

Postprint del artículo publicado en:
Molecular Biology and Evolution
33(1): 174-184 (2016)

ARTICLE (Discoveries section)

Sexual selection of protamine 1 in mammals

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Short title: Sexual selection of protamine 1

Key words: Sexual selection, protamine, mammals, sperm, selective pressures

ABSTRACT

Protamines have a crucial role in male fertility. They are involved in sperm chromatin packaging and influence the shape of the sperm head and, hence, are important for sperm performance. Protamine structure is basic with numerous arginine-rich DNA-binding domains. Postcopulatory sexual selection is thought to play an important role in protamine sequence evolution and expression. Here we analyze patterns of evolution and sexual selection (in the form of sperm competition) acting on protamine 1 gene sequence in 237 mammalian species. We assessed common patterns as well as differences between the major mammalian subclasses (Eutheria, Metatheria) and clades. We found that a high arginine content in protamine 1 associates with a lower sperm head width, which may have an impact on sperm swimming velocity. Increase in arginine content in protamine 1 across mammals appears to take place in a way consistent with sexual selection. In metatherians, increase in sequence length correlates with sexual selection. Differences in selective pressures on sequences and codon sites were observed between mammalian clades. Our study revealed a complex evolutionary pattern of protamine 1, with different selective constraints, and effects of sexual selection, between mammalian groups. In contrast, the effect of arginine content on head shape, and the possible involvement of sperm competition, was identified across all mammals.

INTRODUCTION

Understanding how evolutionary processes generate the large variation observed among species is one of the fundamental questions in evolutionary biology. Sperm competition, as a form of postcopulatory sexual selection, is a unique evolutionary process which acts on sperm of males competing for the fertilization of ova (Parker 1970). This selective process drives the adaptation of sperm form, function and fertility (reviewed in Birkhead and Møller 1998, Simmons 2001, Birkhead et al. 2009). Uncovering how sperm competition can shape phenotypes through changes in gene and regulatory DNA sequences would allow us to draw conclusions about evolutionary process in general, the evolution of function, and causes underlying medical conditions such as sub- or infertility.

Sperm morphology varies considerably among species (Cummins and Woodall 1985, Roldan et al. 1992, Pitnick et al. 2009). Changes in sperm head size and shape are important determinants of sperm swimming velocity that, in turn, is a key determinant of male fertility. Sperm head morphology seems to be greatly influenced by the condensation of nuclear chromatin (Balhorn 2007, Cree et al. 2011), and variation in the process of histone replacement and in the proteins involved can have a great effect on the shape of the sperm head (Balhorn 2007). Among these proteins, protamines, which are basic arginine-rich sperm nuclear proteins, play a crucial role. They are involved in the train of successive

replacements of histones to transition proteins to protamines (Oliva and Dixon 1991). This process results in a tightly packed, efficiently shielded chromatin and an almost complete silencing of expression (Balhorn 2007). Due to the existence of cysteine residues, protamines of eutherian mammals form disulphide bonds within and between protamines stabilizing the chromatin structure (Balhorn et al. 1992, Queralt et al. 1993).

Because protamines are crucial for the process of chromatin condensation, alterations in their expression can affect fertility (Cho et al. 2003, Aoki et al. 2005, Oliva 2006, Carrel et al. 2007). In men, changes in sperm protamine content affects sperm head morphology, as well as sperm motility (Aoki et al. 2005). In mouse models, modification of the protamine content is linked to sperm morphological abnormalities and decreases in sperm motility (Cho et al. 2001). Furthermore, changes in protamine gene sequences and protamine expression ratios affect head size and shape in rodents (Lüke et al. 2014a,b).

Protamines can be found in the sperm of protostomes as well as deuterostomes (Oliva and Dixon 1991). They most likely evolved in chordates from a sperm-specific histone H1 through a shift from a lysine-rich histone H1 to the arginine-rich protamine (Lewis et al. 2004). The selection for an arginine-rich protamine is thought to be driven by selective constraints imposed by internal fertilization (Kansinsky et al. 2011). Arginine richness, as opposed to lysine, results in a higher affinity for the protein to bind to the DNA molecule as well as a greater binding flexibility due to its guanidinium group (Ausio et al. 1984, Cheng et al. 2003). In mammals, two types of protamines have been identified. Protamine 1 is found in all mammals and shows sequence similarities to bird protamine 1 (Oliva and Dixon 1991). Protamine 2 is found in primates and rodents but evidence for the existence of the protamine 2 gene sequence, transcripts and, in some cases, mature protein has been presented for several species of other mammalian clades (Oliva 2006, Balhorn 2007). Mammalian protamines contain three or more DNA anchoring domains which comprise 3-7 arginine residues that are separated by uncharged amino acids (Balhorn et al. 1999). Within mammals in general protamines are thought to be diverse, especially in the C-terminal region, but contain conserved regions that are also found in birds (N-terminal ARYR, SRSRSR phosphorylation site, 3 arginine clusters) (Queralt et al. 1993). In marsupials (and birds) cysteine residues are absent except in the *Planingale* genus.

Several studies claim that protamines are the fastest evolving reproductive proteins, exhibiting high structural heterogeneity, when compared to other sperm nuclear basic proteins, (Oliva and Dixon 1991, Wyckoff et al. 2000). However, evidence of purifying selection acting on the maintenance of the high arginine concentration exists, while the position of arginine residues seems to be variable, leading to the conclusion that protamine 1 may be affected by positive and purifying selection alike (Rooney et al. 2000). Evidence of positive selection in the protamine 1 gene sequence has been detected in a small group of

primates (Rooney et al. 1999, Wyckoff et al. 2000). Other studies have demonstrated different selective constraints in other mammalian species (Martin-Coello et al. 2009, Lüke et al. 2011). Among cricetid rodents we showed that protamine 1 is under conserved selective constraint with signs of positive selection restricted to specific codon sites. On the other hand, the gene sequence of protamine 2 was shown to be under relaxed constraint on the way to degradation.

There is limited evidence for an effect of sperm competition on coding sequences of sperm proteins. The seminal fluid proteins SEMG2 and SVS, the sperm surface proteins ADAMs 2 and 18, and the acrosomal proteins Zonadhesin and SPAM1 have all been found to be positively affected by level of sperm competition in primates (Dorus et al. 2004, Herlyn and Zischler 2007, Ramm et al. 2008, Finn and Civetta 2010, Prothmann et al. 2012). For butterflies, Walters and Harrison (2011) were able to show a relaxation of purifying selection on seminal fluid proteins in the absence of sperm competition. Similarly, sexual selection on protamine 2 in rodents has been reported to increase selective constraint (Lüke et al. 2011). The evolution of protamine genes and regulatory sequences, as well as their expression, is affected by sperm competition in different groups of rodents. Contrary to expectations, sexual selection could not be detected to act on protamine 1 gene in cricetids or murids. In contrast, sperm competition was shown to reduce the relaxation acting on the gene sequence of protamine 2, resulting in a more conserved state of the gene in species with high levels of sperm competition (Lüke et al. 2011).

Until now sexual selection on protamine gene sequences and its effect on sperm head phenotype has only been studied in rodents. Thus, in this study we aimed to analyze evolutionary patterns of protamine 1 coding sequence and their effects on sperm head dimensions on a broader scale across mammals. The extensive availability of data on protamine 1 sequence in Genbank allowed us to study the evolution and selection of protamine 1 across major mammalian clades. Here we analyze the evolution and selection of protamine 1 in 237 mammal species. We examined the evolutionary rate on whole sequence and site level and tested for sexual selection and possible effect on head dimensions. Additionally, we tested for sexual selection acting on arginine content. We expected to find differences in selective constraints between clades especially in clades with high diversity in levels of sperm competition. We hypothesized that protamine 1 would be sexually selected across mammals and that changes in protamine 1 coding sequence and arginine content may influence head dimensions.

RESULTS

Sequence properties

A summary of all data used in this study is shown in Table S1. Amino acid alignments for all

mammalian species (Eutheria and Metatheria) are shown in Figures S1-S3.

Amino acid frequency and sequence similarity within mammals, as well as within eutherians and marsupials, and within different clades, were compared. Results are shown in Table S2.

Metatheria showed significantly higher relative arginine content than Eutheria ($t(219.33)=-17.26$, $p<0.001$). Within Eutheria, a one-way ANOVA revealed significant differences in arginine content between clades ($F(4,144)=100$, $p<0.001$). Post-hoc comparisons showed that Chiroptera and Rodentia exhibit significantly higher content of arginine in the coding sequence of protamine 1 (Fig. 1A, Table S3). Within Metatheria, Diprotodontia showed a significantly higher arginine content than Dasyuromorpha ($t(52.8) = 9.42$, $p < 0.001$)(Fig. 1B).

Measures of sequence divergence and amino acid frequency within clades are shown in Table S2. Considering the differences in sequence properties between Eutheria and Metatheria all subsequent analyses which included correlation with arginine content were done separately for eutherian and metatherian clades.

Selective pressures across mammals

The phylogenetic tree used in evolutionary analyses is shown in Figure S4. We tested for the general mode of selection acting on protamine 1 in mammals. To obtain the background pressure acting on the whole sequence across all mammals we calculated the evolutionary rate (ω) for the whole tree on the whole protamine 1 sequence (Codeml (PAML4) model M0 as explained in Supplementary materials and methods). The evolutionary rate calculated across mammals in model M0 was $\omega=0.38$.

Comparison of selective pressures

To assess the comparative selective pressures for the entire protamine 1 sequence and the directed selective pressures on codon sites we employed the branch analysis and the branch-site analysis (see Supplementary materials and methods), alternatively marking metatherians as foreground against eutherians as background, and then eutherians as foreground against metatherians as background. The branch analysis comparing Eutheria and Metatheria suggests a significantly stronger selective constraint on metatherians than eutherians (Eutheria: LRT M0 vs MC non significant, M0 ω considered, Metatheria: LRT M0 vs MC significant, MC ω considered). In any case, both clades seem to be evolving under purifying selection (MCfixed vs MC significant, ω is significantly lower than 1) (Table 1). The branch-site test showed no directed selection on codon sites for Eutheria or Metatheria (BSfixed vs BS non significant)(Table 1).

Secondly, we tested for differences between mammalian clades by employing

branch- and branch-site analyses for each group, marking the clade under analysis as foreground against their corresponding eutherian or metatherian background. In the comparison of eutherian clades, we found that Primates, Rodentia and Cetacea showed selective constraints different from the background (M0 vs MC significant, MC ω considered). In contrast, Chiroptera and Artiodactyla showed similar selective constraint as their background (M0 vs MC non significant, M0 ω considered). Primates and Cetacea showed signs of relaxed selective constraint (MCfixed vs MC non significant, ω did not differ significantly from 1), while Rodentia, Chiroptera and Artiodactyla showed evidence for purifying selection (MCfixed vs MC significant, ω is significantly lower than 1). Branch-site analysis revealed evidence for positive selection on codon sites for Chiroptera and Cetacea (BSfixed vs BS significant, PSS detected). For Artiodactyla BSfixed vs BS is significant while no positively selected sites were detected. This might be interpreted as evidence for high purifying selection on codon sites. Primates and Rodentia showed no evidence for positive selection at the site level (BSfixed vs BS non significant) (see Table 1).

In the comparison of metatherian clades neither Dasyuromorpha nor Diprotodontia showed selective constraints differing from the background. The selective constraint is therefore considered the same for both groups (M0 vs MC non significant, M0 ω considered). Diprotodontia showed evidence of purifying selection acting on the protamine 1 coding sequence (MCfixed vs MC significant, ω is significantly lower than 1) while the likelihood ratio test of MCfixed vs MC was not significant for Dasyuromorpha, likelihood ratio test M0 vs MC shows this clade to be evolving under the same selective constraints as the background and Diprotodontia. We therefore consider purifying selection to be the most probable mode of selection acting on this clade. The branch-site test showed directed positive selection on codon sites for Dasyuromorpha and Diprotontia (BSfixed vs BS significant)(Table 1).

The ω calculated by using COEVOL (see below) for each mammalian species is shown in Table S1.

Relationships with a proxy of sexual selection

Rationale for analyses

To examine the possible associations between relative testes mass (a sperm competition proxy) and protamine 1 coding sequence within all mammals and, subsequently, for each clade, we employed COEVOL (a Bayesian Markov Chain Monte Carlo sampling software for comparative analyses) to test for correlations between genotype and phenotype data.

Furthermore we calculated the clades' median absolute deviation of residual testes mass and arginine content to test for an effect of variability of sperm competition ("median absolute deviation of residual testes mass") on the clades' ω value (Codeml (PAML4) model M3, see Supplementary materials and methods), arginine content, and arginine content

variability ("median absolute deviation of arginine content"). The median absolute deviation (or absolute deviation around the median) is a robust measure of variability. It is calculated as the median of the absolute deviations from the data's median (i.e., the median of each absolute value minus the median) and it is not sensitive to the presence of outliers (Huber 1981).

Relationships with relative testes mass across mammals

COEVOL correlation analyses showed no significant correlations between relative testes mass and protamine 1 ω in mammals. A nearly significant positive correlation was found for arginine content with relative testes mass (Fig. 2, Table S4). Arginine content was significantly and negatively correlated with ω (Fig. 2, Table S4). For a comparison between amino acid substitution according to PAML codeml marginal ancestral reconstruction and evolutionary rate as computed by codeml see Figure S5. For Eutheria a significant positive correlation was found between arginine content and relative testes mass (Fig. 2, Table S4).

Relationships with relative testes mass within clades

COEVOL analysis showed significantly positive correlations between arginine content (as percent of sequence length) and relative testes mass for Rodentia and Cetacea (Fig. 2, Table S4). Tests for sexual selection could not be done for Artiodactyla or Chiroptera due to limited data for testes mass.

Median absolute deviation of relative testes mass for clades correlated positively with the clade's ω (M3) and with the clade's median absolute deviation of arginine frequency. On the other hand, it correlated negatively with the clade's mean arginine frequency (Table S3, Table S4).

Because the sequence of protamine 1 was significantly longer in marsupials than in eutherians ($t(227.5) = -72.69$, $p < 0.001$) (Fig. 3A) we tested for possible relationships between relative testes mass and sequence length within this subclass. Due to the presence of distinct sequence length groups we chose to test differences between length groups for corrected testes mass using ANOVA, followed by visual analysis of a trend when plotted against corrected testes mass. We found significant differences between sequence length groups for relative testes mass ($F(43)=7.73$, $p<0.001$) in Metatheria. Post-hoc comparisons showed that the lowest sequence length group (183bp) has significantly lower residual testes mass than the two highest sequence length groups (192bp, 195bp), and that the intermediate length group (189bp) has a significantly lower residual testes mass than the 192bp length group (Fig. 3B, Table S3).

Associations with sperm head dimensions

In order to test for the effect of changes in the coding sequence of protamine 1 on sperm head dimensions, the evolutionary rate and the arginine content were used in COEVOL post-analysis to test for correlation with relative head length (HL), relative head width (HW) and head elongation (HL/HW). For Chiroptera, Artiodactyla and Dasyuomorpha data available for sperm head dimensions were insufficient for regression analyses. For Primates data available for relative HW were insufficient for regression analysis. We examined possible relationships across mammals and within clades.

Across mammals and within eutherians we found a significant negative correlation between arginine content and relative HW (Fig. 2, Table S4) as well as a trend for positive correlation between arginine content and head elongation. In Eutheria the negative correlation between arginine content and relative HL was nearly significant. In Rodentia COEVOL correlation analysis showed a significant negative correlation between arginine content and relative HW (Fig. 2, Table S4) as well as a negative trend for arginine content with relative HL and a positive trend for head elongation. In addition, a trend for a negative correlation was found in Cetacea for arginine content and HW (Fig. 2, Table S4).

DISCUSSION

In this study we analyzed the evolution of protamine 1 in mammals and the possibility that postcopulatory sexual selection may be an important selective driver for this protein. Our results suggest that postcopulatory sexual selection drives maintenance of high arginine content in protamine 1 amino acid sequences across mammals and, in turn, that higher arginine content associates with reduced sperm head width. There was also evidence that higher protamine 1 evolutionary rates are associated with lower arginine content across mammals. On the other hand, the relationship throughout mammals between postcopulatory sexual selection and the arginine content of protamine 1 could not be confirmed within all the mammalian clades but was observed only in two of them, rodents and cetaceans. Within metatherians, the length of protamine 1 seems to be important as it was positively associated to sperm competition levels. Finally, we found that higher variability in inferred sperm competition levels seems to be related to a higher evolutionary rate and, in addition, a higher variability in arginine content (see Fig. 4 for a summary of the relationships found).

