Working Title: Comparative genomic analysis of the pheromone receptor Class 1 family (V1R) reveals extreme complexity in mouse lemurs (genus, *Microcebus*) and chromosomal hotspots across mammals

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Abstract

Sensory gene families are of special interest, both for what they can tell us about molecular evolution, and for what they imply as mediators of social communication. The vomeronasal type-1 receptors (V1Rs) have often been hypothesized as playing a fundamental role in driving or maintaining species boundaries given their likely function as mediators of intraspecific mate choice, particularly in nocturnal mammals. Here, we employ a comparative genomic approach for revealing patterns of V1R evolution within primates, with a special focus on the small-bodied nocturnal mouse and dwarf lemurs of Madagascar (genera *Microcebus* and *Cheirogaleus*, respectively). By doubling the existing genomic resources for strepsirrhine primates (i.e., the lemurs and lorises), we find that the highly-speciose and morphologically-cryptic mouse lemurs have experienced an elaborate proliferation of V1Rs that we argue is functionally related to their capacity for rapid lineage diversification. Contrary to a previous study that found equivalent degrees of V1R diversity in diurnal and nocturnal lemurs, our study finds a strong correlation between nocturnality and V1R elaboration, with nocturnal lemurs showing elaborate V1R repertoires and diurnal lemurs showing less diverse repertoires. Recognized subfamilies among V1Rs show unique signatures of diversifying positive selection, as might be expected if they have each evolved to respond to specific stimuli. Further, a detailed syntenic comparison of mouse lemurs with mouse (genus *Mus*) and other mammalian outgroups shows that orthologous mammalian subfamilies, predicted to be of ancient origin, tend to cluster in a densely populated region across syntenic chromosomes that we refer to as V1R "hotspots."

Introduction

The evolutionary dynamics of sensory gene families are of fundamental interest as a model for how molecular evolutionary processes can shape the content and structure of genomes and for their ability to characterize the life history and ecological traits of organisms. Vomeronasal type-1 receptor genes (V1Rs) comprise one such gene family and have been the subject of increasing interest in both the molecular genetics (e.g., Adipietro et al. 2012) and evolutionary genetics (e.g., Yohe and Brand 2018) communities. Vomerolfaction is a form of chemosensation that mediates semiochemical detection and occurs in the vomeronasal organ (VNO) of mammals (Leinders-Zufall et al. 2000). V1Rs are expressed on vomeronasal sensory neurons in the VNO and have been demonstrated to detect pheromones in mice (Boschat et al. 2002; Haga-Yamanaka et al. 2014). For example, impaired vomeronasal function in mice, either through a knockout of V1Rs or removal of the VNO, alters appropriate chemosensory behaviors such as conspecific avoidance of sick animals, interspecies defensive cues, male sexual behavior, and maternal aggression (Del Punta et al. 2002; Papes et al. 2010; Boillat et al. 2015). Thus, the evolution of V1Rs can have direct consequences for both the emitter and the receiver of pheromone signals, with ample evidence indicating that molecular evolution of V1Rs is associated with the speciation process (Lane et al. 2002; Kurzweil et al. 2009; Hohenbrink et al. 2012; Nikaido et al. 2014).

V1Rs are ideally suited for study within the context of "sensory drive" wherein mate preferences in communication systems diverge in the face of novel environmental opportunity (Boughman 2002). Communication mechanisms for mate recognition have been recognized as an important component for driving rapid reproductive isolation (Mendelson 2003; Dopman et al. 2010; Servedio and Boughman 2017; Brand et al. 2019) and with the reinforcement of species

45 boundaries (Servedio and Noor 2003). Sensory drive can affect diverging populations in two
46 ways by targeting the pheromone receptors and/or their signaling molecules. As examples,
47 adaptation of a likely V1R signal in mice, androgen binding protein, is associated with
48 assortative mating between *Mus musculus* subspecies (Karn et al. 2010; Chung et al. 2017; Hurst
49 et al. 2017) just as fixation of nonsynonymous polymorphisms among V1Rs is associated with
50 the speciation of *Mus spretus* and *Mus musculus* (Kurzweil et al. 2009). Moreover, it has been
51 shown that differential expression of vomeronasal and olfactory receptor genes, including V1Rs,
52 is associated with assortative mating in a pair of house mouse subspecies (Loire et al. 2017) and
53 is likely reinforcing the subspecies along their hybrid zone.

54 The V1R gene family has experienced many duplications and losses in the evolutionary
55 history of mammals, and the availability of duplicate copies can allow for divergence among
56 sequences, gene expression, and ultimately function (e.g. Lynch and Conery 2000; Des Marais
57 and Rausher 2008). Though not directly addressed in this study, it is worth noting that changes in
58 gene expression often occur rapidly after gene duplication events (Makova and Li 2003; Keller
59 and Yi 2014; Guschanski et al. 2017) and are often accompanied by shifts in rates of molecular
60 evolution (Chen et al. 2010; Yang and Gaut 2011). Although the mechanisms that explain
61 variable rates of molecular evolution, specifically the nonsynonymous to synonymous
62 substitution rate ratio (*dN*/*dS*), are complex, there is some interdependence on expression levels
63 (O'Toole et al. 2018) and genome architecture (Dai et al. 2014; Xie et al. 2016). The V1R gene
64 family demonstrates structural complexity (Ohara et al. 2009; Yohe et al. 2018), and gene family
65 expansions and directional selection acting on duplicate copies may be important for the
66 maintenance of species boundaries where vomerolfaction is linked with assortative mating (Luo
67 et al. 2003; Isogai et al. 2011; Fu et al. 2015).

68 Here, we present a comparative genomic study of V1R evolution within the lemuriform
69 primates, primarily focusing on the mouse lemurs of Madagascar (genus *Microcebus*). Mouse
70 lemurs are perhaps the most species-rich clade of living primates (Hotaling et al. 2016), and are
71 well-known for high levels of interspecific genetic divergence though with nearly uniform
72 morphological phenotypes. They have thus come to be regarded as a classic example of a
73 cryptic species radiation, perhaps related to their nocturnal lifestyle (Yoder et al. 2016). Mouse
74 lemurs, and the closely-related dwarf lemurs, have elaborate olfactory communication behaviors
75 that are associated with adaptive strategies such as predator recognition (Sündermann et al.
76 2008), fecundity (Drea 2015), and even biased sex ratios (Perret 1996; Perret and Colas 1997).
77 V1Rs take on particular interest in mouse lemurs as we hypothesize that their observed role in
78 both speciation and in the maintenance of species boundaries within *Mus* may also apply to this
79 speciose clade of primates (Smadja et al. 2015; Loire et al. 2017). We hypothesize that among
80 primates, mouse lemurs will show signatures of sensory drive via genomic elaboration of the
81 V1R complex and evidence of positive selection acting on V1R genes.
82 There are numerous lines of evidence to lead us to this hypothesis: 1) Previous studies
83 have indicated that V1Rs within the lemuriform clade have evolved under pervasive positive
84 selection 5/13/19 2:41:00 PM, 2) that the majority of gene copies are intact (Young et al. 2010;
85 Larsen et al. 2014), and 3) that the differential expression of a large number of vomeronasal
86 receptors in both the VNO and main olfactory epithelium of mouse lemurs are associated with
87 different behaviors and chemical signals (Hohenbrink et al. 2014). In fact, along with murids,
88 opossums, and platypus, mouse lemurs have been reported to have among the largest V1R
89 repertoires found in mammals (Young et al. 2010). Even so, numerous obstacles such as
90 complexities of chemical background, chemical signals, and the genetic basis of chemosensation

complicate both ecological and experimental approaches for differentiating between cause and effect in the speciation process (Yohe and Brand 2018). This is particularly problematic for studies of mouse lemurs given their remote geographic distribution, nocturnal life history, and endangered status. Thus, we take a comparative genomic approach for reconstructing the evolutionary dynamics of the V1R gene family within the small-bodied and nocturnal mouse and dwarf lemurs (family, Cheirogaleidae).

