japanese squirrel (*S. lis*). **d** cDNA from red squirrel (*S. vulgaris*) brain; +*RT* and −*RT* indicates the presence or the absence, respectively, of reverse transcriptase during cDNA synthesis

different Eurasian locations, as well as from two supposed close relatives, the North American grey squirrel (*S. carolinensis*) and the Japanese squirrel (*S. lis*) (Oshida and Masuda 2000; Grill et al. 2009). It turned out that the 2R and 5R bands were present in all investigated red squirrels (Fig. 1b), while the two other squirrel species only had the 5R band (Fig. 1c). The identity of the amplification products of *S. carolinensis* and *S. lis* was again confirmed by sequencing (Fig. 2). It is thus most likely that the *PRNP* gene duplication occurred in the red squirrel lineage, after its divergence from the other two squirrel species, but before the geographic dispersal of the red squirrels.

Given the uninterrupted open reading frame of the red squirrel 2r *PRNP* gene, it was expected that it would be expressed next to the 5r gene. However, RT-PCR with *PRNP*-specific primers on squirrel brain cDNA yielded only a single band with the expected size of the 5r product (Fig. 1d). This indicated that the 2r *PRNP* gene is not transcribed in the brain and may in fact be a pseudogene. To further assess this possibility, we performed 5′-flanking-PCR on the 2r *PRNP* gene. The 5′ UTR sequence aligns well with the corresponding sequences of the mouse *PRNP* cDNA and with ground squirrel *PRNP* ESTs retrieved in the databases (Fig. 4), and thus established the absence of the two introns that are normally present in the rodent *PRNP* gene. This characterizes the 2r genomic *PRNP* sequence as a retroposed pseudogene. Unfortunately, no sequences could be obtained to assess the presence of other characteristics of such processed pseudogenes, i.e., flanking repeats and a 3′ poly-T track. We did not perform flanking-PCR on the 3r *PRNP* pseudogene of the red squirrel, but the available sequence data of the orthologous 3r pseudogene of *Sp. tridecemlineatus* also shows the absence of introns (Fig. 4).

A phylogenetic analysis of all available squirrel *PRNP* gene and pseudogene sequences confirms that the 2r pseudogene of *S. vulgaris* most likely originated after the divergence of this species from the two other *Sciurus* species (Fig. 5). The tree also confirms that the 3r pseudogenes of *S. vulgaris* and *Sp. tridecemlineatus* are orthologs, and have a much older origin. This 3r *PRNP* pseudogene might therefore be present in other sciurid rodents as well.

Discussion

Reports that *PRNP* alleles with two repeats occur in lemurs (Gilch et al. 2000) and red squirrel (van Rheede et al. 2003) might imply that prion proteins with only two repeats can be functionally viable. It is therefore important that we here demonstrate that the squirrel two-repeat *PRNP* sequence actually represents a non-expressed retroposed pseudogene. This not only means that any concerns about consuming squirrel brains (Berger et al. 1997; Sekercioglu 2004) can not be related to the expression of a two-repeat PrP, but, more importantly, also raises interesting questions about prion structure, function and evolution. In the case of the reported lemur two-repeat *PRNP* genomic sequence, it can not be decided from the available data whether it is actually expressed, or whether it shows any further differences with the lemur five-repeat *PRNP* sequence (Gilch et al. 2000). Since the lemur two-repeat *PRNP* sequence reportedly occurred in two individuals from different genera, it might be expected to be a more common feature in lemurs. We