Protamine sequence length is sexually selected in Metatheria but not in Eutheria

In this study we analyzed protamine 1 gene sequences of 237 mammalian species of eutherians and metatherians. Marsupial protamine 1 differs from the eutherian gene in several important ways and, in fact, is more similar in structure to bird protamines (Retief et al. 1995a). Whereas eutherian protamine 1 contains 5 to 9 cysteine residues, which allow

protamines to form complex tertiary structures by building disulfide bridges between and within protamines (Balhorn 1982, Oliva and Dixon 1991, Balhorn et al. 1995), metatherian protamine sequences, like those of birds and fishes, lack these residues. An exception is the *Planigale* genus, which seems to have obtained up to 7 cysteine residues by convergent evolution (Retief et al. 1995a). The lack of cysteine residues in most metatherians results in a less stable chromatin which is more easily decondensed compared to that of eutherian mammals (Cummins 1980). Metatherian protamine 1 is significantly longer than eutherian's and contains more serine and tyrosine. N- and C-terminal serine residues, as well as the C-terminal threonine residues, are known phosphorylation targets in protamines (Balhorn 2007). It seems plausible that the higher serine content in metatherian protamine 1 might lead to a higher degree of phosphorylation. Due to these differences, metatherians are likely to bind chromatin in a slightly different manner than eutherians (Balhorn 1985). In fishes and birds the size of protamines was found to be an important factor in chromatin condensation (Oliva et al. 1987, Oliva and Dixon 1991). Since metatherians, as birds and fishes, cannot rely on disulfide bridges to stabilize the chromatin, a longer protamine sequence might be necessary for efficient chromatin condensation. We therefore tested if sexual selection might be affecting protamine 1 sequence length within metatheria and found significant differences in sperm competition levels between sequence length groups across metatherians following a positive trend. Additional analysis including a greater variety of metatherian genera will be necessary to confirm this relationship. However, our results suggest sequence length to be a key factor in stabilizing chromatin in metatherian mammals as shown by its selection towards an increase of sequence length in species with higher levels of sperm competition.

Sperm competition maintains high arginine content of protamine 1 through sequence conservation

Protamine 1 is crucial for correct sperm chromatin condensation. Alterations in protamine content are linked to morphological abnormalities of the sperm head, increases in DNA damage, and decreases in sperm motility (Belokopytova et al. 1993, Cho et al. 2001, Aoki et al. 2005), and strongly affect male fertility (Cho et al. 2003, Aoki et al. 2005, Oliva 2006, Carrel et al. 2007).

It was known that protamines with higher arginine content form more stable chromatin complexes, replace histones more efficiently and are more efficient in chromatin decondensation following fertilization (Ohtsuki et al. 1996). We found arginine content of protamine 1 to be correlated positively with a proxy of sperm competition levels in mammals. This correlation was significant when Metatheria was not included in the analysis; metatherians have a significantly higher arginine content than eutherian mammals and might therefore affect the regression slope. However, when metatherians were included a clear

trend was still observed. Species experiencing higher selective pressure through sperm competition showed higher arginine content in the protamine 1 amino acid sequence. Additionally, we found that an elevated evolutionary rate is generally associated with less arginine in mammals. However an association between sperm competition proxy and evolutionary rate was not found within mammalian clades. Sexual selection on protamine 1 therefore seems to be specifically concentrated on arginine content. Rooney et al (2000) described an unusual form of purifying selection on protamine 1. While the arginine content seemed to be conserved throughout mammals the distribution of arginine residues varies to a great extent. Here we were able to provide evidence for sexual selection as a factor in this unusual form of selection acting on protamine 1. Arginine content seems to be under even stronger selective constraint in highly promiscuous species. These relationships are clear throughout mammals, but we are presented with a more complex scenario when we examine these associations within clades. Evidence of sexual selection on arginine content was only found in two eutherian clades (rodents and cetaceans). If high arginine content is beneficial for species with high levels of sperm competition, and high evolutionary rate shows a general trend towards lower arginine content throughout mammals, why do we not find the same trend when clades are examined?

The gene sequence of protamine 1 seems to be under purifying selective constraint across mammals and within all clades, with the exception of primates and cetaceans, which show evidence of relaxation. We analyzed the evolutionary rate not only on whole sequences but also performed an additional study calculating the possible positive selection at site level. We found signals for positive selection on site level in Chiroptera, Cetacea and in both metatherian clades. Previous studies show that, in rodents, changes in the protamine gene sequences and protamine expression ratio are associated with head size and shape changes (Lüke et al. 2014a,b). Within the coding sequence of protamine 1 in mammals highly variable, as well as highly conserved regions can be found (Oliva and Dixon 1991, Rooney et al. 2000, Wyckoff et al. 2000). In primates the coding sequence was shown to be positively selected (Wyckoff et al. 2000), while within rodents it has been shown to be functionally conserved, with directed positive selection on a few C-terminal sites (Lüke et al. 2011). It seems that the unusual form of selection affecting protamine 1 is driven by adaptations to sexual selection (Oliva and Dixon 1991, Rooney et al. 2000, Wyckoff et al. 2000). Due to the importance in sperm form and function, as well as the protection of sperm DNA, protamine 1 might be affected by different types of selection resulting in a careful balance between conservation of function and adaptations to high sperm competition levels.

The main reason for sexual selection acting on protamine 1 is proposed to be its effect on sperm head shape. Here we found arginine content to be positively associated with slimmer sperm heads including a possible positive effect on sperm head elongation. Yet

also here the relationships seem to be present as a general trend across mammals but not to be as straightforward when focusing on clades. As an adaptation to high levels of sperm competition protamines might affect the head shape to become more hydrodynamically efficient thus favoring higher sperm velocity. However, which type of sperm head is the most beneficial to ensure high sperm velocity is presently unknown. Considering the high variety of sperm head shapes and sperm sizes in mammals (Cummins and Woodall 1985, Roldan et al. 1992, Pitnick et al. 2009) it is plausible that a complex interplay between sperm metabolism, pattern of flagellar movement, total sperm size, hook shape and nucleus shape determine the ability of the sperm to swim faster (Malo et al. 2006, Gómez-Montoto et al. 2011, Tourmente et al. 2013). For example, while a previous study found that relative reduction in protamine 2 content seems to lead to a more beneficial head shape in mice in terms of reproductive fitness (Lüke et al. 2014b), we might not be able to extrapolate this relationship to other groups of species in which, for example, sperm heads do not have a hook or the flagellum shows a different beating pattern. Broader sperm heads might be beneficial for certain sperm morphologies, especially taking into account sperm head thickness. Sexual selection might therefore be affecting the arginine content and evolutionary rate of protamine 1 to varying degrees in different groups of species.

In general terms high evolutionary rate seems to be associated with lower arginine content. We found this relationship across mammals but not within clades. Sexual selection was found to act on cetacean and rodent arginine content. Rodents show high arginine when compared to other eutherian clades which could be proposed to be the result of sexual selection driving high arginine content in this clade. Cetaceans however contradict this theory since this clade shows a comparably low arginine level in protamine 1 sequence. A family level analysis revealing possible differential selection on deeper organizational level might be useful to understand the role sexual selection plays on protamine 1 in this clade. Bats show a high level of arginine when compared to other eutherian mammals and we might expect this to be a result of sexual selection acting to maintain, or promote, a high arginine content. However, not enough data are available on body mass and testes mass in the literature to test for associations in this clade. Thus, further studies are needed to understand the role of protamine 1 in bats.

Less selective constraints in clades with highly variable sperm competition levels?

When comparing selective constraints on protamine coding sequences between clades we observed conserved constraints in all groups except for primates and cetaceans, which showed a comparatively relaxed constraint. This seems to coincide with a comparatively higher variability in inferred levels of sperm competition in both groups. The clades' evolutionary rates correlated positively with clades' variability of sperm competition. While the

clades' variability in arginine content seemed to increase with clades' variability of sperm competition, the clade mean arginine content decreased. Similarly, when looking at eutherians and metatherians in general, the lower selective constraints in metatherians seemed to coincide with lower variability in levels of sperm competition compared to eutherians. A group of species exhibiting big differences in sperm competition therefore also show bigger differences in their coding sequences resulting in the detection of a higher evolutionary rate and less selective constraints in the group. As a result, clades with high variability in sperm competition show higher variability and lower mean arginine content. Therefore, while the effect of sexual selection on evolutionary rate or arginine content seems to be difficult to detect by analyzing the relationship directly through regression analysis, the simple existence of an effect of sexual selection on protamine 1 evolutionary rate might be detected by analyzing levels of variation. This might lead to the conclusion that protamine 1 is sexually selected in primates and cetaceans while the nature and direction of the selection is complex and could be different between primate and cetacean families.

Conclusions

Adaptation to high levels of sperm competition seems to be a factor in the unusual form of purifying selection of protamine 1 as described by Rooney et al (2000). We found evidence for sexual selection, in the form of sperm competition, maintaining a high arginine content across mammals, while changes in the protamine 1 coding sequence seem to be associated with less arginine. A higher arginine content seems to be associated with a reduction in sperm head width and was previously found to result in more efficient chromatin condensation. Due to a lack of cysteine residues, metatherian protamine 1 cannot rely on disulfide bridges to stabilize chromatin; this may be compensated by an increase in sequence length, which is sexually selected. Within mammalian clades sexual selection on arginine content was detected within Rodentia and Cetacea, while we found differences in selective pressures on sequences and codon sites between mammalian clades. Due to the wide variety of sperm head morphologies within mammals changes in arginine content and changes in protamine coding sequence might be beneficial in some mammalian groups and disadvantageous in others. This would result in a complex evolutionary pattern of protamine 1 and difficulties in detecting sexual selection within mammalian groups. However, due to general sequence conservation, and the importance of arginine in chromatin condensation and head shaping, a general trend for sexual selection acting on arginine content, and its possible effect of sperm head width, has been identified in mammals. Since the interplay between evolutionary rate, arginine content, sperm head morphology, and swimming speed seems to be very complex, the effect of sexual selection on protamine 1 might not be readily detected in some groups. However, if an effect of sexual selection exists in general, it might

be revealed when testing for an association between the level of variability in sperm competition and evolutionary rate.

MATERIAL AND METHODS

Sequence data and phylogenetic tree

Gene sequences of mammalian protamine 1 for which at least 10 species were available were obtained from NCBI Genbank and previous publications (Lüke et al. 2011) (Table S1), visualized with Geneious 5.5.9 (Biomatters, <http://www.geneious.com/>) and trimmed to coding sequence based on NCBI Genbank information. Sequences were manually checked to ensure correct trimming. Translation alignments based on the muscle alignment algorithm implemented in Geneious 5.5.9 were performed and checked manually. In addition to an alignment including all 237 mammalian species (translated alignment see Fig. S1) we performed separate alignments for each mammalian clade studied (Primates, Rodentia, Chiroptera, Cetacea, Artiodactyla, Dasyuromorpha, Diprotodontia) as well as separate alignments for eutherians and metatherians (translated alignment see Figs. S2 and S3). Amino acid frequencies, pairwise percent identity and percentage of identical sites were calculated using Geneious 5.5.9 for each alignment.

The phylogenetic tree of 237 included mammalian species was constructed as a consensus of phylogenies available in literature (Fig. S4 and references therein).

Phenotype data

Data on body mass, testes mass and sperm dimensions were obtained from the literature (Table S1 and references therein). Testes mass corrected for body mass ("relative testes mass", is a well recognized proxy for sperm competition; Birkhead et al. 2009, Gomendio et al. 1998, Soulsbury and Dornhaus 2010). Testes and body mass data were available for 132 of the 237 species for which protamine 1 information was available. Data on sperm head width were found for 65 species, and sperm head length for 87 species. Residual testes mass data were obtained from a regression analysis including body mass as independent and testes mass as dependent variable. Residual testes mass was obtained for each clade separately due to previously reported differences of body mass and testes mass regression slopes between clades (MacLeod 2010). Residual testes mass was used for graphical representation of multiple regression results and comparisons between clades. Because total sperm length varies greatly among-species, and drag resulting from head size should be analyzed taking into account the length of the flagellum (Humphries et al. 2008), sperm head length and head width were each employed in analyses as proportions of total sperm length (TSL) (hereafter, relative HL and relative HW).

Analysis of selective pressures

The nonsynonymous/synonymous substitutions rate ratio ($\omega = dN/dS$) is an indicator of selective pressure at the protein level, with $\omega=1$ indicating neutral evolution, $\omega<1$ purifying selection, and $\omega>1$ diversifying positive selection (Goldman and Yang 1994). To estimate rates of sequence evolution across mammals and within clades we used the application Codeml implemented in PAML 4 (Yang and Rannala 1997, Yang 2007). Applied models are explained in Supplementary materials and methods. Likelihood-ratio-tests (LRT) were performed to test if the alternative model presents a better fit to the dataset against the null model. For the Codeml codon frequency setting, as well as the setting for number of categories, we used the setting with the best fit for each analysis according to the preliminary likelihood-ratio-analysis. Branch lengths calculated in the model M0 “one-ratio” (see Supplementary materials and methods) were used as input for subsequent models. For estimation of rates of sequence evolution per branch for subsequent correlation with phenotypic data we employed COEVOL (Lartillot and Poujol 2011).

Association between evolutionary rate and phenotype data

In order to test for associations between protamine 1 and relative testes mass as well as its effect on sperm head dimensions we employed the program COEVOL a Bayesian Markov Chain Monte Carlo sampling software for comparative analyses, to test for correlation between genotype and phenotype data. It allows for joint estimation of evolutionary rate for the input alignment and changes in the phenotypic input variables. It allows for correlation and multiple regression analyses. Unlike previous approaches this software allows for detection of associations between genotypic and phenotypic data taking into account all parameter estimates of internal nodes. Additionally, COEVOL can be used to estimate ancestral traits and evolutionary rates (Lartillot and Poujol 2011). We ran two independent chains per analysis providing an input nucleotide alignment, data-table of quantitative traits and phylogeny without branch-lengths (Fig. S4). Chains were run until discrepancy between summary statistics was below 0.02 and effective size larger than 300. After runs are completed correlations, divergence times and ancestral traits were estimated in COEVOL. To test for associations between testes mass and other variables, correlations were corrected for body mass computed by COEVOL using a multiple regression approach. For correlation analyses we used a burn-in of 300. Branch lengths were calculated by COEVOL. The program additionally produces trees and tabulated lists of ancestral rates and traits. These data were used for visualization of results (see Fig. 2).

Phylogenetically corrected regression analysis (PGLS)

To test for correlations between clades' evolutionary rates and sperm competition variability

we employed the phylogenetic generalized least squares approach (PGLS) (Freckleton 2002). Associations between genetic and morphometric traits should also take into account that such traits are not independent from their phylogenetic history (Harvey and Pagel 1991). The PGLS approach has been shown to be a powerful tool to detect associations of this kind (Rohlf 2001), and it has been used in earlier studies in combination with the root-to tip dN/dS method showing genetic-morphometric associations (Lüke et al. 2011, Montgomery et al. 2011, 2012, Pointer et al. 2012). We performed PGLS analysis using CAPER v0.5 (Orme et al., 2012) package for R (v3.0.1; R Foundation for Statistical Computing 2013).

ACKNOWLEDGEMENTS

We thank Francois Serra and Hernan Dopazo for help with preliminary work on rodent and primate protamine 1 sequence evolution. We are very grateful to Nicolas Lartillot for his help and support with COEVOL analysis. This work was supported by funds from the Spanish Ministry of Economy and Competitiveness (grant number CGL2011-26341). L.L. was funded by a predoctoral studentship from the CSIC's JAEpredoc program. M.T. was supported by a Juan de la Cierva postdoctoral fellowship from the Spanish Ministry of Economy and Competitiveness.