A Comparative Genomic Approach

V1R loci are highly repetitive and they, along with their surrounding regions, are notoriously challenging for genome assembly. Though previous studies have used targeted sequencing or short-read sequencing to examine the evolutionary dynamics of V1R expansions in a limited number of species (Young et al. 2010; Hohenbrink et al. 2012; Yoder et al. 2014), strepsirrhine primates have until recently remained woefully underrepresented in genomic databases (Perry et al. 2012; Meyer et al. 2015; Larsen et al. 2017; Hawkins et al. 2018). Here, we take advantage of the chromosome-level assembly of the gray mouse lemur, *Microcebus murinus*, along with short-read sequencing in related species, to characterize the V1R repertoires for lemuriform primates. Recent improvements using long-read sequencing of the mouse lemur genome (Larsen et al. 2017) improve our ability to characterize the V1R repertoire (Larsen et al. 2014) and allow for comparisons of the genomic architecture of V1R-containing regions in expanded and contracted V1R repertoires across mammals.

In this study, we have sequenced and assembled seven new cheirogaleid genomes, with a particular focus on the mouse lemurs. Further, to explore intraspecific copy number variation and evaluate the effects of assembly error on V1R repertoire counts, we resequenced and *de novo*

114 assembled genomes from eight *M. murinus* individuals from a captive breeding colony. Our
115 study thus serves as a timely companion to two recent overviews of comparative genomic studies
116 for illuminating the evolutionary and life-history dynamics of chemosensory gene family
117 evolution in vertebrates (Bear et al. 2016; Hughes et al. 2018). A comparative genomic approach
118 allows us to explore classic predictions of gene-family evolution, namely, that genomic drift can
119 operate at very fine scales to produce high intraspecific copy number variation (Nozawa et al.
120 2007) and that gene-family evolution is often marked by a strong birth-death process over
121 phylogenetic time scales (Nei et al. 1997; Csűrös and Miklos 2009; Hughes et al. 2018). The
122 latter question is of particular interest for V1R evolution given that adaptive pressures on these
123 genes makes them highly vulnerable to pseudogenization in cases of relaxed selection, thus
124 yielding the observed correlations between levels of V1R ornamentation and diverse adaptive
125 regimes. An overview of primates shows that those with elaborate representation of subfamilies
126 have a strong reliance on chemosensory communication whereas those with depauperate V1R
127 representation rely on alternative mechanisms for inter- and intra-specific communication (Yoder
128 and Larsen 2014).

129 These new genomic resources have also allowed us to address a number of questions
130 regarding rates of molecular evolution in V1Rs. Divergent gene function following gene
131 duplication predicts that some signature of positive selection should be evident in the gene
132 sequences (Zhang et al. 1998), but it remains unknown if selection has acted pervasively over
133 time or has occurred in episodic bursts prior to the diversification of mouse lemurs. We might
134 anticipate episodic positive selection to be the primary mechanism if purifying selection has been
135 operating at more recent time scales to preserve gene function among duplicate copies. For
136 strepsirrhine primates (i.e., the lemurs and lorises), pervasive positive selection has been detected

137 at the interspecific level (Hohenbrink et al. 2012; Yoder et al. 2014), while strong purifying
138 selection has been found within populations. Here we disentangle pervasive versus episodic
139 positive selection among V1Rs and show that both gene duplication and rates of molecular
140 evolution have been active in shaping expanded V1R repertoires among the dwarf and mouse
141 lemurs. Moreover, through comparison with *Mus* and other mammals, we show that orthologous
142 subfamilies tend to cluster in a densely populated region on syntenic chromosomes that we refer
143 to as V1R "hotspots."
144

145 **Results and Discussion**

146 **Novel genome assemblies of several strepsirrhine primates**

147 We *de novo* assembled seven novel strepsirrhine genomes: *Microcebus griseorufus, M.*
148 *ravelobensis, M. mittermeieri, M. tavaratra, Mirza zaza, Cheirogaleus sibreei* and *C. medius*.
149 These efforts have doubled the number of publicly available genomes for the Strepsirrhini with a
150 specific focus on the dwarf and mouse lemur clade. Excluding *C. medius,* the seven genomes
151 were sequenced to an average depth of coverage between 26x and 45x with scaffold N50s of 17-
152 76kb (Supplementary Table S1). The *C. medius* reference genome was assembled using Dovetail
153 Genomics to an average depth of coverage of 110x and a scaffold N50 of approximately 50Mb
154 (Williams et al. 2019). We evaluated assembly completeness using the Benchmarking Universal
155 Single-Copy Orthologs tool, BUSCO (Simão et al. 2015), which assesses genomes for the
156 presence of complete near-universal single-copy orthologs (Supplementary Figure S1). The
157 assemblies recovered between 77.2% and 92.3% of the mammalian BUSCO gene set. We also
158 resequenced eight *M. murinus* individuals, with one duplicate individual (Campbell et al. 2019),
159 and here have *de novo* assembled genomes for each individual with 21x-29x effective coverage

using the 10x Genomics Supernova pipeline. The additional scaffolding information provided by the 10x Genomics linked-reads resulted in scaffold N50s of 0.6-1.2 Mb. BUSCO analyses revealed that the resequenced assemblies recovered between 89.9% and 95.5% of the mammalian gene set. A denser sampling of genomes within Cheirogaleidae not only provides an opportunity for illuminating patterns of V1R gene family evolution but also promotes greater understanding of the molecular evolution of primate and strepsirrhine-specific genomes. Genome resequencing of *M. murinus* individuals has allowed investigation of intraspecific V1R copy number variation as well as questions regarding microevolutionary processes and gene family evolution (Park et al. 2011).

The monophyletic genus *Microcebus* contains 24 named species (Hotaling et al. 2016), and our results clearly demonstrate that the clade has a uniquely complex V1R repertoire compared to other primates thus far characterized (Figure 1A and B). Contrary to a previous study suggesting that V1R expansion is ubiquitous across the lemuriform clade (Yoder et al. 2014), increased sampling reveals that expansion has been profound in the nocturnal dwarf and mouse lemurs. This is consistent with the original hypothesis that local V1R expansions may play a role in forming or maintaining speciation boundaries within *Cheirogaleus* and *Microcebus* as might be predicted given their nocturnal lifestyle. Phylogenetic analyses revealed that expanded V1R repertoires in mouse lemurs demonstrate a remarkably higher rate of duplicate gene retention in comparison to other primates (Figure 1A and B; Table 1). The common ancestor of mouse lemurs is not associated with novel subfamily birth though the diversity and number of V1R gene copies is striking (Figure 1A; Table 1). Although genomes generated exclusively from short-read data are vulnerable to collapsing loci in assemblies (Larsen et al. 2014), our inference of increased V1R retention in *M. murinus* relative to non-cheirogaleid

183 primates was robust to assembly strategies and data sources (Supplementary Table S2). Further,
184 the resequenced *M. murinus* individuals reveal low intraspecific variation in copy number
185 (Figure 2), suggesting that the observed differences in repertoire size between mouse lemurs and
186 other non-nocturnal lemurs is not an artifact of individual sampling or assembly error (Figure 3).
187 The expansion dynamics of V1Rs within Cheirogaleidae do not support a simple linear
188 correlation between species richness and repertoire size. Although all cheirogaleid repertoires
189 had full primate subfamily membership, there was variation in subfamily proportions between
190 species, which is consistent with our hypothesis that species-specific V1R repertoires and
191 chemosensation may be important for species diversity of cheirogaleids in comparison to diurnal
192 strepsirrhines. Dwarf lemurs, genus *Cheirogaleus*, are hypothesized to have as many as 18
193 species (Lei et al. 2014) though have the smallest V1R repertoires within the cheirogaleids
194 examined here. Conversely, the genus *Mirza,* with only two recognized species, has a repertoire
195 size that is nearly equal to that of *Microcebus murinus*. It is notable, however, that the *Mirza*
196 genome's expanded repertoire is primarily enriched for subfamily III (Figure 1A). The
197 differential subfamily enrichment among species suggests that despite the similarity in size to
198 *Microcebus* repertoires, the V1R repertoire of *Mirza* has experienced independent selective
199 pressures on gene retention and may ultimately fulfill a different functional role compared to
200 *Microcebus*.
201
202 **V1R repertoire estimation across primates**
203 We estimated V1R repertoire size evolution across strepsirrhine primates as well as for
204 several well-annotated primate and mammalian genomes for outgroup comparison. Notably,
205 repertoire estimates of extant primates are comparable to previous studies that used trace archive