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                                                      I          II          III          IV           V
S. carolinensis (r5)          ......GQGSPGGNRYP  PQGGGXWGQ  PHGGGWGQ  PHGGGGWGQ  PHGGGGWGQ  PHGGGWXQG  -GTHNQWGKPSKPK-TNMKHVAG
S. lis (r5)                   ........GSPGGNRYP  PQGGGGWGQ  PHGGGWGQ  PHGGGGWGQ  PHGGGGWGQ  PHGGGWGQG  -GTHNQWGKPNKPK-TNMKHVAG
S. vulgaris (r5)              GGSRYPGQGSPGGNRYP  PQGGGGWGQ  PHGGGWGQ  PHGGGGWGQ  PHGGGGWGQ  PHGGGWGQG  -GTHNQWGKPNKPK-TNMKHVAG
S. vulgaris (r2)Ψ             GGSRYPGQGSPGGNRYP  PQGGGGWGQ  --------  ---------  ---------  PHGGGWGQG  -GTHNQWGKPNKPK-TNMKHVAG
S. vulgaris (r3)Ψ             GGSCYPEKGSPGGNYYP  PQGGSGWGQ  --------  ---------  PHGGSG*GQ  PHGGSCSQG  GGTHNQWNKSNKPK?NNMKHVAG
Sp. tridecemlineatus (r3)Ψ    VGSCYPEQGSP?GNHYP  PHGGGGWGQ  --------  ---------  LHGSSD*GQ  PHSSS*GEG  DGNHNQWNKLNKSK-NNIKQVAG
Sp. tridecemlineatus (r4)     GGSRYPGQGSPGGNRYP  PQ-------  --GGGWGQ  PHGGGGWGQ  PHGGGGWGQ  PHGGGWGQG  -GTHNQWGKPVKPK-TSMKHVAG
Sp. lateralis (r4)            GGSRYPGQGSPGGNRYP  PQ-------  --GGGWGQ  PHGGGGWGQ  PHGGGGWGQ  PHGGGWGQG  -GTHNQWGKPVKPK-TSMKHMAG
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Fig. 2 Alignment of the deduced repeat regions in PrP of grey squirrel (*S. carolinensis*), Japanese squirrel (*S. lis*), Eurasian red squirrel (*S. vulgaris*), thirteen-lined ground squirrel (*S. tridecemlineatus*) and golden-mantled ground squirrel (*S. lateralis*). PrP sequences of grey squirrel, Japanese squirrel and Eurasian red squirrel were deduced from sequenced PCR products (see Fig. 1), whereas the ground squirrel PrP sequences were extracted from public databases as either genomic (*Sp. tridecemlineatus r3* and *r4*) or EST sequences (*Sp. lateralis r4*). *r5*, *r4*, *r3* and *r2* indicate the number of repeats (*I–V*) in the different PrP sequences; *dotted line*, missing data; *X*, ambiguous amino acid; *asterisk*, stop codon; *question mark*, out-of-frame deletion; *dashed line*, indel; *psi*, pseudogene. The consensus sequence is *white-in-black*. Accession numbers of the sequences used are: *S. carolinensis* r5, FN678793; *S. lis* r5, FN678794; *S. vulgaris* r5, AY133037; *S. vulgaris* r2, AY133038; *S. vulgaris* r3, FN678795; *Sp. tridecemlineatus* r3, AAQQ01193410; *Sp. tridecemlineatus* r4, AAQQ01175405; and *Sp. lateralis* r4, CO73759