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FIGURE LEGENDS

Figure 1. Boxplots for arginine content (as percentage of total sequence length). (A) Eutherian clades. (B) Metatherian clades. Clades with comparatively high arginine content within each subclass are shown as hatched bars. Comparisons are statistically significant between all clades except between Artiodactyla and Primates.

Figure 2. Visualization of associations identified using COEVOL. (A) Phylogenetic visualization of COEVOL computed protamine 1 dN/dS reconstruction for mammalian species. (B) Phylogenetic visualization of COEVOL computed reconstruction of protamine 1 arginine content (%) for mammalian species. (C) Scatterplot showing relationship between protamine 1 arginine content and dN/dS including reconstructed ancestral node data. (D) Scatterplot showing correlation between residual testes mass and protamine 1 arginine content (as percentage of total sequence length) in mammals (Eutheria and Metatheria highlighted). (E) Correlations between protamine 1 arginine content and testes mass in mammals (Rodentia and Cetacea highlighted). (F) Correlations between protamine 1 arginine content (as percentage of total sequence length) and relative sperm head width in mammals (Eutheria and Metatheria highlighted). (G). Correlations between protamine 1 arginine content (as percentage of total sequence length) and relative sperm head width in mammals (Rodentia and Cetacea highlighted). *P* values are shown for all mammals and for highlighted clades and represent level of significance according to COEVOL analysis.

Figure 3. Sequence length in Metatheria. (A) Boxplot visualizing significant difference in protamine 1 coding sequence length between Eutheria and Metatheria. (B) Visualization of associations between metatherian protamine 1 coding sequence length groups and residual testes mass.

Figure 4. Schematic presentation of relationships found in this study. Arrows represent relationships found across mammals. Dashed arrows represent relationships not found across mammals and proposed to be diverse between groups. Positive correlations are marked with (+) and negative correlations are marked with (-). Diverse or unknown relationships are marked with (?).

Table 1. Results of branch analyses and branch-site analyses for eutherian and metatherian clades, as well as Eutheria and Metatheria.

Foreground branches	LRTs for branch analysis		LRTs for branch-site analysis				Proportion of sites in ω site classes (BS):				Positively selected sites (BEB $p < 0.05$)	interpretation		
	2Δ (M0-MC)	P	2Δ (MCfixed-MC)	P	2Δ (BSfixed-BS)	P	ω	0	1	2a		2b	Selection over whole sequece	Directed selection on sites
Primates	13.77	0.01	1.09	ns	3.02	ns	0.791	0.53	0.15	0.25	0.07		relaxed	non significant PSS
Rodentia	12.38	0.01	71.95	0.00	2.12	ns	0.226	0.65	0.32	0.02	0.01		purifying	no signal
Chiroptera	-0.15	ns	30.26	0.00	15.34	0.00	0.385	0.65	0.27	0.06	0.02	54R, 55Y	purifying	positive
Artiodactyla	0.28	ns	6.15	0.05	5.48	0.05		0.68	0.32	0.00	0.00		purifying	purified
Cetacea	15.57	0.00	-5.70	ns	5.65	0.05	0.94	0.43	0.20	0.26	0.12	5R, 15C, 28C, 32R, 35R, 55Y, 60R	relaxed	positive
Dasyuromorphia	-10.37	ns	2.19	ns	18.05	0.00	0.237	0.73	0.24	0.02	0.01	27V, 28R	purifying	positive
Diprotodontia	2.57	ns	100.76	0.00	23.16	0.00	0.237	0.74	0.12	0.12	0.02	41R, 42R, 43R, 45R, 47K, 48G	purifying	positive
Eutheria	2.71	ns	134.76	0.00	-2.80	ns	0.376	0.44	0.28	0.17	0.11		purifying	non significant PSS
Metatheria	5.67	0.05	146.75	0.00	-3.87	ns	0.273	0.57	0.26	0.12	0.05		purifying	non significant PSS

LRT= Likelihood ratio test, ω =clade's ω as calculated by branch analysis; if LRT of M0 versus MC significant MC, ω is reported; if LRT is non significant, M0 ω is reported. PSS=positively selected sites. ω site classes: 0: $0 < \omega < 1$ for foreground and background branches, 1: $\omega = 1$ for foreground and background branches, 2a: $0 < \omega < 1$ for background and $\omega > 1$ for foreground, 2b: $\omega = 1$ for background and $\omega > 1$ for foreground.

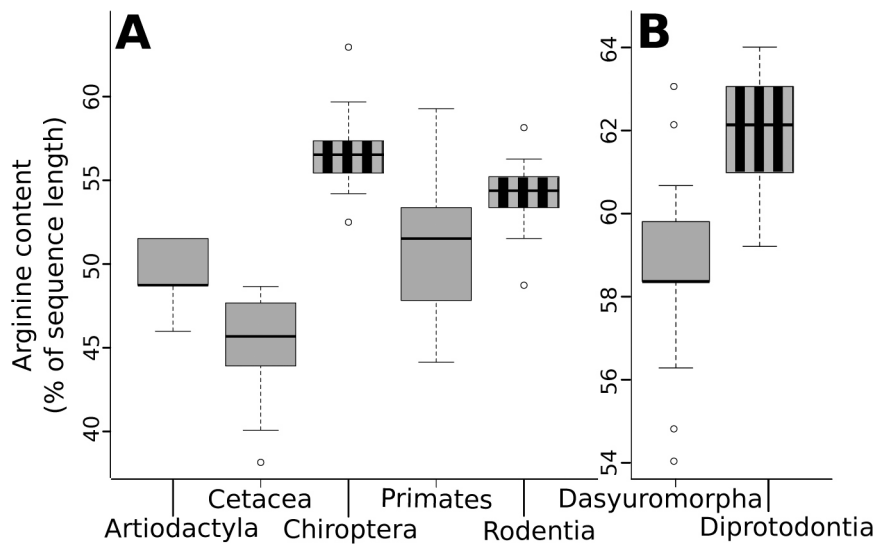


Figure 1

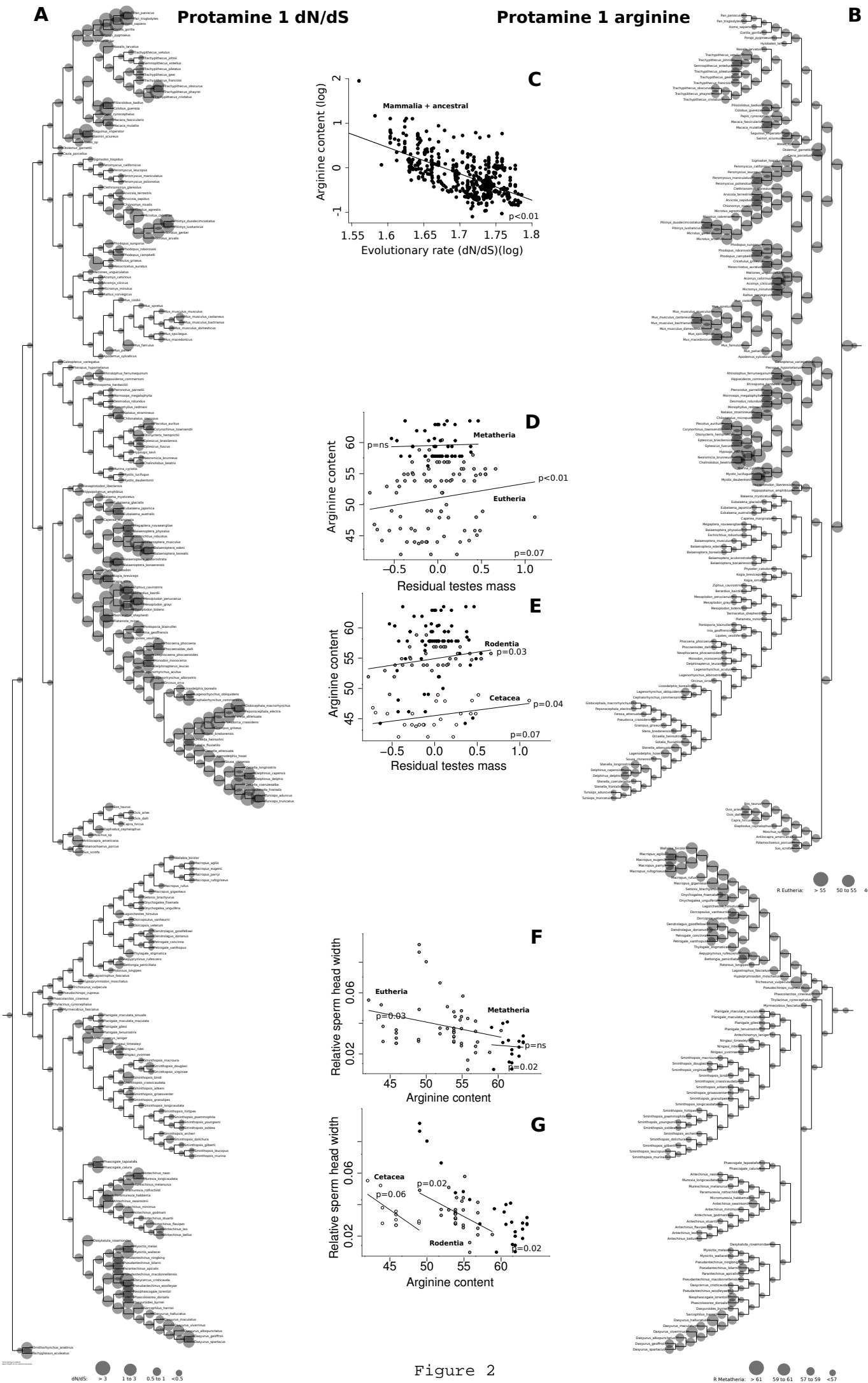


Figure 2

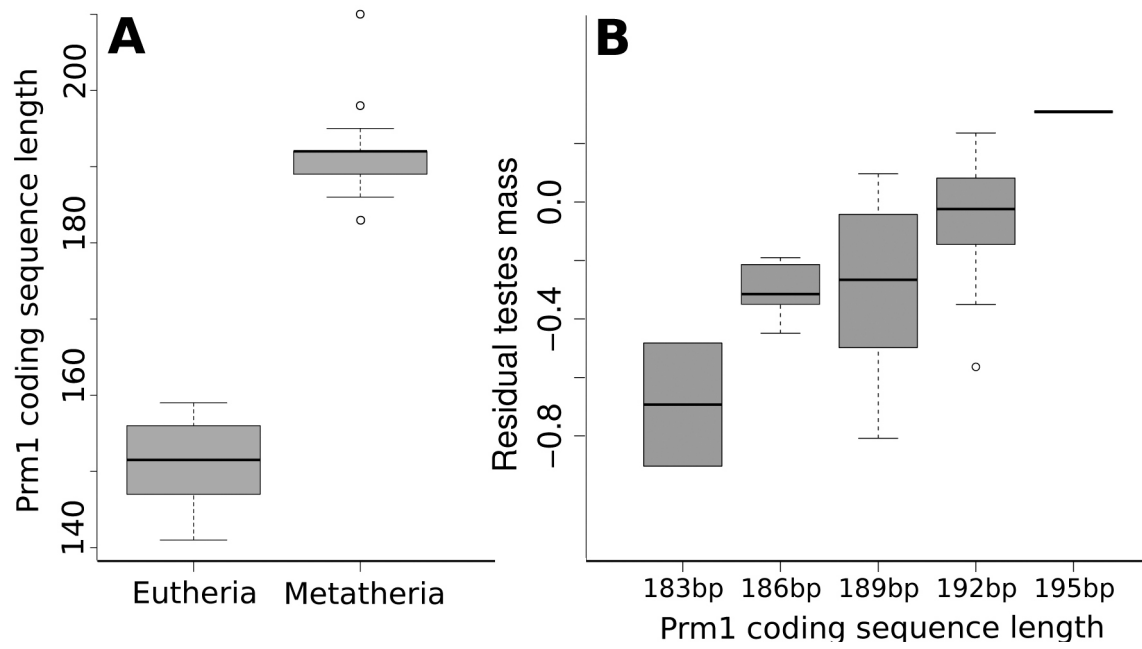


Figure 3

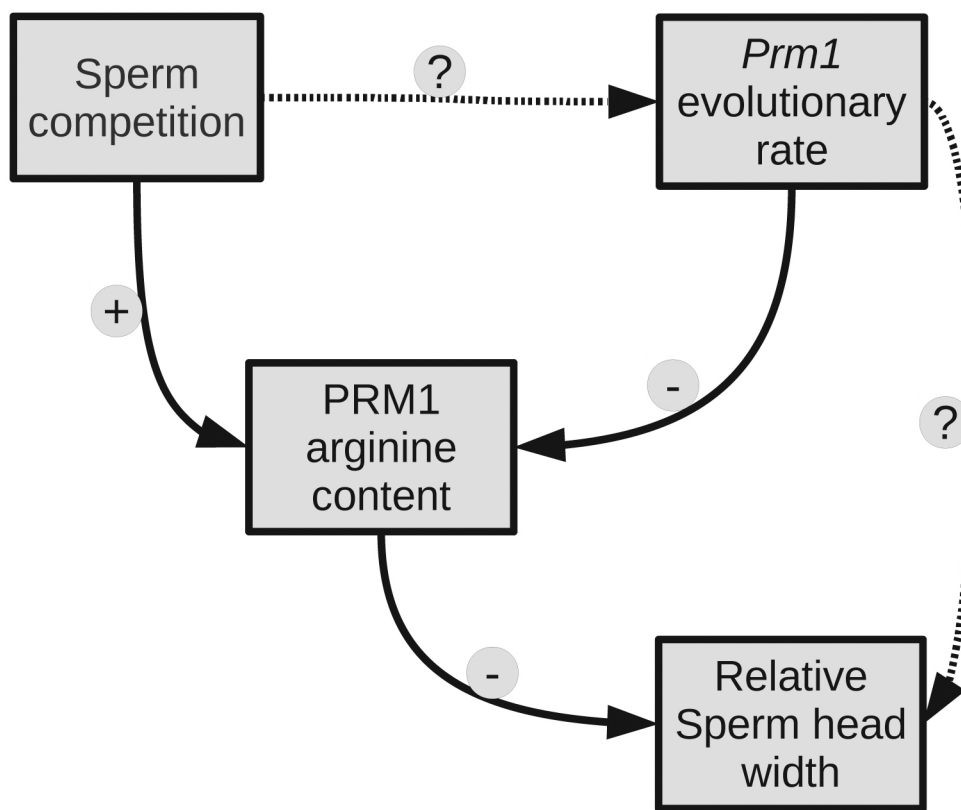


Figure 4

Supplementary Information for

Sexual selection of protamine 1 in mammals

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Table S1. Data included in this study: sperm head width, sperm head length, total sperm length, body mass, testes mass; residual testes mass calculated separately for each clade.

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Figure S2. Multiple sequence alignment for eutherian species based on muscle algorithm.

Figure S3. Multiple sequence alignment for metatherian species based on muscle algorithm

Table S2. Clade descriptive statistics of residual testes mass and arginine content data and amino acid alignment statistics.

Table S3. Results for Tukey HD post-hoc comparisons for one-way anovas.

Figure S4. Phylogenetic tree constructed as consensus of phylogenetic data available in the literature.

Table S4. Results of COEVOL correlation analysis and phylogenetically controlled regression analyses (PGLS).

Figure S5. Comparison of amino acid substitutions along branches and dN/dS branch values.

Supplementary Material and Methods

I. EVOLUTIONARY MODELS APPLIED IN CODEML (PAML4)

A. Branch analysis

In order to obtain the evolutionary rate of clades and groups of species we computed the clade model comparing marked foreground branches against the unmarked background in the analysed phylogenetic tree. Three models were computed: M0 “one ratio” in which all branches were constrained to evolve at the same rate; M_{Cfixed} “two-ratio, foreground fixed” where the background clade ω was allowed to be estimated freely while the foreground ω was restrained to a value of $\omega = 1$; and M_C “two ratio” model which estimates for both background and the Cricetidae clade a free and independent ω . To test if the foreground evolves at a significantly different rate than the background we compare M0 versus M_C by means of LRT. If foreground omega is significantly higher than 1 (LRT significant for M_{Cfixed} vs M_C and $\omega > 1$) we assume positive selection acting on the foreground branches on whole sequence level. If foreground omega is significantly lower than 1 (LRT significant for M_{Cfixed} vs M_C and $\omega > 1$) we report purifying selection acting on the branch on whole sequence level. Relaxed selective constraint for the foreground branch is assumed if foreground evolves at a significantly different ω than the background (M0 vs M_C), and this ω was not significantly different from 1 (M_{Cfixed} vs M_C) (Yang 1998).