206 fragments and earlier draft genome versions (Figure 3; Supplemental Table S2; Young et al.
207 2010; Moriya-Ito et al. 2018). The expanded V1R repertoire within the gray mouse lemur is not
208 ubiquitous across the Strepsirrhini, however. Rather, repertoire size expanded gradually from a
209 reduced set in the strepsirrhine common ancestor to its peak in the mouse lemur clade. This
210 expansion is characterized by a reduced repertoire in the early diverging aye-aye lineage (genus
211 *Daubentonia*), moderate repertoires among diurnal lemurs, and an expansion that likely occurred
212 in the common ancestor of Cheirogaleidae (Figure 3). If the origins of many V1R copies in
213 mouse lemur date to the Cheirogaleidae common ancestor, this means that at least some of those
214 duplicates have remained functional and intact since their origins 30 million years ago, as would
215 be consistent with divergence time estimates for the cheirogaleid radiation (Yang and Yoder
216 2003; dos Reis et al. 2018).

217 Within Cheirogaleidae, repertoire sizes ranged from a low of 58 intact V1Rs in *C. medius*
218 to highs between 102-143 intact V1Rs in the genus *Microcebus*. The mouse lemurs have
219 universally large V1R repertoires (102-146 intact genes) with notable intragenus variation. Prior
220 to this study, *M. murinus* had been identified as having one of the largest V1R repertoires within
221 mammals (Young et al. 2010). Additional sampling from *Microcebus* reveals, however, that
222 among the five mouse lemur species here characterized, *M. murinus* actually has the smallest
223 repertoire with only 102 intact V1Rs. We also estimated the percent of intact V1Rs contained
224 within the total repertoire for each species. Most haplorrhine primates (Anthropoidea plus
225 *Tarsius*) species have repertoires with low percentages of intact receptors (<37% intact). Within
226 Lemuroidae, the diurnal lemurs also have small and pseudogenized repertoires (26% to 49%
227 intact) containing only 22-27 intact V1Rs. In contrast, among nocturnal species excluding aye-

228 aye, we observe intact repertoires between 58% to 66% within Cheirogaleidae, and 61% for the
229 nocturnal lorisiform *Otolemur garnetti*.

230 These comparisons do not, however, provide definitive evidence that expanded V1R
231 repertoires in primates are strictly associated with nocturnal life history (Wang et al. 2010;
232 Moriya-Ito et al. 2018). Although *Otolemur garnetti* shows a proportion of intact V1R copies
233 similar to dwarf and mouse lemurs (Figure 3), subfamilies VII and IX are absent from *O.*
234 *garnetti* (Figure 1B). By comparison, the genomes of both the aye-aye and the tarsier (Schmitz et
235 al. 2016) contain low numbers of intact V1R gene copies, which appears to contradict the
236 hypothesis that a nocturnal life history alone is sufficient for explaining V1R elaboration in
237 mouse lemurs. Though it is true that both aye-aye and tarsier have more V1R copies than the
238 diurnal primates compared here, they also show a high proportion of putative pseudogenes and
239 an absence of some V1R subfamilies found in Cheirogaleidae (Figure 1A and B).

240 Our phylogenetic approach reveals a pattern of gene family evolution compatible with
241 active gene birth and death (Nei et al. 1997; Csűrös and Miklos 2009; Hughes et al. 2018) with
242 an independent V1R expansion isolated to Cheirogaleidae with three subfamily gains rather than
243 a single more ancient expansion followed by losses in diurnal lineages (Table 1). Although the
244 gain and loss dynamics of V1Rs over time is complex with uncertainty in the origins of specific
245 subfamilies, variation in subfamily membership among species suggests that nocturnal primates
246 possess more diverse repertoires than their diurnal counterparts (Figure 1A and B). These results
247 are suggestive of an association between nocturnal life histories and V1R repertoire evolution, as
248 well as the importance of chemosensation generally among nocturnal primates. Our findings are
249 not conclusive, however, as the pattern observed in aye-aye deviates from this expectation,
250 though it must be noted that the quality of the aye-aye genome assembly is considerably poorer

than the others compared with the lowest contig/scaffold N50 and most incomplete BUSCO results (Supplementary Figure S1and Table S1). An improved genome for aye-aye, a notably solitary primate (Sterling and Richard 1995), as well as genomes for species within the diurnal nest-dwelling genus, *Varecia*, will allow for more formal tests of how life history traits are correlated with V1R copy number.

Subfamily membership and ancestral repertoire reconstruction

For each genome analyzed, we classified repertoire subfamily membership based on homology inferred from a maximum likelihood (ML) tree (Figure 4) and previously-described subfamily designations (Hohenbrink et al. 2012). During alignment, sequences that introduced excessive gaps to transmembrane regions were iteratively removed, resulting in alignments of increasing conservatism (see Materials and Methods "V1R repertoire estimation and ancestral count reconstruction"). We tested whether these varying alignments affected our estimates of subfamily composition and found little impact. Regardless of the number of sequences removed from the alignment, the relative proportions of subfamily membership within each species remained constant (Supplementary Figure S2). Although topological errors may contribute to uncertainty in gene count reconstructions, the ML tree shows 70% or greater bootstrap support for 63% of nodes (Figure 4), with little additional improvement possible due to the limitations of a single-exon gene family (Supplementary Table S3). Our results suggest that both the ancestral primate and the ancestral lemur had repertoires more limited in size and diversity than many living strepsirrhine primates, further supporting the controversial hypothesis that the ancestral primate was diurnal rather than nocturnal (Tan et al. 2005; Borges et al. 2018).

Subfamily membership varies among the other extant strepsirrhines examined (Figure 1A and B). While *Otolemur garnetti* contains a very diverse repertoire, it lacks subfamily VII and IX membership. The diurnal lemurs lack receptors belonging to a few subfamilies, most consistently IV, VIII, and IX. The basal lineage within the lemuriform radiation, *Daubentonia madagascariensis,* lacks membership for most subfamilies, including *Strep*/I, II, IV, V, and VII. Subfamily I, referred to as "V1R*strep*" in Yoder et al. (2014), is used synonymously here for distinction from the mouse subfamily "I". The repertoires of cheirogaleids are highly enriched for subfamily III, V, and IX membership, while the diurnal lemurs are enriched for subfamilies *Strep*/I, II, and III. In haplorrhine primates, repertoires contain only one or a few subfamilies. Ancestral state reconstruction with asymmetric parsimony (Csűrös and Miklos 2009; Csűrös 2010) revealed that the stem primate possessed only a subset of now extant V1R subfamilies, *Strep*/I, II, III, IV and VIII (Figure 1B). Subfamily IX has undergone a notable expansion in Cheirogaleidae, but the aye-aye repertoire also contains members from subfamily IX thus, subfamily IX is the only subfamily exclusive to nocturnal strepsirrhines, despite its absence in *Otolemur garnetti* (Figure 1A and B).