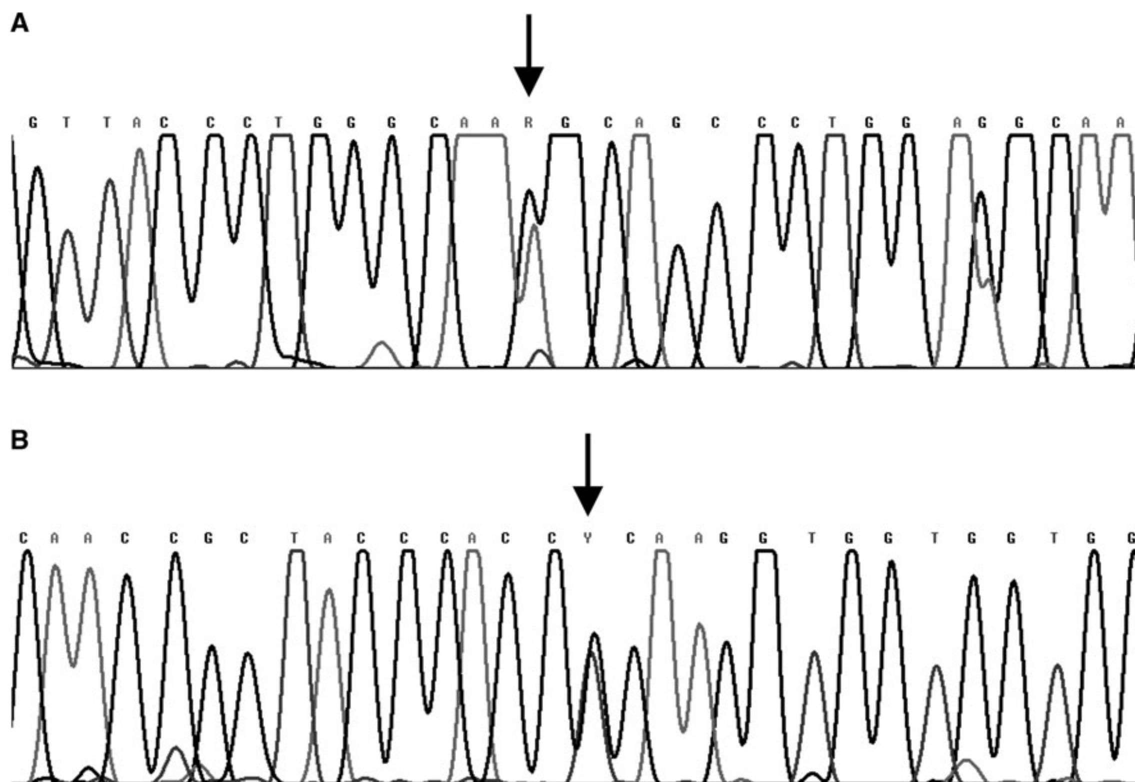

Fig. 3 Examples of single base pair heterozygosity (*arrows*) in 5-repeat (**a**) and 2-repeat (**b**) red squirrel sequences

therefore attempted to retrieve and analyse the nature of such a two-repeat *PRNP* sequence in six lemur species, including one from the same genus *Eulemur* as studied by Gilch et al. (2000). However, we have been only able to amplify five-repeat *PRNP* sequences, and did not find any two-repeat sequence, using a variety of primers and PCR conditions. It thus remains uncertain whether a two-repeat PrP protein actually exists in lemurs, and whether such a PrP is viable at all.

The most interesting observation in this study is that the two *PRNP* pseudogenes in *S. vulgaris* have only two and three repeats, rather than the original five. Mammalian *PRNP* alleles with three repeats have only rarely been reported (Goldmann et al. 1998; Beck et al. 2001; Capellari et al. 2002), and the existence of functional alleles with two repeats has not been documented at all. Is such loss of repeats a more common phenomenon in *PRNP* pseudogenes? Unfortunately, very few *PRNP* pseudogenes can be retrieved in the databases and the literature. We performed searches for *PRNP*-like sequences in 35 mammalian genomic resources present in the Ensembl and Genbank databases but never recovered more than a single and

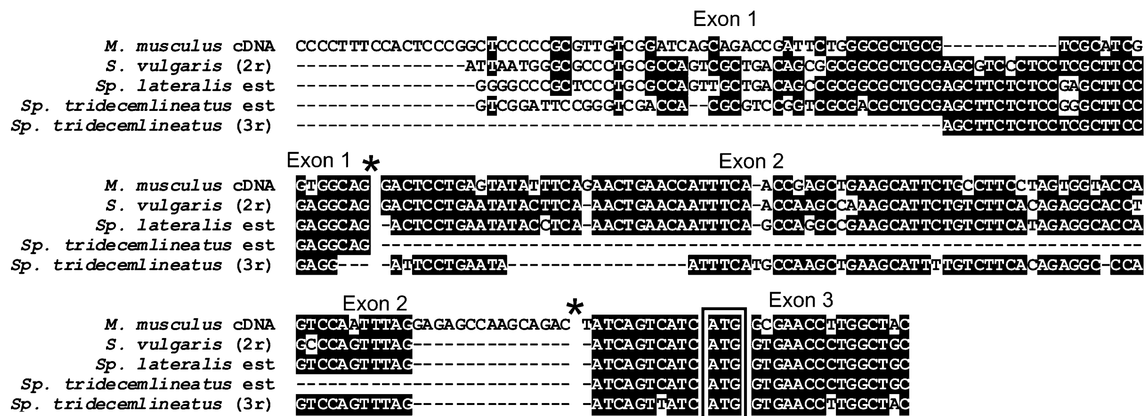

Fig. 4 Alignment of the 5′ flanking sequence of the 2-repeat *PRNP* pseudogene of red squirrel (*S. vulgaris* 2r) with the prion cDNA sequence of mouse (*M. musculus*; acc. nr. BC032085), prion EST sequences of thirteen-lined ground squirrel (*Sp. tridecemlineatus*; EL772736) and golden-mantled ground squirrel (*Sp. lateralis*; CO739249), and with the 5′ flanking sequence of the 3-repeat *PRNP* pseudogene of thirteen-lined ground squirrel (*Sp. tridecemlineatus*; AAQQ01193410). *Asterisks* indicate the positions of the two introns in the mouse *PRNP* gene; the start codon is *boxed*. The absence of exon 2 in the *Sp. tridecemlineatus* EST is probably the result of exon skipping, as has also been reported for the mouse *PRNP* transcript (Haigh et al. 2007)