B. Branch-site analysis

Similarly, to test evolution along coding sequences and infer amino acids under positive selection for marked foreground branches in contrast to the unmarked background two models were computed. B_{Sfixed} “branch-site model A, foreground fixed” in which the codon site ω for background branches is allowed to be computed freely and B_S “branch-site model A” in which codon sites in both foreground and background are computed freely (Zhang et al. 2005). Evidence of the existence of positively selected codon sites (PSS) is reported if LRT between B_{Sfixed} and B_S is significant and sites significantly belonging to the positive selected site category are reported by the model.

II. EXPERIMENTAL DESIGN

A. Comparison of sequence properties

Amino acid frequency and sequence similarity within mammals as well as within eutherian and marsupialian mammals and within the 7 clades were compared and described (Table S1).

B. Selective pressures across mammals

In a first instance we test for the general mode of selection acting on protamine 1 in mammals. To obtain the background pressure acting on the whole sequence across all mammals we calculate an ω for the whole tree on the whole sequence (M0 as explained above).

C. Comparison of selective pressures

In order to obtain comparative selective pressures for the whole sequence and for directed selective pressure on codon sites we employ the branch analysis and the branch-site analysis as explained above, once marking metatherians as foreground against eutherians as background and once marking eutherians as a foreground against metatherians as background. Secondly we test for differences between mammalian clades by employing a branch- and branch-site analysis for each group marking the respective clade as foreground against either eutherian or metatherian background.

D. Testing for sexual selection

To test for sexual selection on protamine coding sequence within mammals and subsequently in clades, we determined evolutionary rate for each branch in the included phylogenetic tree followed by a analysis of associations with testes mass corrected for body mass (computed in COEVOL a Bayesian Markov Chain Monte Carlo sampling software for comparative analyses, to test for correlation between genotype and phenotype data. It allows for joint estimation of evolutionary rate for the input alignment and changes in the phenotypic input variables). Additionally we calculated the clades median absolute deviation of residual testes mass and arginine content to test for an effect of variability of sperm competition (median absolute deviation of residual testes mass) on the clades ω (M3), arginine content and arginine content variability (median absolute deviation of arginine content).

E. Effect on sperm head dimensions

In order to test for the effect of changes in the coding sequence of protamine 1 on sperm head dimensions, the evolutionary rate or arginine content were examined for associations with relative HL, relative HW and head elongation (HL/HW) by using COEVOL. We tested for effects on sperm head dimensions across mammals and within clades.

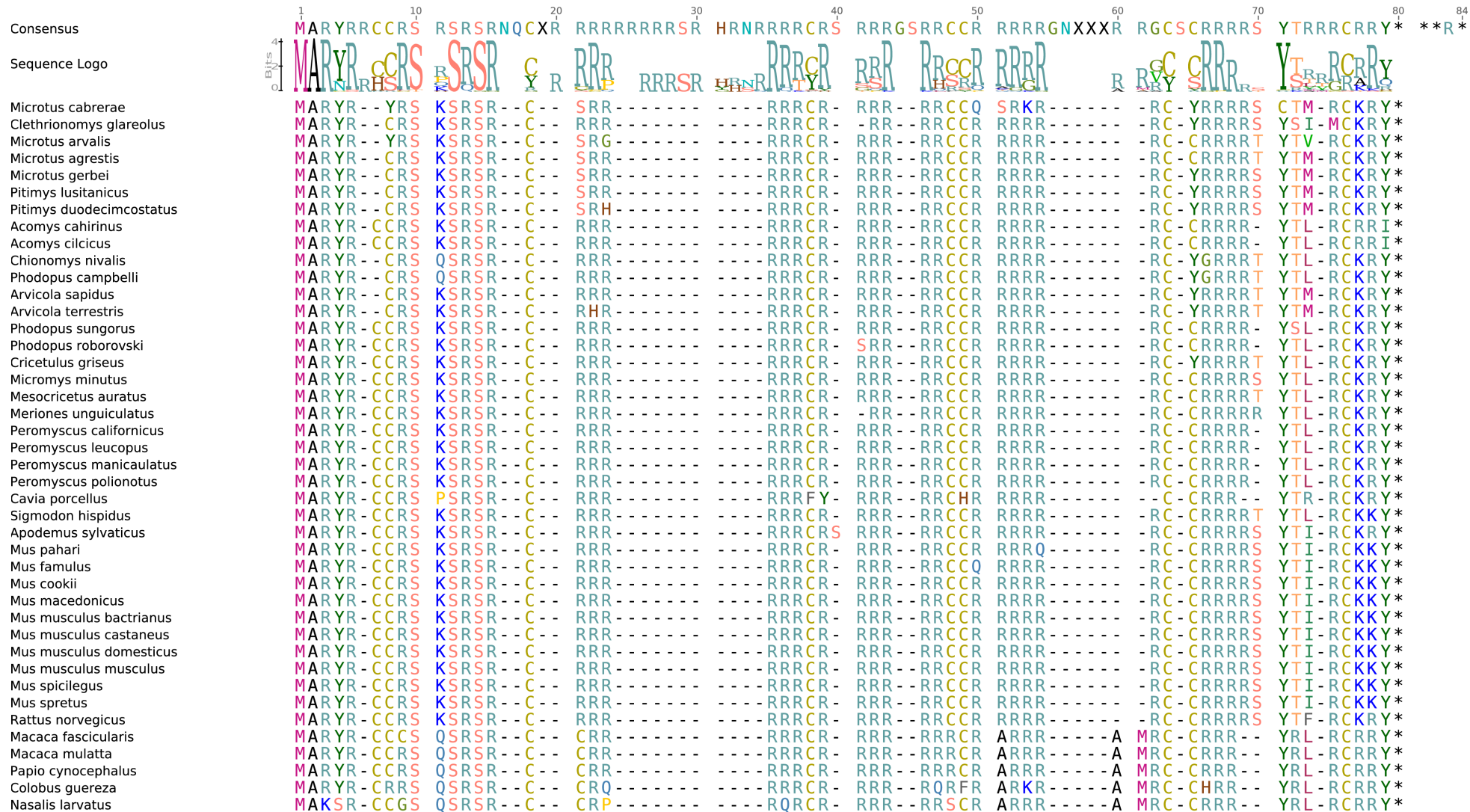
<i>Smigodon hispidus</i>	Eutheria	Rodentia	Cricetidae	0.345	156	54	3.20	6.10	87.00	0.04	0.07	1.91	223.00	1.73	2.35	0.24	-0.13	EU980395	Cummins and Woodall 1985	Kenagy and Trombulak 1986
<i>Smynthopsis aitkeni</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.449	192	58							18.00	0.11	1.26	-0.96	-0.15	AF089871		Taggart et al 1998
<i>Smynthopsis archeri</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.189	192	58												AF089872		
<i>Smynthopsis binali</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.166	192	58							15.30	0.11	1.18	-0.97	-0.11	AF089873		Taggart et al 1998
<i>Smynthopsis crassicaudata</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.411	192	58			12.80	264.90	0.05		14.60	0.17	1.16	-0.78	0.09	L32743	Gage 1998	Taggart et al 1998
<i>Smynthopsis dolichura</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.462	192	58							16.00	0.14	1.20	-0.85	0.00	AF089874		Taggart et al 1998
<i>Smynthopsis douglasi</i>	Metatheria	Dasyuromorpha	Dasyuridae	1.010	192	58												AF089875		
<i>Smynthopsis gilberti</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.485	192	58												AF089876		
<i>Smynthopsis granulipes</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.535	192	58							18.50	0.28	1.27	-0.55	0.25	AF089877		Taggart et al 1998
<i>Smynthopsis oriseoventer</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.539	192	59							20.00	0.15	1.30	-0.82	-0.04	AF089878		Taggart et al 1998
<i>Smynthopsis hirtipes</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.477	192	58							16.50	0.16	1.22	-0.81	0.03	AF089879		Taggart et al 1998
<i>Smynthopsis leucopus</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.441	192	58							22.50	0.23	1.35	-0.63	0.11	AF089880		Taggart et al 1998
<i>Smynthopsis longicaudata</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.935	189	59												AF089881		
<i>Smynthopsis macroura</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.349	192	58							20.00	0.24	1.30	-0.62	0.16	AF001586		Taggart et al 1998
<i>Smynthopsis murina</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.401	192	58							20.50	0.27	1.31	-0.58	0.20	AF001585		Taggart et al 1998
<i>Smynthopsis ooldea</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.424	192	58							12.00	0.11	1.08	-0.97	-0.04	AF089882		Taggart et al 1998
<i>Smynthopsis psammophila</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.408	192	58												AF089883		
<i>Smynthopsis virginiae</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.407	192	58							31.00	0.25	1.49	-0.60	0.05	AF089884		Taggart et al 1998
<i>Smynthopsis youngsoni</i>	Metatheria	Dasyuromorpha	Dasyuridae	0.315	192	58							11.00	0.12	1.04	-0.94	0.02	AF089885		
<i>Sotalia fluviatilis</i>	Eutheria	Cetacea	Delphinidae	0.597	141	49												JF505012		
<i>Sousa chinensis</i>	Eutheria	Cetacea	Delphinidae	0.530	144	48												JF505010		
<i>Stenella attenuata</i>	Eutheria	Cetacea	Delphinidae	0.506	141	49							80500.00	1029.03	4.91	3.01	0.04	JF505009		MacLeod 2010
<i>Stenella coeruleoalba</i>	Eutheria	Cetacea	Delphinidae	2.229	144	46												EU697409		
<i>Stenella frontalis</i>	Eutheria	Cetacea	Delphinidae	1.209	141	49												JF505008		
<i>Stenella longirostris</i>	Eutheria	Cetacea	Delphinidae	0.507	141	49												JF505007		
<i>Steno bredanensis</i>	Eutheria	Cetacea	Delphinidae	1.129	144	48		2.00	3.60			1.80						EU697407	Meisner et al 2005	
<i>Sus scrofa</i>	Eutheria	Artiodactyla	Suidae	0.168	153	49	5.00	8.50	54.60	0.09	0.16	1.70	39700.00	128.20	4.60	2.11	-0.12	NM_214253	Cummins and Woodall 1985; Gage 1998	Almeida et al 2006
<i>Tasmacetus shepherdi</i>	Eutheria	Cetacea	Ziphiidae	2.356	150	42												GQ368521		
<i>Thylacynus cynocephalus</i>	Metatheria	Dasyuromorpha	Thylaciniidae	0.374	192	58												UB7140		
<i>Thylagale stigmatica</i>	Metatheria	Diprotodontia	Macropodidae	5.803	189	62	2.20	7.20	110.30	0.02	0.07	3.27						AF187534	Cummins and Woodall 1985	
<i>Trachypithecus cristatus</i>	Eutheria	Primates	Cercopithecidae	2.232	156	52												AF294861		
<i>Trachypithecus francoisi</i>	Eutheria	Primates	Cercopithecidae	0.159	156	52												AF119234		
<i>Trachypithecus geei</i>	Eutheria	Primates	Cercopithecidae	0.226	156	52												AF294857		
<i>Trachypithecus johnii</i>	Eutheria	Primates	Cercopithecidae	0.219	156	54												AF294853		
<i>Trachypithecus obscurus</i>	Eutheria	Primates	Cercopithecidae	1.458	156	52												AF119238		
<i>Trachypithecus phayrei</i>	Eutheria	Primates	Cercopithecidae	1.291	153	51												AF294858		
<i>Trachypithecus pileatus</i>	Eutheria	Primates	Cercopithecidae	0.212	156	52												AF294856		
<i>Trachypithecus vetulus</i>	Eutheria	Primates	Cercopithecidae	0.360	153	53												AF119236		
<i>Trichosurus vulpecula</i>	Metatheria	Diprotodontia	Phalangeridae	0.183	189	63	2.58	5.53	94.17	0.03	0.06	2.14	3350.00	8.26	3.53	0.92	0.11	L32744	Cummins and Woodall 1985	Taggart et al 1998
<i>Tursiops aduncus</i>	Eutheria	Cetacea	Delphinidae	0.329	144	46												JF505006		
<i>Tursiops truncatus</i>	Eutheria	Cetacea	Delphinidae	5.738	144	46	2.13	4.41	70.16	0.03	0.06	2.07	146250.00	756.40	5.17	2.88	-0.26	GQ368508	Kita et al 2001; Plen and Bernard 2006	MacLeod 2010
<i>Wallabia bicolor</i>	Metatheria	Diprotodontia	Macropodidae	12.746	189	63	3.05	7.80	109.50	0.03	0.07	2.56	31500.00	14.75	4.50	1.17	-0.38	L35328	Cummins and Woodall 1985	Taggart et al 1998
<i>Ziphius cavirostris</i>	Eutheria	Cetacea	Delphinidae	5.363	150	42							3000000.00	8001.00	6.48	3.90	-0.08	EU444928		MacLeod 2010

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Figure S1. Multiple sequence alignment for all 237 included mammal species based on muscle algorithm (implemented in Geneious 5.5.9.)