Copy number variation in intraspecific *Microcebus murinus* repertoires

We resequenced eight *M. murinus* individuals of known pedigree from the colony at the Duke Lemur Center in Durham, North Carolina. Using these genomes, we estimated intraspecific variation in V1R repertoire size (Figure 2). For the eight resequenced *M. murinus* individuals, we observed low levels of intraspecific V1R repertoire size variation relative to size variation between taxonomic families with individual repertoires ranging from 86 to 105 intact V1R loci. Though one might expect that levels of intraspecific variation in V1R repertoire size in

299 a captive population may be reduced relative to wild populations of *M. murinus,* the colony at the
300 Duke Lemur Center shows signs of admixture from two distinct evolutionary lineages, *M.*
301 *murinus* and *M. ganzhorni* (Larsen et al., 2017), presently recognized as distinct species
302 (Hotaling et al., 2016). Therefore, the intraspecific variation observed here may actually be
303 exaggerated, rather than reduced, which increases our confidence in the robustness of repertoire
304 size estimates among species through sampling of single individuals. To test for the potentially
305 confounding effects of sequencing and assembly error, one individual, DLC7033, was sequenced
306 twice as a technical replicate. The duplicate genome assemblies respectively contained 92 or 96
307 intact loci indicating that sequencing and assembly error likely play a measurable role in
308 generating variation among observed repertoire counts, though the effect appears to be modest.
309 Thus, taking the results of the pedigree analysis as largely accurate, this emphasizes the highly
310 dynamic nature of V1R repertoire size evolution, even over generational timescales.
311
312 **Complex history of diversifying positive selection in the dwarf and mouse lemurs**
313 Our results agree with previous studies in finding that selection has acted pervasively
314 across the V1R gene family over time (Hohenbrink et. al. 2012). Pervasive positive selection was
315 revealed for all subfamilies identified in this study, even when analyzing the genus *Microcebus*
316 alone (Supplementary Tables S4 and S5) and additional genome sequences for dwarf and mouse
317 lemurs have likely increased the power of the sites tests. For example, positive selection was not
318 evident for subfamily VII in a previous study limited to only *Microcebus murinus* (Hohenbrink
319 et al. 2012). Furthermore, some subfamilies have unique profiles of sites under selection (Figure
320 5). Although lineage-specific rate variation is a confounding factor in V1R gene family evolution

321 (Yoder et al. 2014), our analyses, spanning a range of taxon sampling schemes, show that our
322 ability to characterize the V1R selection profiles are robust to such rate variation (Figure 5).
323 We performed two different model comparisons to differentiate between hypotheses of
324 neutrality versus selection, and for the latter, for differentiating between the effects of rate
325 constancy versus heterogeneity among sites. The M7 and M8 model comparisons always
326 recovered more sites under selection than the more conserved M1a and M2a comparisons, but
327 individual sites under selection detected by Bayes empirical Bayes with M2a were subsets of
328 those detected by M8. Both model comparisons use likelihood ratio tests (LRTs) to detect
329 positive selection and assume *dN*/*dS* is constant across branches, but the M2a and M1a
330 comparison (Zhang et al. 2005) uses three finite mixtures of *dN*/*dS* while the M8 and M7
331 comparison (Yang et al. 2000) accounts for heterogeneity in *dN*/*dS* among nearly neutral sites
332 with a beta distribution. Tests of pervasive positive selection were also performed on data
333 realigned by subfamily, and similar estimates of proportions of sites under positive selection
334 suggested that our site models were not misled by alignment errors (Supplementary Tables S6
335 and S7). Most individual sites under positive selection are unique to different subfamilies
336 (Supplementary Figure S3) and reflect biases in selective pressures across different loop and
337 transmembrane domains (Supplemental Figure S4). However, some selection profiles were more
338 differentiated than others, such as *Strep*/I, II, V, and IX (Figure 5). The divergent selection
339 profiles among subfamilies leads us to interpret positive selection acting on V1R genes in
340 primates to be largely diversifying. Differentiated selection profiles among subfamilies are
341 explained by biases among transmembrane and loop domains (Supplementary Material;
342 Supplementary Figure S5; Supplementary Tables S8-S10).

343 Previous studies have indicated that extracellular loops have been primary targets of
344 positive selection in V1Rs (Hohenbrink et al. 2012), and our results agree with these findings.
345 Positive selection acting on extracellular loops two and three from Hohenbrink et al. (2012),
346 identified here simply as loops three and five respectively, is evident (Supplementary Figure S5).
347 These specific domains are probable regions where V1Rs bind to semiochemicals (Hohenbrink
348 et al. 2012). Our results also show the transmembrane domains themselves, whether directly or
349 by linkage, have also been under variable levels of positive selection (Supplementary Material;
350 Supplementary Table S8). We find limited evidence for an enriched number of sites under
351 positive selection in transmembrane domains four and five, and a depletion in transmembrane
352 domain three, which have been previously predicted to form the ligand binding pocket of V1Rs
353 (Kobilka et al. 1988; Pilpel and Lancet 1999; Palczewski et al. 2000; Yoder et al. 2014). These
354 results prompt us to hypothesize that pervasive diversifying positive selection has accompanied
355 selection for divergent function among V1R subfamilies, although additional evidence is needed
356 for hypothesis testing.
357 Branch-site models detected evidence of episodic positive selection in the evolution of all
358 V1R subfamilies except for lemur VIII (Supplementary Figure S6; Supplementary Table S11).
359 Tests of episodic positive selection across the V1R subfamilies in the house mouse have been of
360 little interest (Karn et al. 2010) and our tests of episodic selection here are generally not
361 associated with the expansion of V1Rs in dwarf and mouse lemurs. However, subfamily IX
362 would be a candidate for further investigation, given that it was the only subfamily to show
363 notable levels of episodic positive selection, and is the only subfamily specific to nocturnal
364 strepsirrhines. Further, many of the sites identified to be under positive selection correspond to
365 the previously identified ligand binding domains (Supplementary Figure S6, Supplementary

366 Table S12). Exploration of alternative topologies revealed that branches showing episodic
367 positive selection were likely not due to topological errors (Supplementary Material;
368 Supplementary Figure S6).
369
370 **Comparative evolution of V1R repertoires and genome architecture across Mammalia**
371 Here we take advantage of the recently published chromosome-level assembly for *M.*
372 *murinus* and other chromosome-level mammalian assemblies in an effort to identify genomic
373 features that are generally associated with V1R expansion. The molecular environment of V1Rs
374 is predicted to play a role in their regulation and has previously been studied only in mouse, rat,
375 and pig (Lane et al. 2002; Stewart and Lane 2007; Kambere and Lane 2009; Michaloski et al.
376 2011; Dinka and Le 2017). We compared the expanded V1R repertoires of mouse and mouse
377 lemur with the putatively contracted V1R repertoires of horse, cow, dog, and human. As
378 predicted from previous studies (Kambere and Lane 2007; Kambere and Lane 2009), enrichment
379 for repetitive LINE elements is associated with expansion of V1Rs in mammals (Supplementary
380 Figure S7). We find that mouse lemur V1Rs primarily cluster by subfamily at chromosomal
381 locations across the genome as is also characteristic of the V1R repertoire in mouse. Only mouse
382 lemur subfamily VIII does not form a cluster but is instead uniquely dispersed across three
383 different chromosomes (Figure 6). We also analyzed the locations of all regions demonstrating
384 V1R homology to determine if there are any potential pseudogenized subfamilies or clusters in
385 the genome and found no evidence for pseudogenized clusters of V1Rs in mouse lemur
386 (Supplemental Figure S12). Both LINE enrichment and physical clustering of V1R loci have
387 been predicted to be associated with proper regulation of V1Rs (Lane et al. 2002; Kambere and
388 Lane 2007) and may be characteristic of expanded V1R repertoires in general.