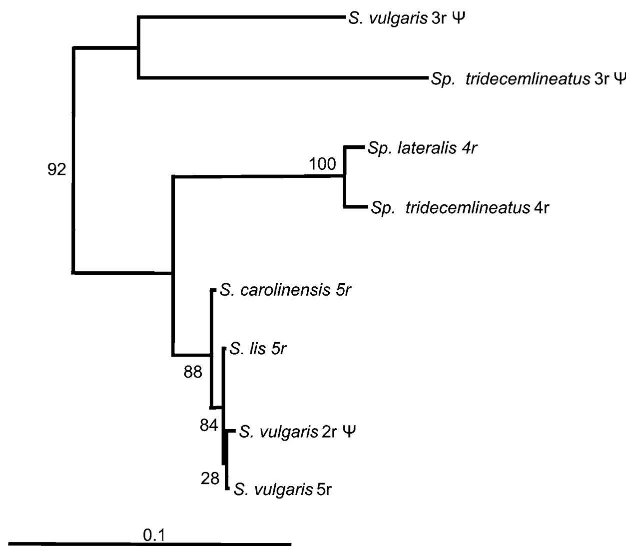

Fig. 5 Unrooted maximum likelihood tree (−ln L = 1191.87880) based on a 479-bp alignment of the different squirrel *PRNP* sequences. Bootstrap support values are indicated. The *bar* corresponds with 0.1 substitutions per site. *2r*, *3r*, *4r* and *5r* indicates the number of repeats; *psi*, pseudogenes. Accession numbers of the used sequences are given in the legends of Fig. 2

apparently normal *PRNP* gene per species, apart from the 3r *PRNP* pseudogene of *Sp. tridecemlineatus*, which is in fact the orthologue of the *S. vulgaris* 3r *PRNP* pseudogene (Fig. 5). In the literature two additional *PRNP* pseudogenes have been reported. The anteater (*Cyclopes didactylus*) has a *PRNP* pseudogene with several frameshift and nonsense mutations (van Rheede 2003; acc. nr. AF545183). The deduced protein sequence is 66% identical to the functional PrP, and has only three repeats, against four or five in the functional anteater PrP. It is not known whether this is a retroposed or a duplicated pseudogene. In mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*) a retroposed *PRNP* pseudogene has been characterized, apparently orthologous in the two species (Brayton et al. 2004; O'Rourke et al. 2004). This pseudogene still has an open reading frame, and the deduced amino acid sequence differs at only one position (138 S → N) from that of the corresponding functional PrP. The functional *PRNP* gene has five repeats in these deer species, while the pseudogene has alleles with five or six repeats.

Thus, in the four retrieved independent instances of *PRNP* pseudogene formation in mammals, the repeat number has in two cases been reduced to three, and in one case to two, in sharp contrast to the extreme scarcity or absence of such contractions in case of functional *PRNP* genes. If this observation may be generalized, it raises the question why such repeat contraction readily occurs in *PRNP* pseudogenes, but not in the functional gene. Expansion and contraction of repeats is a frequent mutational process in the eutherian *PRNP* gene. The mechanisms involved can be unequal crossing-over and replication slippage, and contraction would be expected to be as likely to occur as expansion (Collinge 2001). The fact that the repeat number in functional *PRNP* genes is maintained between four and seven, depending on the species, strongly suggests that higher but also lower repeat numbers are deleterious. In the case of repeat numbers higher than seven this is clearly related to increased susceptibility for TSE (Stevens et al. 2009). Why reduction to three and certainly two repeats apparently is selectively deleterious is perhaps more difficult to understand in view of the fact that PrP knockout mice have no obvious

phenotype (reviewed by Steele et al. 2007), and PrP knockout mice transgenic for PrP without the repeat region are apparently healthy (Flechsig et al. 2000). However, PrP is a well-conserved protein in mammals, indicating the importance of maintaining its structural and functional integrity (e.g., Wopfner et al. 1999; van Rheede et al. 2003; Rongyan et al. 2008; Premzl and Gamulin 2009). The expression of a PrP with three and especially two repeats might frustrate pathways and processes in which PrP is normally involved, most likely relating to its copper binding properties, and eventually making it evolutionarily disadvantageous.

Acknowledgments We are very grateful to Mieke Holtslag (Squirrel Rescue Centre, De Meern, The Netherlands) for providing the Dutch red squirrel samples. We also like to thank Drs Rob Ogden, Juan Carlos Illera, Luc Wauters, Alexei Kryukov and Anna Hundsdörfer for providing the samples of Welsh, Spanish, Italian, Russian and German red squirrels, respectively. Samples of *S. lis* and *S. carolinensis* DNA were kindly provided by Drs Noriko Yaekashiwa and Scott Steppan, respectively. We thank Dr. Guido Kappé for useful bioinformatical suggestions.

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