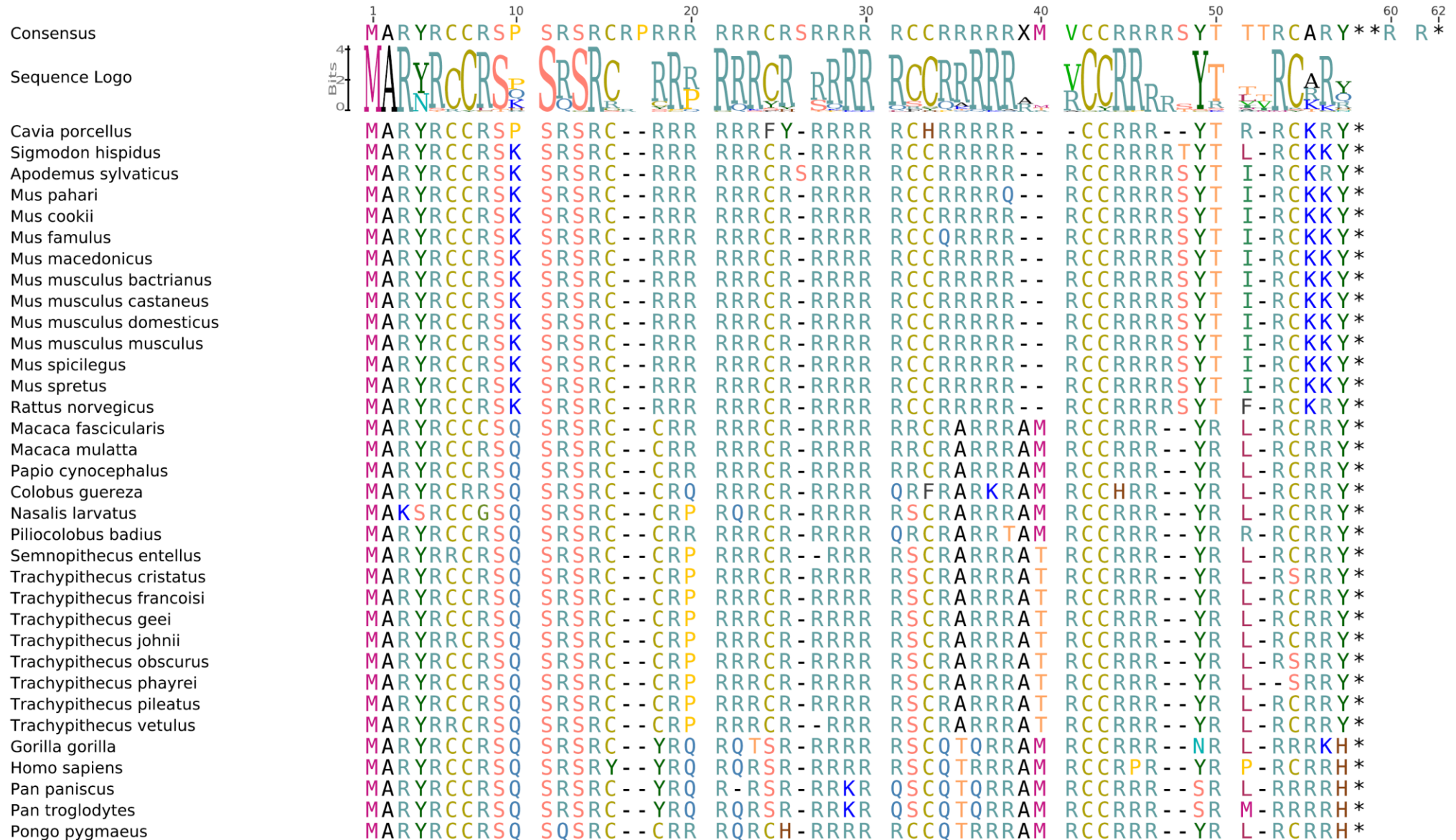
<i>Ptilocolobus badius</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RQRCR	ARRT-----A	MRC-CRRR--	YRR-RCRRY*
<i>Semnopithecus entellus</i>	MARYR-RCRS	QSRSR--C--	CRP-----	RRRCR--RR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RCRRY*
<i>Trachypithecus cristatus</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RSRRY*
<i>Trachypithecus obscurus</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RSRRY*
<i>Trachypithecus phayrei</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL--SRRY*
<i>Trachypithecus francoisi</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RCRRY*
<i>Trachypithecus geei</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RCRRY*
<i>Trachypithecus pileatus</i>	MARYR-CCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RCRRY*
<i>Trachypithecus vetulus</i>	MARYR-RCRS	QSRSR--C--	CRP-----	RRRCR--RR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RCRRY*
<i>Trachypithecus johnii</i>	MARYR-RCRS	QSRSR--C--	CRP-----	RRRCR--RRR--RRSCR	ARRR-----A	TRC-CRRR--	YRL-RCRRY*
<i>Gorilla gorilla</i>	MARYR-CCRS	QSRSR--C--	YRQ-----	RQTSR--RRR--RRSCQ	TQRR-----A	MRC-CRR--	RNRLRRRKH*
<i>Homo sapiens</i>	MARYR-CCRS	QSRSR--Y--	YRQ-----	RQRSR--RRR--RRSCQ	TRRR-----A	MRC-CRPR--	YRP-RCRRH*
<i>Pan paniscus</i>	MARYR-CCRS	QSRSR--C--	YRQ-----	RRSR--RRK--RQSCQ	TQRR-----A	MRC-CRR--	RSRLRRRRH*
<i>Pan troglodytes</i>	MARYR-CCRS	QSRSR--C--	YRQ-----	RQRSR--RRK--RQSCQ	TQRR-----A	MRC-CRR--	RSRMRRRRH*
<i>Pongo pygmaeus</i>	MARYR-CCRS	QSRSR--C--	YRQ-----	RQRSR--RRR--RRSCQ	TRRR-----A	MRC-CRPR--	YRL-RRRRH*
<i>Hylobates lar</i>	MARYR-CCRS	RSRSR--C--	YRQ-----	RPRCR--RRR--RRSCR	RRRG-----	SRC-CRRR--	YRL-RRRRY*
<i>Ateles sp.</i>	MARYR-CCRS	QSRSR--C--	YRQ-----	RRRGR--RRR--RRTCR	RRRA-----	SRC-CRRR--	YKL-TCRRY*
<i>Saguinus imperator</i>	MARYR-CCRS	RSRSR--C--	YRR-----	RRRCR--TRR--RRCCR	RRRA-----	RRC-CRRR--	YKL-RCRRY*
<i>Saimiri sciureus</i>	MARYR-CCRS	QSRSR--C--	RRR-----	RRRCR--RRR--RRCCR	RRRR-----	-RC-CRRR--	YRL-RCRRY*
<i>Otolemur garnettii</i>	MARNR-CCRS	QSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARR*
<i>Balaena mysticetus</i>	MARNR-CCRS	QSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARR*
<i>Eubalaena australis</i>	MARNR-CCRS	QSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARR*
<i>Eubalaena glacialis</i>	MARNR-CCRS	QSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARR*
<i>Eubalaena japonica</i>	MARNR-CCRS	QSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARR*
<i>Balaenoptera physalus</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRRR--SOR--RRCCQ	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Balaenoptera musculus</i>	MARNT-CCRS	PSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Balaenoptera acutorostrata</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCAGQ*
<i>Balaenoptera bonaerensis</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCAGQ*
<i>Balaenoptera borealis</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Balaenoptera edeni</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Megaptera novaeangliae</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRRR--SOR--RRCCQ	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Eschrichtius robustus</i>	MARNS-CCRS	PSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Caperea marginata</i>	MARNR-CCRS	QSRSR--R--	RRP-----	RQRCR--SOR--RRCCR	RRRR-----	-VC-CRR--	YTTVRCARQ*
<i>Cephalorhynchus commersonii</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YATTRCARQ*
<i>Delphinus capensis</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCAR--*
<i>Delphinus delphis</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCAR--*
<i>Feresa attenuata</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRYR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Globicephala macrorhynchus</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRYR--RR--RQCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Grampus griseus</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRYR--RR--RQCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Lagenodelphis hosei</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCAR--*
<i>Lagenorhynchus acutus</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCL--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCAR--*
<i>Lagenorhynchus obliquoidens</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCAR--*
<i>Lagenorhynchus albirostris</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCASQ*
<i>Lissodelphis borealis</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Orcaella heinsohni</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Orcinus orca</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--IRCCR	RQRR-----	-VC-CRR--	YTTTRCARQ*
<i>Peponocephala electra</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRYR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Pseudorca crassidens</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRYR--RR--RQCCR	RRRR-----	-VC-CRR--	YTTTRCARQ*
<i>Sotalia fluviatilis</i>	MARNR--CRS	PSQSR--C--	RRP-----	RRRCR--RR--RRCCR	RRRR-----	-VC-CRR--	YTTTRCAR--*

Sousa chinensis	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Stenella attenuata	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTTTRCAR-	*
Stenella frontalis	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTTTRCAR-	*
Stenella longirostris	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTTTRCAR-	*
Stenella coeruleoalba	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	-RLCCR	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Steno bredanensis	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Tursiops aduncus	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	-RLCCR	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Tursiops truncatus	MARNR	--CRS	PSQSR	--C--	RRP	-----	---	RRRCR	-RR	-RLCCR	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Inia geoffrensis	MARNR	--CRS	PSQSR	--G--	RRP	-----	---	RRRYR	SRR	--RRCCQ	RRRR	-----	-VC	-CRR	---	YTTVRCARQ*	
Lipotes vexillifer	MARNR	--CRS	PSQSR	--G--	RRP	-----	---	RRKYR	SRR	--RRCCQ	RRRR	-----	-VC	-CRR	---	YTTMRCAKQ*	
Delphinapterus leucas	MARNR	--CRS	PSQSR	--G--	RRP	-----	---	RRRYR	SKR	--RRCCQ	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Monodon monoceros	MARTR	--CRS	PSQSR	--G--	RRP	-----	---	RRRYR	SKR	--RRCCQ	RRRR	-----	-VC	-CRR	---	YTTTRCARQ*	
Neophocaena phocaenoides	MARNR	--CRS	PSQSR	--G--	RCP	-----	---	RRRYR	SKR	--RRCCQ	RRRR	-----	-VC	-RRR	---	YTR-RCARQ*	
Phocoena phocoena	MARNR	--CRS	PSQSR	--G--	RCP	-----	---	RRRYR	SKR	--RRCCQ	RRRR	-----	-VC	-RRR	---	YTR-RCARQ*	
Phocoenoides dalli	MARNR	--CRS	PSQSR	--G--	RCP	-----	---	RRRYR	SKR	--RRCCQ	RRRR	-----	-VC	-RRR	---	YTR-RCARQ*	
Kogia breviceps	MARNR	--CCRS	QSQRG	--C--	RRP	-----	---	RRRYR	SPR	--RRRYQ	RRRR	-----	-VC	-CRR	---	STTMRCASQ*	
Kogia sima	MARNR	--CCRS	QSQRG	--C--	RRP	-----	---	RRRCR	SPK	--RRRYQ	RRRR	-----	-VC	-CRR	---	SATMRCASQ*	
Physeter catodon	MARNR	--CCRS	QSQRG	--C--	RRP	-----	---	RRRCR	SPR	--RRRYQ	RRRR	-----	-VC	-CRR	---	YTTVRCARQ*	
Platanista minor	MARNR	--CCRS	QSQRG	--C--	RRP	-----	---	KRGC	SRR	--RRCYQ	RRRR	-----	-VC	-CRR	---	YTTIRCARQ*	
Pontoporia blainvillei	MARNR	--CRS	PSQNR	--G--	RRP	-----	---	RRRYR	SRR	--RRCCQ	RRRR	-----	-VC	-CRR	---	YTSVRCARQ*	
Berardius bairdii	MARNR	--CCRS	QSQRG	--R--	RRP	-----	---	RRRNR	SRR	--RQCCQ	RRRR	-----	-VC	-CRR	---	YTAIRCARQ*	
Mesoplodon grayi	MARNT	--CCRS	QSQRG	--R--	RRP	-----	---	RRRYR	SRR	--KQCCQ	KRRR	-----	-VC	-CRR	---	YTAIRCARQ*	
Mesoplodon bidens	MARNR	--CCRS	QSQRG	--R--	RRP	-----	---	RRRYR	SRR	--RQCCQ	KRRR	-----	-VC	-CRR	---	YTAIRCARQ*	
Mesoplodon peruvianus	MARNR	--CCRS	QSQRG	--R--	RRP	-----	---	RRRYR	SRR	--KQCCQ	KRRR	-----	-VC	-CRR	---	YTAIRCARQ*	
Tasmacetus shepherdi	MARNR	--CCRS	QSQRG	--R--	RRP	-----	---	RRRYR	SRR	--RQCCQ	KRRR	-----	-VC	-CRR	---	YTAIRCARQ*	
Ziphius cavirostris	MARNR	--CCRG	QSQRG	--R--	RRP	-----	---	RRRYR	SRR	--RQCCQ	KRRR	-----	-VC	-CRR	---	YTAIRCARQ*	
Hexaprotodon liberiensis	MARYR	--CCRS	PSRSR	--C--	RRQ	-----	---	RRRCR	RRR	--RRCCR	QRRR	-----	RVC	-CRR	---	YTMVRCTRQ*	
Hippopotamus amphibius	MARYR	--CCRS	PSRSR	--C--	RRQ	-----	---	RRRCR	RRR	--RRCCR	QRRR	-----	RVC	-CRR	---	YTMVRCTRQ*	
Antilocapra americana	MARYR	--CCLT	HSRSR	--CRP	RRR	-----	---	RRRCR	KLR	--RRFCR	RPRR	-----	RVC	-CRR	---	YTAIRCTR-	*
Bos taurus	MARYR	--CCLT	HSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRSGR	RRRR	-----	RVC	-CRR	---	YTVIRCTRQ*	
Capra hircus	MARYR	--CCLT	HSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRFGR	RRRR	-----	RVC	-CRR	---	YTVVRCTRQ*	
Ovis aries	MARYR	--CCLT	HSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRFGR	RRRR	-----	RVC	-CRR	---	YTVVRCTRQ*	
Ovis dalli	MARYR	--CCLT	HSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRFGR	RRRR	-----	RVC	-CRR	---	YTVVRCTRQ*	
Elaphodus cephalophus	MARYR	--CCLT	HSRSR	--C--	RRR	-----	---	RRRCR	RRR	--KRFGR	RRRR	-----	RVC	-CRR	---	YTVVRCTRQ*	
Moschus sp. JEG-2007	MARYR	--CCLT	HSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRFCR	RRRR	-----	RVC	-CRR	---	YTVVRCTRQ*	
Potamochoerus porcus	MARYR	--CCRS	HSRSR	--C--	RPR	-----	---	RRRCR	RRR	--RRCCP	RRRR	-----	AVC	-CRR	---	YTVIRCRRC*	
Sus scrofa	MARYR	--CCRS	HSRSR	--C--	RPR	-----	---	RRRCR	RRR	--RRCCP	RRRR	-----	AVC	-CRR	---	YTVIRCRRC*	
Pteropus hypomelanus	MARYR	--CCRS	QSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTV-RCRRR*	*
Mormoops megalophylla	MARYR	--CCRS	PSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRSCR	RRRR	-----	-VC	-CRR	---	YTV-RCRRR*	*
Pteronotus parnellii	MARYR	--CCRS	PSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTV-RCRRR*	*
Chilonatalus micropus	MARYR	--CCRS	QSRSR	--C--	RRR	-----	---	RRRCR	TRR	--RRCCR	RRRR	-----	RVC	-CRR	---	YTVVRCRRR*	*
Natalus stramineus	MARYR	--CCRS	QSRSR	--C--	RPR	-----	---	RRRCR	TRR	--RRCCR	RRRR	-----	RVC	-CRR	---	YTVVRCRRR*	*
Desmodus rotundus	MARYR	--CCRS	PSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTV-RCRRR*	*
Monophyllus redmani	MARYR	--CCRS	PSRSR	--C--	RRR	-----	---	RRRCR	RRR	--RRCCR	RRRR	-----	-VC	-CRR	---	YTV-RCRRR*	*
Hipposideros commersoni	MARYR	--CCRS	HSRSR	--C--	RRR	-----	---	RRRSR	RRR	--RRSCG	RRRR	-----	-AG	-YRR	---	YTVR-RCRRR*	*
Rhinolophus ferrumequinum	MARYS	--CCRS	HSRSR	--S--	RRR	-----	---	RQRCR	RRR	--RRSCG	RRRR	-----	-AC	-YRR	---	YTV-RYRRRR	RRR*
Rhinopoma hardwickii	MARYR	--CCRS	RSRSR	--C--	RPR	-----	---	RRRCR	RRR	--RRCCR	RRRR	-----	-VC	-CRR	---	YSA-RCRRRR	*
Chalinolobus beatrix	MARYR	--CCR-	-SRSR	--C--	RRR	-----	---	RRRSY	RRR	--RRCCR	RRRR	-----	RVC	-CRR	---	YV--RCRRR*	*

Corynorhinus townsendii	MARYR-CCRS	QSRSR--C--	RRR-----	----	RRRCY-	RRR--RRCCR	RRRR-----	RVC-CRR---	YT--RYRR*
Eptesicus brasiliensis	MARYR-CCR-	-SRSR--C--	RRR-----	----	RRRCY-	RRR--RRCCR	RRRR-----	RVC-CRR---	YTVIRCRR*
Eptesicus fuscus	MARYR-CCR-	-SRSR--C--	RRR-----	----	RRRCY-	RRR--RRCCR	RRRR-----	RVC-CRR---	YTVIRCRR*
Hypsugo savii	MARYR-RCR-	-SRSR--C--	RRR-----	----	RRRCH-	RRR--RRCCR	RRRR-----	RAC-CRR---	Y--RCRR*
Murina cyclotis	MARYR-CCR-	-SRSR--C--	RRR-----	----	RRRCH-	RRR--RRCSR	RRRR-----	RVC-CRR---	YTVIRCRR*
Galeopterus variegatus	MARYR-CCR-	-SRSR--C--	RRR-----	----	RRSCR-	-RR--RRCCR	RRAR-----	-RS-CRRR-	YSLRCRRY*
Myotis daubentonii	MARYR-CCR-	-SRSR--C--	RRR-----	----	RRRCY-	RRR--RRCCR	RRRR-----	RVC-CRR---	YS--RCRR*
Myotis lucifugus	MARYR-CCR-	-SRSR--C--	RRR-----	----	RRRCY-	RRR--RRCCR	RRRR-----	RVC-CRR---	YS--RCRR*
Neoromicia brunneus	MARYR-RCR-	-SRSR--C--	RRR-----	----	RRRCH-	RRR--RRCCR	RRRR-----	RAC-CRR---	Y--RCRR*
Otonycteris hemprichii	MARYR-CCRS	RSRSR--C--	RRR-----	----	RRKCY-	RRR--RRCSR	KRRR-----	RVC-CRR---	YTVMRCRR*
Plecotus auritus	MARYR-CCRS	QSRSR--C--	RRR-----	----	RRRCY-	RRR--RRCCR	RRRR-----	RVC-CRR---	YTVVRCRR*
Antechinomys laniger	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	RHHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus bellus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus flavipes	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus godmani	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus leo	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus minimus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus naso	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus stuartii	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Antechinus swainsonii	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasyercus cristicauda	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasykaluta rosamondae	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HRNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasyuroides byrnei	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HRNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasyurus hallucatus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSR-RGRR*	
Dasyurus viverrinus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSR-RGRR*	
Dasyurus albopunctatus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasyurus geoffroi	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasyurus spartacus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Dasyurus maculatus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Micromurexia habbema	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Murexchinus melanurus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Murexia longicauda	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Myoictis melas	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HRNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Myoictis wallacei	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HRNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Neophascogale lorentzii	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Ningau ridei	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Ningau timealeyi	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Ningau yvonnae	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Paramurexia rothschildi	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Parantechinus apicalis	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HRNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Phascogale calura	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Phascogale tapoatafa	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Phascosorex dorsalis	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	GR-RRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Planigale maculata maculata	MARYRRHSRS	RSRSR--Y-R	RRR--RRRS	HHNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*	
Planigale maculata sinualis	MARCRRHHSRS	RSRSRNQCQR	RRR-----	R	RYNRRTYR-	RSR--RHSRR	RRGR-----	RGY-SRRR-	YSRRGRRY*
Planigale gilesi	MARCRRHHSRS	RSRSRNQCQR	RRR-----	R	HYNRRTYR-	RSR--RHSRR	RRVR-----	RGCS-CRR-	CSRRRRRC*
Planigale tenuirostris	MARCRRHHSRS	RSRSRNQCQR	RRR-----	RS	HYNRRTYR-	RSR--RHSRR	RRVR-----	RGCS-CRR-	CSRRRRRC*