389 To investigate whether homologous subfamilies have retained chromosomal synteny in
390 species with expanded repertoires and across mammals broadly, we evaluated chromosomal
391 synteny for each species relative to mouse using the SynChro software (Drillon et al. 2014;
392 Figure 6, Figure 7A and B, Supplementary Figures S8-S11). In mouse and mouse lemur, most
393 homologous V1R subfamilies retain chromosomal synteny (Figure 6, Figure 7A and B). Mouse
394 subfamily D is most closely related to mouse lemur subfamily IV, and both subfamilies share
395 mouse chromosome 7 synteny. Subfamilies J/K and V as well as subfamilies G and *Strep*/I also
396 share mouse chromosome 7 synteny. Lemur subfamily III is syntenic with mouse E and F on
397 mouse chromosomes 6 and 7. Lemur subfamilies VI and VII are syntenic with mouse
398 subfamilies H and I on chromosome 13. Lemur subfamilies not sharing synteny with any mouse
399 subfamily include subfamilies II, VIII, and IX. The expanded subfamilies in Cheirogaleidae, IV,
400 VII, and IX, all map to different chromosomal regions of the *M. murinus* genome and were not
401 linked on an ancestral syntenic block based on comparisons between *M. murinus* and mouse.
402 Therefore subfamily expansions have occurred independently and not as tandem duplications of
403 a single genomic region.
404 Interestingly, when comparing all mammalian species examined, our results reveal that in
405 each species, one chromosome contains a very dense block of highly homologous subfamilies on
406 a backbone of mouse chromosome 7 synteny, referred to here as "V1R hotspots" (Figure 7B).
407 These hotspots usually contain receptors of the EF/III, D/IV, JK/V, and Strep/G subfamilies, and
408 cluster order is maintained with a few species-specific subfamily deletions. The chromosomal
409 synteny of the "hotspots" is rarely interrupted, and if interrupted, it is almost exclusively
410 interrupted by a stretch of synteny from another mouse chromosome containing V1Rs. These
411 interleaving regions in hotspots are usually chromosome 13 or 17, indicating that genomic

412 regions where V1Rs cluster are also subject to increased gene duplication rates. Interestingly, the
413 only putative intact members of the contracted human V1R repertoire are also contained within
414 this "hotspot" location and share homology with hotspot subfamilies.

415 Previous studies of Laurasiatheria have predicted that the V1R repertoires of cow, horse,
416 and dog consist mostly of highly orthologous loci with evolutionary conserved functions (Yohe
417 et al. 2018). While conserved function remains to be shown experimentally, retained synteny of
418 these Laurasiatherian V1Rs within hotspots across Mammalia supports the hypothesized ancient
419 origin of these subfamilies and reinforces the idea that V1Rs in these subfamilies are orthologous
420 in function (Ohara et al. 2009; Yohe et al. 2018). Mouse lemur V1Rs show striking structural
421 similarities to the functionally diverse repertoire of mouse and considering the independent gains
422 in copy number and novel subfamily evolution, coupled with variable rates of molecular
423 evolution and selective pressures, V1Rs in mouse lemurs may serve as an ideal system for
424 elucidating pheromone evolution in primates. Similar patterns of deep synteny have been
425 described for ~80 My of odorant receptor evolution in bees (Brand and Ramirez 2017).
426 Considered in this context, our results suggest that chemosensory gene family evolution may
427 follow similar molecular "rules" in organisms with vastly different natural histories, even when
428 evolved independently from different ancestral gene families, as would be the case comparing
429 mammals to insects.

430

431 **Conclusions**

432 We revealed that an expansion of the V1R gene family is shared across the dwarf and
433 mouse lemurs, and that duplicate V1R gene copies have been evolving under strong selective
434 pressures. Divergent patterns of molecular evolution among V1R subfamilies and diversity in

435 subfamily membership and abundance suggests that V1Rs may serve as a test case for studying
436 the evolution of sensory drive in primates. Pheromone detection among nocturnal primates,
437 especially the morphologically cryptic mouse lemurs, may be more important for driving and
438 maintaining species boundaries than previously appreciated. Syntenic analyses with improved
439 genomic resources revealed strikingly similar genetic architecture between the expanded V1R
440 repertoires of mouse and mouse lemur, and that some V1R subfamilies have been maintained in
441 V1R "hotspots" across ~184 million years of mammalian evolution (dos Reis Mario et al. 2012).
442 Characterizing additional features of V1R hotspots across species will be important for future
443 studies translating experimental genetic studies in mice to primates such as mouse lemur.
444

445 **Materials and Methods**

446
447 **Sampling and DNA extraction**

448 To improve the resolution of the V1R repertoire expansion in lemurs, we sequenced the
449 genomes of *Microcebus griseorufus*, *M. mittermeieri*, *M. ravelobensis, M. tavaratra*, and *Mizra*
450 *zaza*. Tissue biopsies were taken from wild individuals in Madagascar from 1997-2015 and from
451 captive individuals at the Duke Lemur Center (Supplemental Table S13). To investigate within
452 species variation in V1R repertoires, we also resequenced eight individuals from the Duke
453 Lemur Center *Microcebus murinus* colony. Blood and tissue samples were collected in 2016 in
454 accordance with IACUC guidelines. For the novel strepsirrhine genomes, DNA was extracted
455 following manufacturer instructions using the Qiagen DNeasy Blood and Tissue kit, while DNA
456 from the *Microcebus murinus* resequenced individuals was extracted using the Qiagen
457 MagAttract Kit (Qiagen, Germantown, MD, USA).

458 **Genome Sequencing and Assembly**

459

460 The genomes of *Microcebus griseorufus*, *M. mittermeieri*, *M. tavaratra,* and *Mizra*
461 *zaza* were sequenced at the Baylor College of Medicine as approximately 400bp insert libraries
462 on a single lane of an Illumina HiSeq 3000 with paired-end 150bp reads. We sequenced
463 the *Microcebus ravelobensis* genome from two libraries, one with an average insert size of
464 570bp on an Illumina HiSeq 2000 at Florida State University and the other with a 500bp insert
465 library on 5.5% of both lanes of an Illumina NovaSeq at the Duke University GCB Sequencing
466 Core. We also generated two additional cheirogaleid assemblies for *Cheirogaleus sibreei* and *C.*
467 *medius* (Williams et al. 2019). *Cheirogaleus sibreei* was sequenced from a 300bp insert library
468 on the Illumina HiSeq 4000 at the Duke University GCB Sequencing Core with paired-end
469 150bp reads. A reference genome was generated and assembled for *Cheirogaleus medius* using
470 Dovetail Genomics. All other genomes were assembled using MaSuRCA v3.2.2 (Zimin et al.
471 2013). We assumed an insert size standard deviation of 15% and used automatic kmer selection.
472 However, we did not use MaSuRCA's scaffolds for annotation and downstream analyses.
473 Scaffolds were obtained from SSPACE (Boetzer et al. 2010), which also attempted to correct
474 assembly errors and extend contigs from MaSuRCA. *De novo* assembly statistics are available in
475 the supplementary material (Supplementary Table S1) as well as annotation details
476 (Supplementary Table S14) and SRA identifiers (Supplementary Table S15).
477 The eight *Microcebus murinus* individuals were resequenced from high molecular weight
478 DNA prepared using the 10X Genomics Chromium platform. Briefly, high-molecular weight
479 molecules of DNA are partitioned into gel beads with unique barcodes then prepared for Illumina
480 sequencing (Weisenfeld et al. 2017). The resulting short-read libraries are barcoded such that
481 individual "linked reads" can be traced back to their molecule of origin assisting the genomic