Pseudantechinus bilarni	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HRNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSR-RGRR*
Pseudantechinus macdonnellensis	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HRNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Pseudantechinus ningbing	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HRNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Pseudantechinus woolleyae	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HRNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sarcophilus harrisii	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	GR-RRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis griseoventer	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRRR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis bindi	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSIR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis longicaudata	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSR-RRRY*
Sminthopsis aitkeni	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis archeri	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis crassicaudata	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis dolichura	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis douglasi	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis gilberti	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis granulipes	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis hirtipes	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis leucopus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis macroura	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis murina	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis ooldea	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis psammophila	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis virginiae	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Sminthopsis youngsoni	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Myrmecobius fasciatus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Thylacinus cynocephalus	MARYRRHSRS	RSRSR--Y-R	RRR--RRRSR	HHNRRRTYR-	RSS--RHSRR	RRGR-----R	RGY-SRRR--	YSRRGRRY*
Dendrolagus doriani	?ARYR-HSRS	R?RSR--Y-R	RRR--R?RSR	YRS?RRRYR-	GRR--RRRSR	RGR--	RGY-SR?R-	YSRRRRRY*
Dendrolagus goodfellowi	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Dorcopsis veterum	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Dorcopsulus vanheurni	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Lagorchestes hirsutus	MARYR-HSRS	RSRSR--Y-R	RQR--RRRSR	YRSRRRYR-	RRQ--RRSR	GRR--GYSR	RGY-SRRR--	YSRRRRRY*
Lagostrophus fasciatus	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GSR--RSRSR	RRGR--	RGY-SRRR--	YSRRRRRY*
Macropus rufus	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSR-RRRY*
Macropus parryi	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--R?RSR	RGR--	RGY-SRRR--	YSRRRRRY*
Macropus agilis	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRSR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Macropus eugenii	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRSR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Macropus rufogriseus	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Macropus giganteus	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSR-RRRY*
Onychogalea fraenata	MARYR-HSRS	RSRS?--Y--	RRR--?RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Onychogalea unguifera	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Petrogale concinna	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	-R--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Petrogale xanthopus	MARYR-HS?S	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Setonix brachyurus	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Thylagale stigmatica	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGYSRR--	YSRRRRRY*
Wallabia bicolor	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GRR--RRRSR	RGR--	RGY-SRRR--	YSRRRRRY*
Trichosurus vulpecula	MARYR-HSRS	RSRSR--Y--	RRRRRRRSR	YRSRRRYR-	RSS--RR-RR	RGR--	RGY-SRRR--	YSRRGRRY*
Phascolarctos cinereus	MARYR-HSRS	RSRSR--Y-Q	RRR--RRRSR	YRSRRRYR-	RRRGSRRRR	RGR--	RGY-RRR--	YS--RRRY*
Aepyprymnus rufescens	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GSR--RRRSR	RRRR--	RGY-SRRR--	YSR-RRRY*
Bettongia penicillata	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRSRRRYR-	GSR--RRRSR	RRGR--	RGY-SRRR--	YSRRRRRY*
Hypsiprymnodon moschatus	MARYR-HSRS	RSRSR--Y-R	RRR--RRRSR	YRGRRRYR-	RSS--RRRSR	RRGR--	RGY-YRRR--	YSRRRRRY Y*
Potorous longipes	MARYR-HSRS	RSR?R--Y-R	RRR--RRRSR	YRSRRRYR-	GSR--RSRSR	RRGR--	RGY-SRRR--	YSR-RRRY*
Pseudocheirops cupreus	MARYR-HSRS	RSRSR--YRR	RRR--RRRSR	YRGRRRYR-	RSS--RRRRR	GRRRGNCLGR	RGY-RRR--	YSRRRRRY Y*
-	MARYR-CCRS	RSRSR--C--	RRR--	---RRRCR-	RRR--RRCCR	RRRR--	RRC-CRRR-	YT--RCRRY X*

Figure S2. Multiple sequence alignment for included eutherian species based on muscle algorithm (implemented in Geneious 5.5.9.)

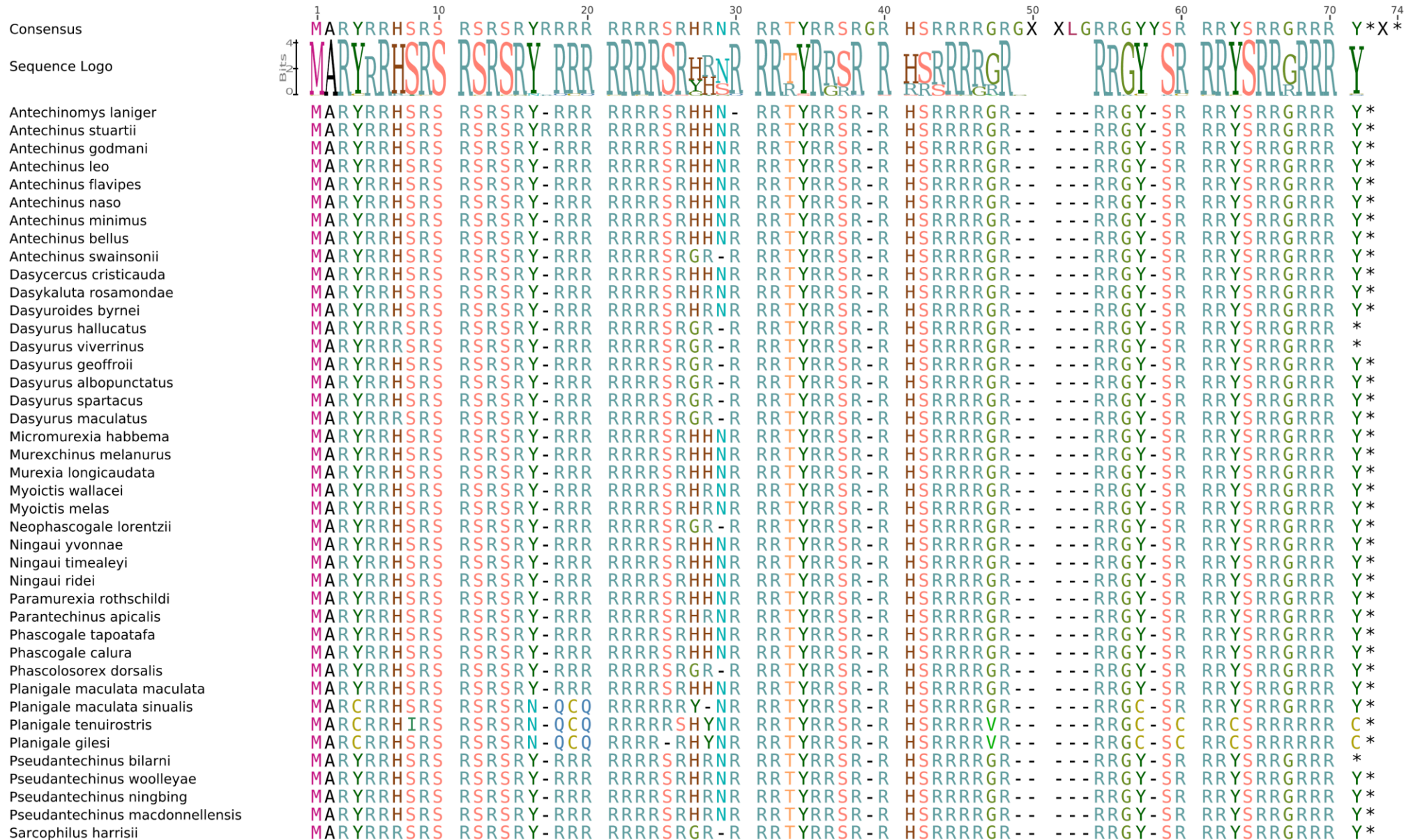


Hylobates lar	MARYRCRSQ	SRSRC	--YRR	GQRSR	-RRRR	RSCQTRRRAM	RCCRPR	--YR	L-RRRRH*
Ateles sp.	MARYRCRSR	SRSRC	--YRQ	RPRCR	-RRRR	RSCRRRRGS-	RCCRPR	--YR	L-RRRRY*
Saguinus imperator	MARYRCRSQ	SRSRC	--YRQ	RRRGR	-RRRR	RTCRRRRAS-	RCCRPR	--YK	L-TCRRY*
Saimiri sciureus	MARYRCRSR	SRSRC	--YRR	RRRCR	-TRRR	RCCRPRRAR-	RCCRPR	--YK	L-RCRRY*
Otolemur garnettii	MARYRCRSQ	SRSRC	--RRR	RRRCR	-RRRR	RCCRPRRRR--	RCCRPR	--YR	L-RCRRY*
Balaena mysticetus	MARNRCRSQ	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARR*
Eubalaena australis	MARNRCRSQ	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARR*
Eubalaena glacialis	MARNRCRSQ	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARR*
Eubalaena japonica	MARNRCRSQ	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARR*
Balaenoptera acutorostrata	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCAGQ*
Balaenoptera bonaerensis	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCAGQ*
Balaenoptera borealis	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Balaenoptera edeni	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Balaenoptera musculus	MARNTCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Balaenoptera physalus	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Megaptera novaeangliae	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Eschrichtius robustus	MARNSCCRSP	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Caperea marginata	MARNRCRSQ	SRSRR	--RRP	RQRQR	-SQR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Cephalorhynchus commersonii	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YA	TTRCARQ*
Delphinus capensis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Delphinus delphis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Feresa attenuata	MARNR-CRSP	SQSRC	--RRP	RRRYR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Globicephala macrorhynchus	MARNR-CRSP	SQSRC	--RRP	RRRYR	-RRR	QCCRPRRRR--	VCCR	--YT	TTRCARQ*
Grampus griseus	MARNR-CRSP	SQSRC	--RRP	RRRYR	-RRR	QCCRPRRRR--	VCCR	--YT	TTRCARQ*
Lagenodelphis hosei	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Lagenorhynchus acutus	MARNR-CRSP	SQSRC	--RRP	RRRCL	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Lagenorhynchus albirostris	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCASQ*
Lagenorhynchus obliquidens	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Lissodelphis borealis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Orcaella heinsohni	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Orcinus orca	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Peponocephala electra	MARNR-CRSP	SQSRC	--RRP	RRRYR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Pseudorca crassidens	MARNR-CRSP	SQSRC	--RRP	RRRYR	-RRR	QCCRPRRRR--	VCCR	--YT	TTRCARQ*
Sotalia fluviatilis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Sousa chinensis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Stenella attenuata	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Stenella coeruleoalba	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	LCCRPRRRR--	VCCR	--YT	TTRCARQ*
Stenella frontalis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Stenella longirostris	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCAR*
Steno bredanensis	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Tursiops aduncus	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	LCCRPRRRR--	VCCR	--YT	TTRCARQ*
Tursiops truncatus	MARNR-CRSP	SQSRC	--RRP	RRRCR	-RRR	LCCRPRRRR--	VCCR	--YT	TTRCARQ*
Inia geoffrensis	MARNR-CRSP	SQSRC	--RRP	RRRYR	-SRR	RCCRPRRRR--	VCCR	--YT	TVRCARQ*
Lipotes vexillifer	MARNR-CRSP	SQSRC	--RRP	RRRYR	-SRR	RCCRPRRRR--	VCCR	--YT	TMRCARQ*
Delphinapterus leucas	MARNR-CRSP	SQSRC	--RRP	RRRYR	-SKRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Monodon monoceros	MARTR-CRSP	SQSRC	--RRP	RRRYR	-SKRR	RCCRPRRRR--	VCCR	--YT	TTRCARQ*
Neophocaena phocaenoides	MARNR-CRSP	SQSRC	--RCP	RRRYR	-SKRR	RCCRPRRRR--	VCCR	--YT	R-RCARQ*

Phocoena phocoena	MARNR - CRSP	SQSRG - - RCP	RRRYR - SKRR	RCCQRRRR - -	VCCRR - - - YT	R - RCARQ *
Phocoenoides dalli	MARNR - CRSP	SQSRG - - RCP	RRRYR - SKRR	RCCQRRRR - -	VCCRR - - - YT	R - RCARQ *
Kogia breviceps	MARNRCCRSQ	SRGRC - - RRP	RRRYR - SPRR	RRYQRRRR - -	VCCRR - - - ST	TMRCASQ *
Kogia sima	MARNRCCRSQ	SRGRC - - RRP	RRRCR - SPKR	RRYQRRRR - -	VCCRR - - - SA	TMRCASQ *
Physeter catodon	MARNRCCRSQ	SRSRC - - RRP	RRRCR - SPRR	RRYQRRRR - -	VCCRR - - - YT	VTRCARQ *
Platanista minor	MARNRCCRSQ	SRSRC - - RRP	KRGCR - SRRR	RCYQRRRR - -	VCCRR - - - YT	TIRCARQ *
Pontoporia blainvillei	MARNR - CRSP	SQNRG - - RRP	RRRYR - SRRR	RCCQRRRR - -	VCCRR - - - YT	SVRCARQ *
Berardius bairdii	MARNRCCRSQ	SQSRR - - RRP	RRRNR - SRRR	QCCQRRRR - -	VCCRR - - - YT	AIRCARQ *
Mesoplodon bidens	MARNRCCRSQ	SQSRR - - RRP	RRRYR - SRRR	QCCQKRRR - -	VCCRR - - - YT	AIRCARQ *
Mesoplodon grayi	MARNRCCRSQ	SQSRR - - RRP	RRRYR - SRRR	QCCQKRRR - -	VCCRR - - - YT	AIRCARQ *
Mesoplodon peruvianus	MARNRCCRSQ	SQSRR - - RRP	RRRYR - SRRR	QCCQKRRR - -	VCCRR - - - YT	AIRCARQ *
Tasmacetus shepherdii	MARNRCCRSQ	SQSRR - - RRP	RRRYR - SRRR	QCCQKRRR - -	VCCRR - - - YT	AIRCARQ *
Ziphius cavirostris	MARNRCCRGQ	SQSRR - - RRP	RRRYR - SRRR	QCCQKRRR - -	VCCRR - - - YT	ATRCARQ *
Hexaprotodon liberiensis	MARYRCCRSQ	SRSRC - - RRQ	RRRCR - RRRR	RCCQRRRR - -	VCCRR - - - YT	MVRCRTRQ *
Hippopotamus amphibius	MARYRCCRSQ	SRSRC - - RRQ	RRRCR - RRRR	RCCQRRRR - -	VCCRR - - - YT	MVRCRTRQ *
Antilocapra americana	MARYRCCRLTH	SRSRCR - RRR	RRRCR - RRRR	RFCRR - RRR	VCCRR - - - YT	AIRCCTR *
Bos taurus	MARYRCCRLTH	SGSRCR - RRR	RRRCR - RRRR	RSR - RRRR	VCCRR - - - YT	VIRCCTRQ *
Capra hircus	MARYRCCRLTH	SRSRCR - RRR	RRRCR - RRRR	RFGR - RRRR	VCCRR - - - YT	VVRCCTRQ *
Ovis aries	MARYRCCRLTH	SRSRCR - RRR	RRRCR - RRRR	RFGR - RRRR	VCCRR - - - YT	VVRCCTRQ *
Ovis dalli	MARYRCCRLTH	SRSRCR - RRR	RRRCR - RRRR	RFGR - RRRR	VCCRR - - - YT	VVRCCTRQ *
Elaphodus cephalophus	MARYRCCRLTH	SRSRCR - RRR	RRRCH - RRRK	RFGR - RRRR	VCCRR - - - YT	VVRCCTRQ *
Moschus sp.	MARYRCCRLTH	SRSGCR - RRR	RRRCR - RRRR	RFCRR - RRRR	VCCRR - - - YT	VVRCCTRQ *
Potamochoerus porcus	MARYRCCRSQ	SRSRC - - RPR	RRRCR - RRRR	RCCP - RRRR	VCCRR - - - YT	VIRCRR *
Sus scrofa	MARYRCCRSQ	SRSRC - - RPR	RRRCR - RRRR	RCCP - RRRR	VCCRR - - - YT	VIRCRR *
Pteropus hypomelanus	MARYRCCRSQ	SRSRC - - RRR	RRRCR - RRRR	RCCRRRR - -	VCCRR - - - YT	V - RCRRR *
Mormoops megalophylla	MARYRCCRSQ	SRSRC - - RRR	RRRCR - RRRR	RCCRRRR - -	VCCRR - - - YT	V - RCRRR *
Pteronotus parnellii	MARYRCCRSQ	SRSRC - - RRR	RRRCR - RRRR	RCCRRRR - -	VCCRR - - - YT	V - RCRRR *
Chilonatalus micropus	MARYRCCRSQ	SRSRC - - RRR	RRRCR - TRRR	RCCRRRR - -	VCCRR - - - YT	VVRCRR *
Natalus stramineus	MARYRCCRSQ	SRSRC - - RPR	RRRCR - TRRR	RCCRRRR - -	VCCRR - - - YT	VVRCRR *
Desmodus rotundus	MARYRCCRSQ	SRSRC - - RRR	RRRCR - RRRR	RCCRRRR - -	VCCRR - - - YT	V - RCRRR *
Monophyllus redmani	MARYRCCRSQ	SRSRC - - RRR	RRRCR - RRRR	RCCRRRR - -	VCCRR - - - YT	V - RCRRR *
Hipposideros commersoni	MARYRCCRSQ	SRSRC - - RRR	RRRSR - RRRR	RSR - RRRR	AGYRR - - - YT	V - RYRRR *
Rhinolophus ferrumequinum	MARYSCRSQ	SRSSS - - RRR	RQRCR - RRRR	RSR - RRRR	ACYRR - - - YT	V - RYRRRRR R *
Rhinopoma hardwickii	MARYRCCRSQ	SRSRC - - RPR	RRRCR - RRRR	RCCRRRR - -	VCCRR - - - YS	A - RCRRR *
Chalinolobus beatrix	MARYRCCR - -	SRSRC - - RRR	RRRSY - RRRR	RCCRRRR - -	VCCRR - - - YV	- - RCRRR *
Corynorhinus townsendii	MARYRCCRSQ	SRSRC - - RRR	RRRCY - RRRR	RCCRRRR - -	VCCRR - - - YT	- - RYRRR *
Galeopterus variegatus	MARYRCCR - -	SRSRC - - RRR	RRSCR - - RRR	RCRRRRAR - -	RSCRRR - - - YS	LRCCRRY *
Eptesicus brasiliensis	MARYRCCR - -	SRSRC - - RRR	RRRCY - RRRR	RCCRRRR - -	VCCRR - - - YT	VIRCRRR *
Eptesicus fuscus	MARYRCCR - -	SRSRC - - RRR	RRRCY - RRRR	RCCRRRR - -	VCCRR - - - YT	VIRCRRR *
Hypsignathos monstrosus	MARYRCCR - -	SRSRC - - RRR	RRRCH - RRRR	RCCRRRRRR - -	ACCRR - - - Y-	- - RCRRR *
Murina cyclotis	MARYRCCR - -	SRSRC - - RRR	RRRCH - RRRR	RCSRRRR - -	VCCRR - - - YT	VIRCRRR *
Myotis daubentonii	MARYRCCR - -	SRSRC - - RRR	RRRCY - RRRR	RCCRRRRRR - -	VCCRR - - - YS	- - RCRRR *
Myotis lucifugus	MARYRCCR - -	SRSRC - - RRR	RRRCY - RRRR	RCCRRRRRR - -	VCCRR - - - YS	- - RCRRR *
Neoromicia brunneus	MARYRCCR - -	SRSRC - - RRR	RRRCH - RRRR	RCCRRRRRR - -	ACCRR - - - Y-	- - RCRRR *
Otonycteris hemprichii	MARYRCCRSQ	SRSRC - - RRR	RRKCY - RRRR	RCSRKRRRR - -	VCCRR - - - YT	VMRCRRR *

Plecotus auritus	MARYRCRSQ	SRSRC--RRR	RRRCY-RRRR	RCCRRRRRR--	VCCRR--YT	VVRCRRR*
Acomys cahirinus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCRI*
Acomys cilicicus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCRI*
Arvicola sapidus	MARYR-CRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCYRRRRTYT	M-RCKRY*
Arvicola terrestris	MARYR-CRSK	SRSRC--RH	RRRCR-RRRR	RCCRRRRRR--	RCYRRRRTYT	M-RCKRY*
Chionomys nivalis	MARYR-CRSQ	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCYGRRRTYT	L-RCKRY*
Clethrionomys glareolus	MARYR-CRSK	SRSRC--RRR	RRRCR--RRR	RCCRRRRRR--	RCYRRRRSYS	I-MCKRY*
Cricetulus griseus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCYRRRRTYT	L-RCKRY*
Meriones unguiculatus	MARYRCRSK	SRSRC--RRR	RRRCR--RRR	RCCRRRRRR--	RCCRRRRRYT	L-RCKRY*
Mesocricetus auratus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRRTYT	L-RCKRY*
Micromys minutus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRRSYT	L-RCKRY*
Microtus agrestis	MARYR-CRSK	SRSRC--SRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRRTYT	M-RCKRY*
Microtus arvalis	MARYR-YRSK	SRSRC--SRG	RRRCR-RRRR	RCCRRRRRR--	RCCRRRRTYT	V-RCKRY*
Microtus cabreræ	MARYR-YRSK	SRSRC--SRR	RRRCR-RRRR	RCCQSRKR--	RCYRRRRSCT	M-RCKRY*
Microtus gerbei	MARYR-CRSK	SRSRC--SRR	RRRCR-RRRR	RCCRRRRRR--	RCYRRRRSYT	M-RCKRY*
Peromyscus californicus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCKRY*
Peromyscus leucopus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCKRY*
Peromyscus maniculatus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCKRY*
Peromyscus polionotus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCKRY*
Phodopus campbelli	MARYR-CRSQ	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCYGRRRTYT	L-RCKRY*
Phodopus roborovski	MARYRCRSK	SRSRC--RRR	RRRCR-SRRR	RCCRRRRRR--	RCCRRRR-YT	L-RCKRY*
Phodopus sungorus	MARYRCRSK	SRSRC--RRR	RRRCR-RRRR	RCCRRRRRR--	RCCRRRR-YS	L-RCKRY*
Pitimus duodecimcostatus	MARYR-CRSK	SRSRC--SRH	RRRCR-RRRR	RCCRRRRRR--	RCYRRRRSYT	M-RCKRY*
Pitimus lusitanicus	MARYR-CRSK	SRSRC--SRR	RRRCR-RRRR	RCCRRRRRR--	RCYRRRRSYT	M-RCKRY*

Figure S3. Multiple sequence alignment for included metatherian species based on muscle algorithm (implemented in Geneious 5.5.9.)



<i>Sminthopsis griseoventer</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRRR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis bindi</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSIRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis longicaudata</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRR-RRR	Y*
<i>Sminthopsis virginiae</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis douglasi</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis hirtipes</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis youngsoni</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis psammophila</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis aitkeni</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis macroura</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis dolichura</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis murina</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis ooldea</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis leucopus</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis archeri</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis granulipes</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis gilberti</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Sminthopsis crassicaudata</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Myrmecobius fasciatus</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Thylacinus cynocephalus</i>	MAR YRRHSRS	RSRSRY-RRR	RRRSRHHR	RRTYRRSR-R	HSRRRRGR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Dendrolagus dorianus</i>	?ARY-RHSRS	R?RSRY-RRR	RR?RSYRS?	RRRYRGRR-R	RRSRRGRR--	---RRGY-SR	?RYSRRRRRR	Y*
<i>Dendrolagus goodfellowi</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---R-GY-SR	RRYSRRRRRR	Y*
<i>Dorcopsis veterum</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Dorcopsulus vanheurni</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RR?YRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Lagorchestes hirsutus</i>	MAR Y-RHSRS	RSRSGY-RRQ	RRRSRYRS	RRRYRRRQ-R	RSRRGRRRGY	S---RRY-SR	RRYSRRRRRR	Y*
<i>Lagostrophus fasciatus</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGS-R	SRSSRRGR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Macropus rufus</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRSQ	RRRYRGRR-R	RRSRRGRR--	---R-GY-SR	RRYSRR-RRR	Y*
<i>Macropus parryi</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	?SRRGRR--	---R-GY-SR	RRYSRRRRRR	Y*
<i>Macropus agilis</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRSRGRR-R	RRSRRGRR--	---R-GY-SR	RRYSRRRRRR	Y*
<i>Macropus eugenii</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRSRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Macropus rufogriseus</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Macropus giganteus</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRR-RRR	Y*
<i>Onychogalea fraenata</i>	MAR Y-RHSRS	RSRS?Y-RRR	?RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---R-GY-SR	RRYSRRRRRR	Y*
<i>Onychogalea unguifera</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---R-GY-SR	RRYSRRRRRR	Y*
<i>Petrogale concinna</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRR?-R-R-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Petrogale xanthopus</i>	MAR Y-RHS?S	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Setonix brachyurus</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---R-GY-SR	RRYSRRRRRR	Y*
<i>Thylogale stigmatica</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---RRGY-SC	RRYSRRRRRR	Y*
<i>Wallabia bicolor</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGRR-R	RRSRRGRR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Trichosurus vulpecula</i>	MAR Y-RHSRS	RSRSRYRRRR	RRRSRYRS	RRRYRS--R	RRRRRGRR--	---RRGY-SR	RRYSRRGRRR	Y*
<i>Phascolarctos cinereus</i>	MAR Y-RHSRS	RSRSRY-QRR	RRRSRYRSQ	RRRYRRRRGS	RRRRRRGR--	---RRGY--R	RRYSRR--RR	Y*
<i>Aepyprymnus rufescens</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGS-R	RRSRRRR--	---RRGY-SR	RRYSRR-RRR	Y*
<i>Bettongia penicillata</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRS	RRRYRGS-R	RRSRRGR--	---RRGY-SR	RRYSRRRRRR	Y*
<i>Hypsiprymnodon moschatus</i>	MAR Y-RHSRS	RSRSRY-RRR	RRRSRYRG	RRRYRRSR-R	RRSRRG--	---RRGY-SR	RRYSRRRRRR	YY*
<i>Potorous longipes</i>	MAR Y-RHSRS	RSR?RY-RRR	RRRSRYRS	RRRYRGS-R	SRSSRRGR--	---RRGY-SR	RRYSRR-RRR	Y*
<i>Pseudochirops cupreus</i>	MAR Y-RHSRS	RSRSRYRRRR	RRRSRYRG	RRRYRRSR-R	RRRRGRRRG	NCLGRRGY-RR	RRYSRRRRRR	YY*

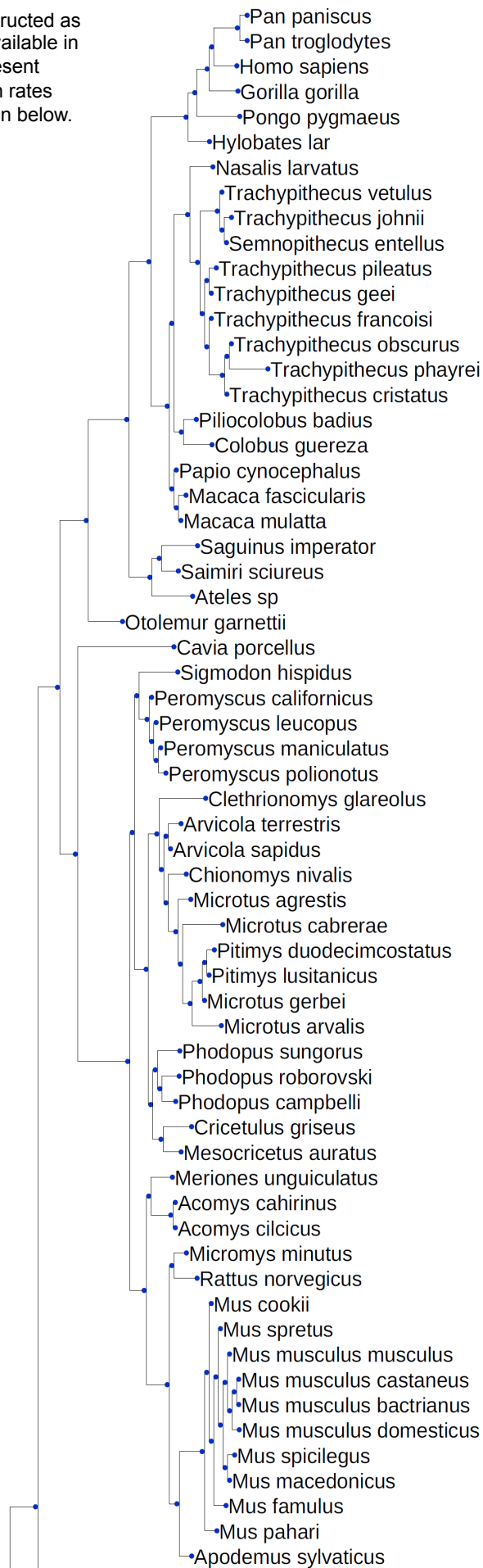
Table S2. Clade descriptive statistics of residual testes mass and arginine content data and amino acid alignment statistics (Geneious 5.5.9). Stdev= standard deviation, mad= median absolute deviation.