482 scaffolding process. The libraries were size selected to approximately 550bp and sequenced on
483 the Illumina HiSeq 4000 system at the Duke University GCB Sequencing Core. We then used
484 the 10X Genomics Supernova assembly software to *de novo* assemble the resequenced genomes
485 (version 2.0.1, 10x Genomics, San Francisco, CA, USA). One replicate individual was
486 sequenced twice, and genomes were assembled *de novo* from each individual library.
487 BUSCO version 3.0.2 and Assemblathon2 scripts were used to assess genome quality
488 statistics (Supplementary Figure S1; Supplementary Table S16; Simão et al. 2015). Additional
489 genomes analyzed in this study were downloaded from the NCBI genome database and include
490 all available Strepsirrhine genomes and additional high-quality primate and mammalian genomes
491 for phylogenetic coverage (Supplementary Table S16).
492
493 **V1R repertoire estimation and ancestral count reconstruction**
494 To assess total V1R repertoires in each species, tblastn searches (e-value cut-off = 0.001)
495 were conducted with the blast+ software suite (version ncbi-blast-2.6.0+; Altschul et al. 1990)
496 using available mouse and mouse lemur V1R query protein sequences downloaded from NCBI
497 GenBank against the genomes analyzed in this study (Camacho et al. 2009). Duplicate protein
498 sequences were removed from the query database using CD-HIT version 4.6 (Li and Godzik
499 2006). Bedtools merge (version 2.27.1) was used to merge overlapping hits within a genome, and
500 bedtools slop and getFasta were used to extract receptor candidate regions longer than 600bp
501 with 50 bp of upstream and downstream surrounding sequence (Quinlan and Hall 2010). For a
502 full list of V1R containing regions analyzed see Supplementary File X).
503 To remove potential pseudogenes from further analyses, we used Geneious version 9.0.5
504 to predict open reading frames (ORFs) and considered sequences intact if they contained an ORF

505 longer than 801bp. We then used MAFFT version 7.187 with the E-INS-i algorithm to align
506 intact sequences from all species using the iterative approach described in Yoder 2014 (Katoh
507 and Standley 2013; Yoder et al. 2014). The MAFFT algorithm is recommended for approaches
508 analyzing ancestral sequence reconstruction (Vialle et al. 2018). A gene phylogeny was
509 constructed using RAxML version 7.2.8 using the GTRGAMMAI nucleotide model with the
510 rapid bootstrapping and search for best ML scoring tree algorithm with 500 bootstraps
511 (Stamatakis 2014). We then assigned primate sequences to the subfamilies *Strep*/I-IX designated
512 in Hohenbrink 2012 (Hohenbrink et al. 2012). The number of intact V1Rs, percentage of intact
513 V1Rs, and the total V1R count were calculated for each species as well as subfamily
514 membership. We then used Count version 10.04 with the Wagner parsimony algorithm and a
515 gain penalty of 2 to infer total ancestral vomeronasal repertoire size and ancestral subfamily
516 membership (Csűrös and Miklos 2009; Csűrös 2010).
517
518 **Establishing synteny of V1Rs across mammalian species**
519 Genomes with chromosome level scaffolding information (*Mus musculus, Microcebus*
520 *murinus, Homo sapiens, Equus caballus, Bos taurus,* and *Canis familiaris*) were used to assess
521 chromosomal synteny of vomeronasal subfamilies among mammalian species. SynChro (Drillon
522 et al. 2014) version SynChro_osx (January 2015) was used to reconstruct synteny blocks
523 between each genome with *Mus musculus* as reference with a delta parameter of 2 using
524 GenBank annotation files from Ensembl release 93 (Figure 6; Supplemental Figures S8-S11;
525 Drillon et al. 2014). Orthologous block information was compared with vomeronasal receptor
526 location for each species (Figure 7A and B).
527

528 **Detecting evidence of positive selection**

529 Evidence for positive selection in V1R repertoires was evaluated with PAML 4.8e (Yang
530 2007). We used two different tests to detect both individual sites under pervasive positive
531 selection throughout the tree (sites models) and individual branches that show an episodic burst
532 of positive selection (branch-site models). For sites models, we applied two tests to each of the
533 nine subfamily trees and alignments: 1) Comparison of the null hypothesis that all sites are a
534 mixture of purifying and neutral rates of molecular evolution (M1a) and the alternative that
535 allows for a third class of sites under positive selection (M2a; Zhang et al. 2005). 2) A null
536 hypothesis that allows for a mixture of ten discretized beta-distributed site classes (M7), while
537 the alternative hypothesis allows an extra component under positive selection (M8;Yang et al.
538 2000). Each of the recognized lemur subfamilies were analyzed separately. The ggtree R
539 package (Yu et al. 2017) was used to extract subtrees for each subfamily and alignments were
540 parsed with Perl scripts. Because signatures of positive selection may be time-dependent
541 (Peterson and Masel 2009; Pegueroles et al. 2013), we explored variation in sites under positive
542 selection using six different taxonomic filters: 1) *Microcebus*, 2) Cheirogaleidae, 3)
543 Lemuriformes, 4) Strepsirrhini, 5) Primates, and 6) Euarchontoglires. For each site test, we
544 assumed the LRT was $\sim X^2_1$ and individual sites were detected using the Bayes empirical Bayes
545 procedure where the posterior probability of selection for each site was determined using the
546 MLE *dN/dS* for the positive selection rate class (Yang et al. 2005). Individual sites were
547 considered to have sufficient evidence for positive selection if the posterior probability was
548 greater than 0.95. Enrichment of sites under selection in transmembrane domains used simple
549 chi-square tests and Fisher exact tests in R (R Core Team 2018) for individual transmembrane
550 and loop domains. Transmembrane domains were predicted using *M. murinus* sequences from

551 subfamilies *Strep*/I through IX with TMHMM (Krogh et al. 2001) through the TMHMM server
552 (http://www.cbs.dtu.dk/services/TMHMM/; last accessed 29 January 2019). Since V1R genes
553 are expected to have seven transmembrane domains (Dulac and Axel 1995), only predicted
554 structures with seven transmembrane domains were used to determine transmembrane domain
555 boundaries in our alignment of the entire V1R repertoire. Predictions that had fewer or more than
556 seven transmembrane domains are assumed to be due to inaccuracies of TMHMM (Melén et al.
557 2003) and not real domain losses or gains.

558 Of important note, the entire V1R repertoire was prohibitively large for ML optimization
559 over the entire tree; we applied tests for selection to individual subfamilies to circumvent this
560 limitation. This strategy also provided a way to evaluate contributions of alignment and
561 topological errors to evidence of positive selection. First, we evaluated if the ML topology
562 estimated from the entire repertoire was a plausible hypothesis using AU tests (Shimodaira
563 2004). First, we estimated the ML topology and branch lengths for each subfamily using the
564 parsed alignments (i.e. the data was not re-aligned) using the same RAxML model and search
565 strategy as the first analysis. We then re-aligned translated amino acid data with MAFFT and
566 estimated phylogeny once more. Site log-likelihoods were then optimized for the three
567 topologies with RAxML and AU p-values computed with CONSEL using the default multiscale
568 bootstrapping strategy (Shimodaira and Hasegawa 2001). Bootstrap trees were also collected for
569 the re-aligned data, but bipartitions were drawn onto the topologies parsed from the entire V1R
570 repertoire tree. The ratio of bootstrap support values was used to identify potential topological
571 errors; bipartitions in the original topology that are absent when the sequences for each
572 subfamily were re-aligned. Site tests were run for both the parsed and re-aligned data to check
573 for consistency in the inference of sites under positive selection across alignments.

Branch-site tests (Zhang et al. 2005) were performed for each branch for each subfamily, except subfamily III, which was still computationally limiting. Each test assumed the LRT was $\sim X^2_1$, but we applied the Benjamini-Hochberg multiple testing correction (Benjamini and Hochberg 1995). With this correction, we do expect some false positives, but the family-wise error rate should be below 5% (Anisimova and Yang 2007) while not underpowering tests towards the tips of the trees (dos Reis and Yang 2011). Tests were only performed on the parsed topology without removing any species, but branches with evidence of episodic positive selection and the bootstrap ratios with re-aligned data were mapped to nodes of the subtree topologies using ggtree to help identify cases where topological errors might lead to false signatures of positive selection (Mendes and Hahn 2016). Individual sites with evidence of episodic positive selection were evaluated using the Bayes empirical Bayes procedure (Yang et al. 2005).

Data Access

Newly sequenced genome data will be made available through NCBI upon publication. Complete record information is given in Supplementary Material (Supplementary Table S15).