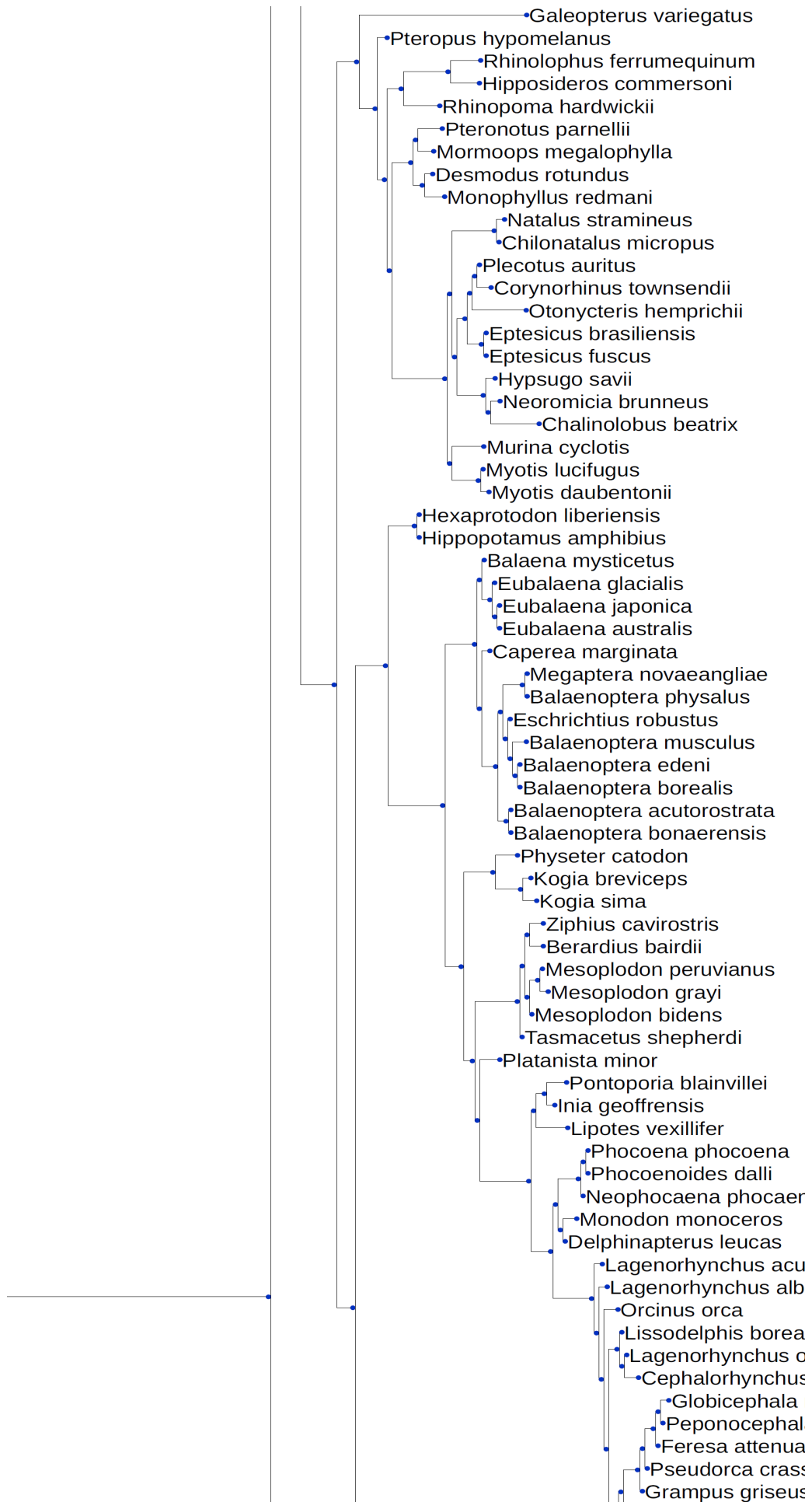
Clade	Evolutionary rate	Residual testes mass						Arginine content (% of sequence length)						Amino acid frequencies (% of clade alignment)												statistics for clade amino acid alignments								
	ω (M3)	n	mean	stdev	median	mad	range	n	mean	stdev	median	mad	range	A	C	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	Y	n	% of identical sites	pairwise sequence identity	mean sequence length
Primates	0.791	17	-0.26	0.46	-0.10	0.56	1.46	25	51.14	4.10	51.92	4.30	15.77	5	16	0	0	1	0	1	2	3	0	1	4	51	8	2	0	6	38	44.40	90.50	51.40
Rodentia	0.226	38	0.17	0.35	0.19	0.34	1.45	37	54.87	1.91	54.90	1.57	9.80	2	16	0	0	1	4	1	2	0	0	0	55	7	2	0	6	25	30.80	83.30	51.60	
Chiroptera	0.362	4	-0.04	0.41	-0.01	0.44	0.90	22	57.45	2.84	57.14	1.51	10.89	3	17	0	0	1	0	0	0	2	0	1	1	57	7	2	3	5	21	53.60	85.60	49.30
Artiodactyla	0.452	5	-0.08	0.37	0.04	0.38	0.92	11	49.82	1.92	49.02	1.45	5.77	3	16	1	1	2	1	0	1	2	0	1	2	50	5	5	5	4	11	54.70	84.40	51.60
Cetacea	0.942	26	0.08	0.48	0.35	0.59	1.54	55	45.46	2.70	45.83	3.09	10.94	4	15	0	1	0	0	1	0	2	2	4	6	45	8	5	3	3	55	38.00	83.90	48.80
Diprotodontia	0.198	10	-0.38	0.31	-0.39	0.26	1.00	26	61.90	1.51	61.90	1.67	5.20	2	0	0	5	2	0	0	0	2	0	0	0	62	15	0	0	11	26	52.70	90.80	62.90
Dasyuromorpha	0.391	38	-0.08	0.30	-0.04	0.19	1.02	61	58.44	1.70	57.81	0.00	9.78	2	0	0	5	5	0	0	0	2	1	0	0	58	14	2	0	9	61	67.70	96.10	63.70

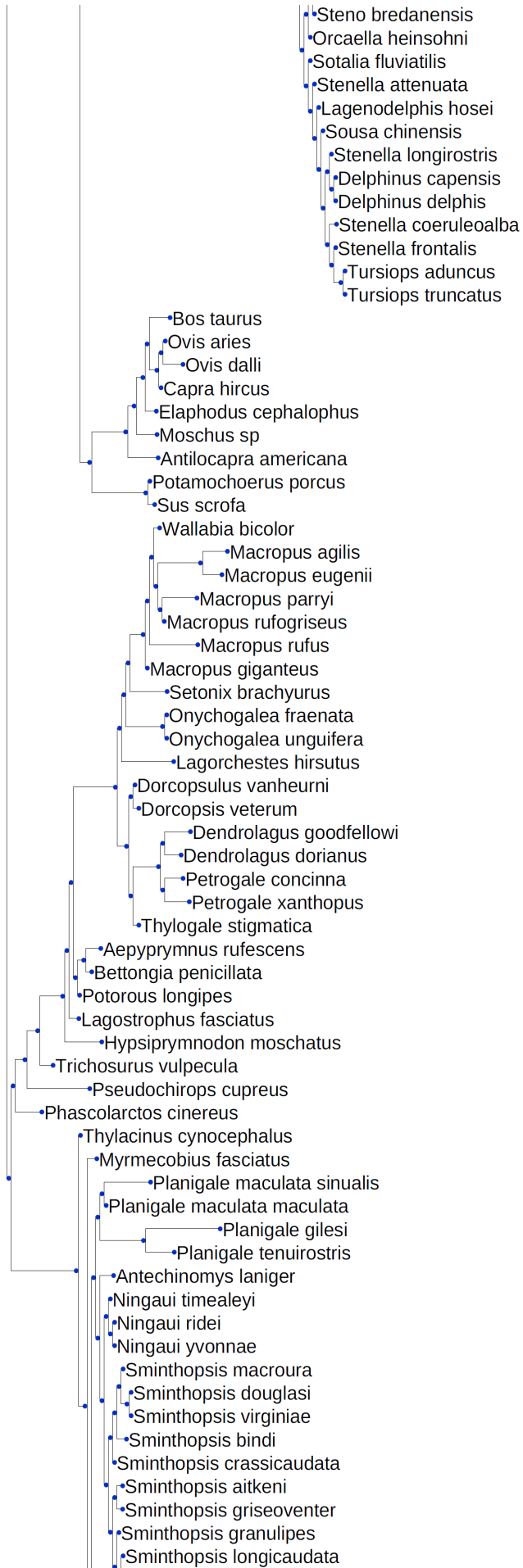
Table S3. Results for Tukey HD post-hoc comparisons for one-way anovas. Diff = difference in the observed means, lwr = lower end point of the interval, upr = upper end point, p adj = p-value after adjustment for the multiple comparisons.

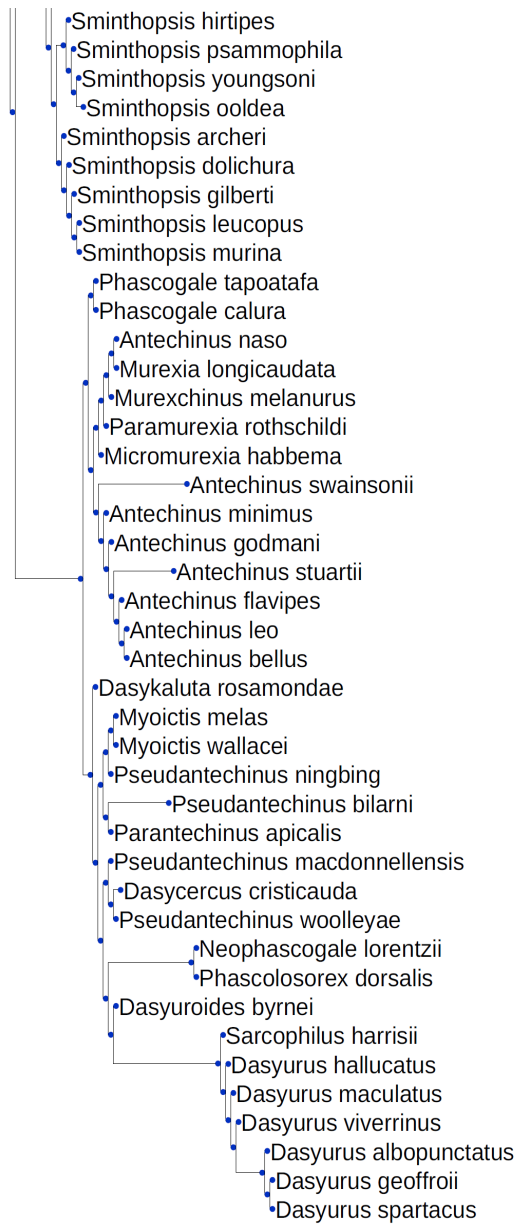
	Comparison	diff	lwr	upr	<i>p adj</i>
Arginine content (% of sequence length) / Clade (Eutheria)	Chiroptera-Rodentia	0.859	0.165	1.552	0.007
	Artiodactyla-Rodentia	-1.685	-2.569	-0.800	0.000
	Primates-Rodentia	-1.244	-1.911	-0.577	0.000
	Cetacea-Rodentia	-3.138	-3.686	-2.591	0.000
	Artiodactyla-Chiroptera	-2.543	-3.494	-1.592	0.000
	Primates-Chiroptera	-2.103	-2.856	-1.350	0.000
	Cetacea-Chiroptera	-3.997	-4.647	-3.347	0.000
	Primates-Artiodactyla	0.440	-0.492	1.372	0.688
	Cetacea-Artiodactyla	-1.454	-2.304	-0.603	0.000
	Cetacea-Primates	-1.894	-2.515	-1.273	0.000
Residual testes mass / Protamine sequence length group (Metatheria)	186bp-183bp	0.390	-0.124	0.904	0.215
	189bp-183bp	0.404	-0.072	0.880	0.130
	192bp-183bp	0.648	0.200	1.097	0.002
	195bp-183bp	1.002	0.249	1.754	0.004
	189bp-186bp	0.015	-0.322	0.351	1.000
	192bp-186bp	0.259	-0.038	0.556	0.114
	195bp-186bp	0.612	-0.061	1.285	0.090
	192bp-189bp	0.244	0.020	0.468	0.027
	195bp-189bp	0.598	-0.047	1.242	0.081
195bp-192bp	0.354	-0.271	0.978	0.498	

Figure S4. Phylogenetic tree constructed as consensus of phylogenetic data available in the literature. Branch lengths represent protamine 1 nucleotide substitution rates (PAML4, M0). References are given below.









0.31

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Table S4. Results of COEVOL correlation analysis and phylogenetically controlled regression analyses (PGLS). Correlations with relative testes mass are corrected for body mass (see material and methods section), ω =dN/dS substitution rate computed by COEVOL, superscripts following the λ value indicate significance levels (n.s., $p > 0.05$; * $p < 0.05$) in likelihood ratio tests against models with $\lambda = 0$ (first superscript) and $\lambda = 1$ (second superscript), n = number of species in analysis, significant and nearly significant results are shown in boldface.

COEVOL	variable 1	variable 2	n	covariances	correlation coefficient	posterior probability			
Mammalia	ω	relative testes mass	132	-0.47	-0.16	0.22			
	arginine content	relative testes mass	132	0.01	0.13	0.07	.		
	arginine content	ω	237	-0.06	-0.34	0.00		**	
	relative head length	ω	87	-0.01	-0.01	0.46			
	relative head width	ω	65	-0.01	-0.01	0.50			
	Head elongation	ω	72	0.00	0.00	0.48			
	relative head length	arginine content	87	0.00	-0.13	0.10			
	relative head width	arginine content	65	-0.01	-0.23	0.02		**	
Eutheria	Head elongation	arginine content	72	0.00	0.18	0.07		.	
	arginine content	relative testes mass	83	-0.02	0.27	0.02		*	
	arginine content	ω	150	-0.05	-0.56	0.00		**	
	relative head length	arginine content	47	-0.01	-0.29	0.07		.	
	relative head width	arginine content	66	0.00	-0.20	0.03		*	
Metatheria	Head elongation	arginine content	52	0.01	0.22	0.09		.	
	arginine content	relative testes mass	49	0.01	0.15	0.80			
	arginine content	ω	87	0.14	0.37	0.07		.	
	relative head length	arginine content	21	0.00	0.00	0.48			
Primates	relative head width	arginine content	18	0.00	-0.15	0.23			
	Head elongation	arginine content	20	0.00	0.14	0.23			
	ω	relative testes mass	13	-0.02	-0.04	0.40			
	arginine content	relative testes mass	13	-0.02	0.15	0.28			
	arginine content	ω	25	-0.01	-0.21	0.34			
Rodentia	relative head length	ω	11	0.01	0.04	0.59			
	relative head length	arginine content	11	-0.01	0.38	0.09		.	
	ω	relative testes mass	35	-0.08	-0.17	0.21			
	arginine content	relative testes mass	35	0.01	0.30	0.03		*	
	arginine content	ω	35	0.00	-0.28	0.15			
	relative head width	ω	27	0.11	0.67	0.11			
	relative head length	ω	33	0.03	0.39	0.20			
	Head elongation	ω	27	-0.07	-0.56	0.15			
Chiroptera	relative head length	arginine content	33	0.00	-0.25	0.08		.	
	relative head width	arginine content	27	0.00	-0.35	0.02		*	
	Head elongation	arginine content	27	0.00	0.26	0.08		.	
	arginine content	ω	22						
	Artiodactyla	arginine content	ω	11	0.00	-0.01	0.50		
		ω	relative testes mass	26	0.00	0.00	0.50		
		arginine content	relative testes mass	26	0.04	0.35	0.04		*
arginine content		ω	55	0.00	-0.04	0.46			
relative head length		ω	15	0.00	0.00	0.50			
relative head width		ω	11	0.01	0.06	0.55			
Head elongation		ω	14	-0.01	-0.10	0.46			
relative head width		arginine content	11	-0.01	-0.39	0.06		.	
Dasyuromorpha	relative head length	arginine content	15	0.00	-0.24	0.17			
	Head elongation	arginine content	14	0.01	0.24	0.17			
	ω	relative testes mass	38	-0.55	-0.14	0.31			
Diprotodontia	arginine content	relative testes mass	38	0.00	-0.11	0.24			
	arginine content	ω	61	0.07	0.18	0.35			
	ω	relative testes mass	10	0.00	-0.02	0.48			
	arginine content	relative testes mass	10	0.01	0.19	0.25			
	arginine content	ω	26	0.00	-0.01	0.49			
	relative head width	ω	15	0.00	0.016	0.51			
	relative head length	ω	15	0.00	0.0272	0.52			
	Head elongation	ω	15	0.00	0.0129	0.51			
	relative head length	arginine content	15	0.00	0.0625	0.55			
	relative head width	arginine content	15	0.00	0.0486	0.54			
Head elongation	arginine content	15	0.00	-0.00829	0.49				
PGLS	dependent variable	independent variable	n	slope	F	R2	λ	p	
Mammalian clades	clade ω (M3)	relative testes mass variability	7	1.40	2.91	0.11	0(ns,ns)	0.03	*
	mean arginine content	relative testes mass variability	7	-29.16	-2.88	0.63	0(ns,ns)	0.03	*
	arginine content variability	relative testes mass variability	7	9.93	4.82	0.82	1(ns,ns)	0.01	*

Figure S5: Comparison of amino acid substitution along branches (according to PAML, CodeML marginal ancestral reconstruction) and dN/dS branch values (according to PAML CodeML branch model).