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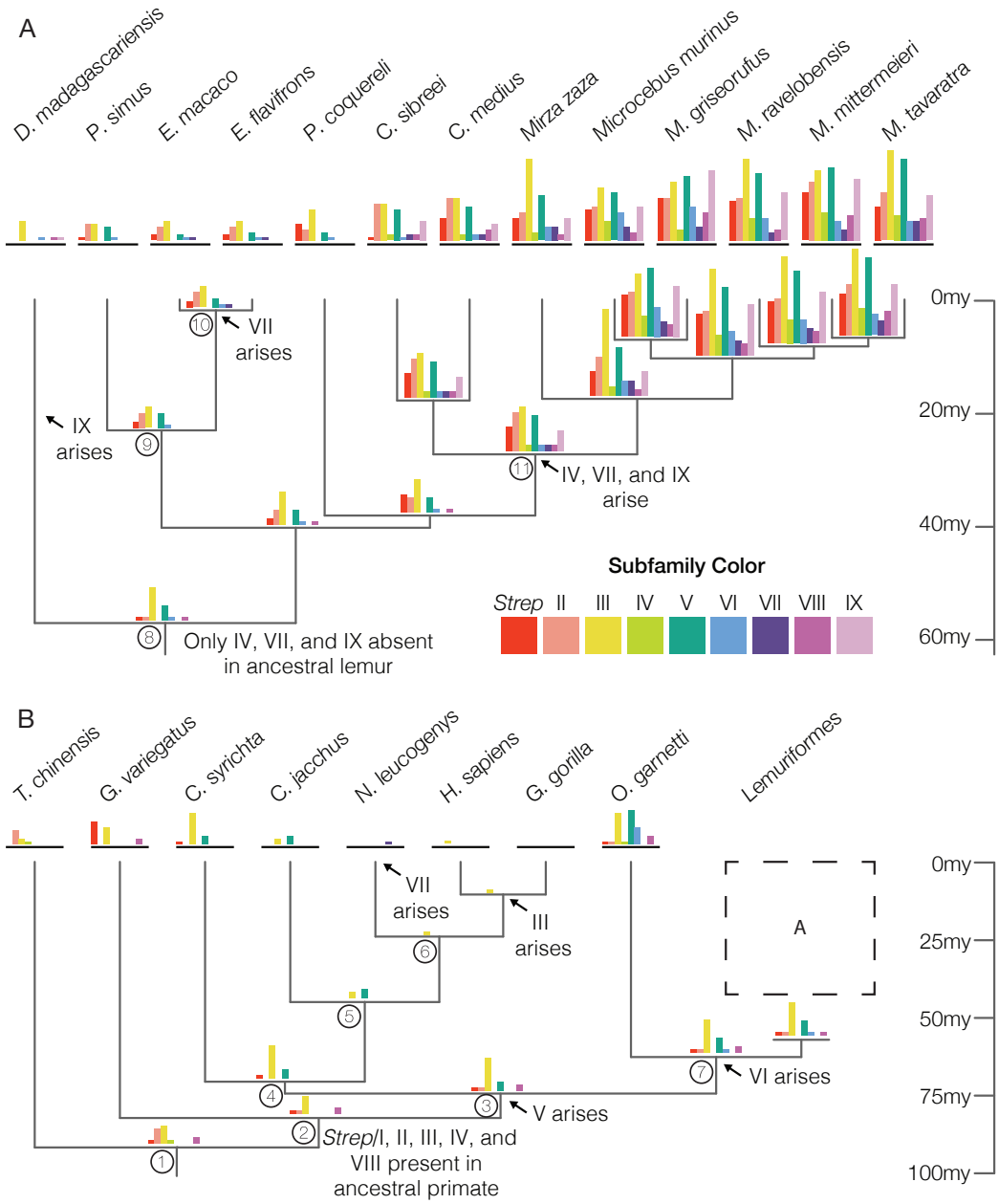


Figure 1. V1R subfamily membership across the primate phylogeny. Membership was assessed for available strepsirrhine genomes (A) and for select primate outgroups (B) and estimated using the Count software for ancestral lineages. Bar graphs show absolute gene count for each subfamily. Predicted gene subfamily origins are annotated with arrows. Tree adapted from dos Reis 2018 (dos Reis et al. 2018). Circled node numbers correspond with Table 1.

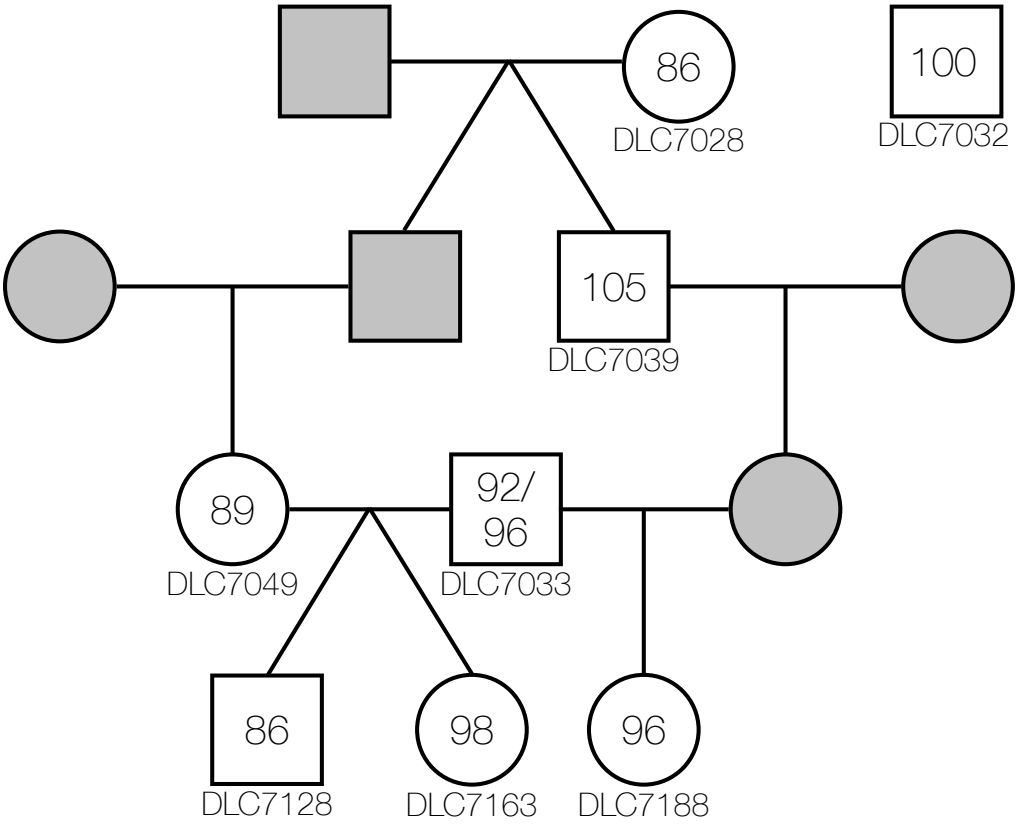
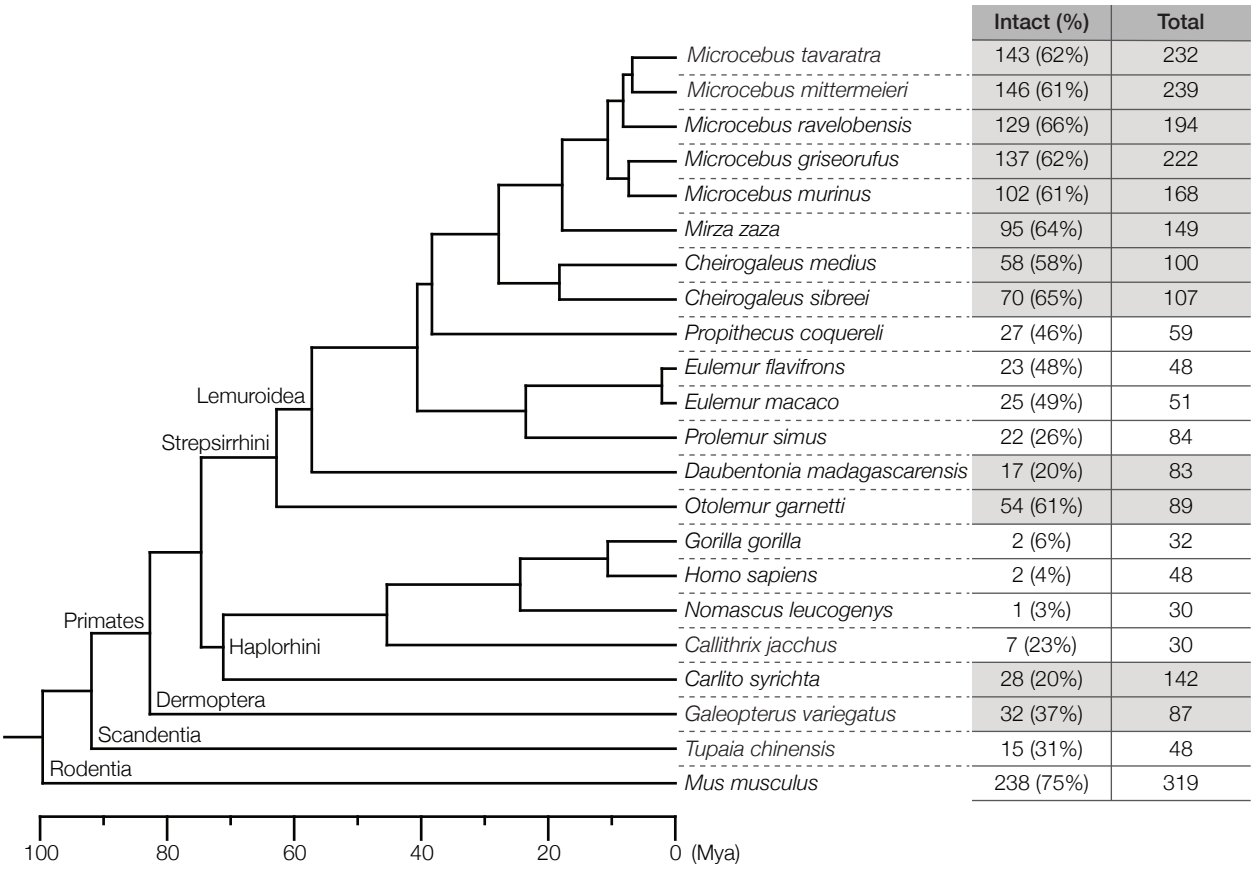


Figure 2. Intraspecific variation in V1R repertoire size estimates across eight closely-related *Microcebus murinus* individuals. Genomes were *de novo* assembled and mined for loci with significant V1R homology and an ORF longer than 801bp. Individual DLC7033 was sequenced twice and repertoire size estimates are reported for both assemblies. Squares represent males and circles represent females. Horizontal lines indicate mate pairs (mother and father) and vertical or slanted lines indicate parent to offspring relationshiop. Numbers inside the symbols represent repertoire size estimates. Individuals represented by grey symbols were not sequenced.



	Intact (%)	Total
Microcebus tavaratra	143 (62%)	232
Microcebus mittermeieri	146 (61%)	239
Microcebus ravelobensis	129 (66%)	194
Microcebus griseorufus	137 (62%)	222
Microcebus murinus	102 (61%)	168
Mirza zaza	95 (64%)	149
Cheirogaleus medius	58 (58%)	100
Cheirogaleus sibreei	70 (65%)	107
Propithecus coquereli	27 (46%)	59
Eulemur flavifrons	23 (48%)	48
Eulemur macaco	25 (49%)	51
Prolemur simus	22 (26%)	84
Daubentonia madagascarensis	17 (20%)	83
Otolemur garnetti	54 (61%)	89
Gorilla gorilla	2 (6%)	32
Homo sapiens	2 (4%)	48
Nomascus leucogenys	1 (3%)	30
Callithrix jacchus	7 (23%)	30
Carlito syrichta	28 (20%)	142
Galeopterus variegatus	32 (37%)	87
Tupaia chinensis	15 (31%)	48
Mus musculus	238 (75%)	319

Figure 3. V1R repertoire size estimates across the strepsirrhine phylogeny. Sequences with V1R homology were mined from available strepsirrhine and select outgroup genomes. Total V1Rs consist of all genomic regions with V1R homology that are ≥ 600bp in length. Intact genes are defined by vomeronasal homology and a ≥ 801bp ORF. Nocturnal species are highlighted in gray. Tree adapted from dos Reis 2018 (dos Reis et al. 2018).

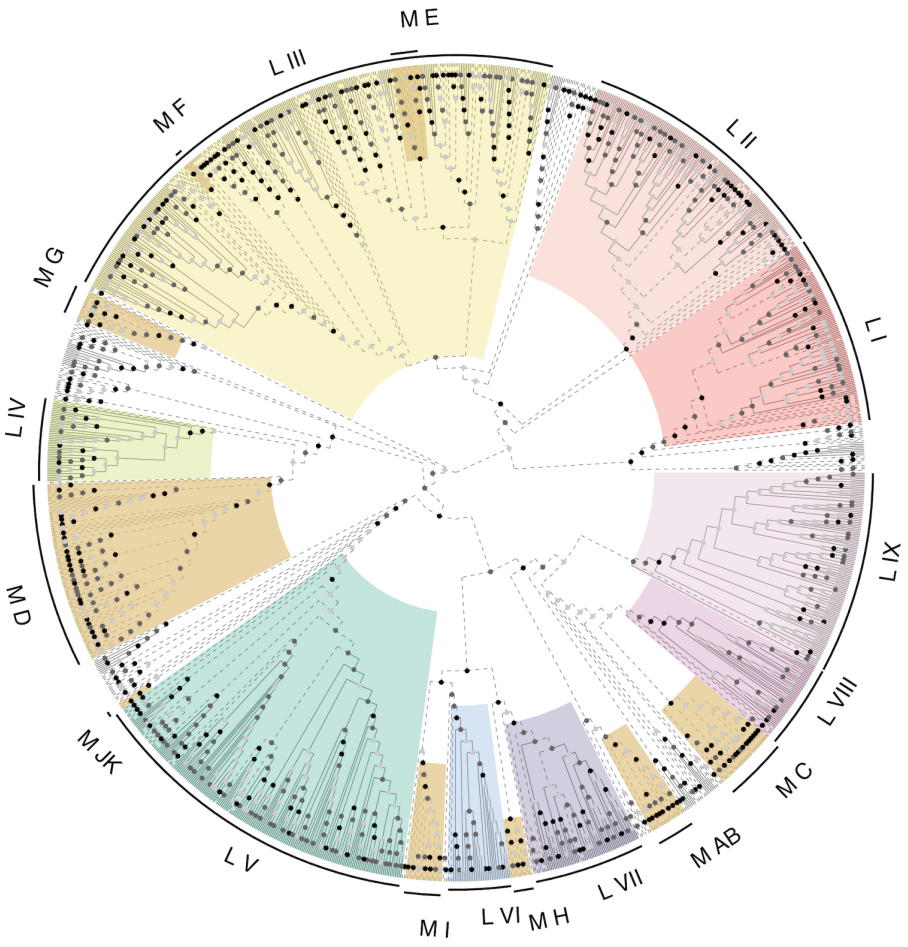


Figure 4. ML topology of V1R repertoire. V1R subfamilies in primates are highlighted and circumscribed based on Hohenbrink et al. (2012). There are nine described subfamilies in lemurs, L *Strep*/I through L IX, although not all lemur sequences fall into these subfamilies. Clades of V1R subfamilies with known function in mice are shown in burnt orange (M AB through M JK). Circles at nodes represent bootstrap support. Black nodes have 100% bootstrap support, dark grey nodes are supported with 70% or more bipartitions from bootstrap trees, and light grey nodes are weak or unsupported with less than 70% of bipartitions across bootstrap replicates. The topology is arbitrarily rooted for visualization. Solid lines represent dwarf and mouse lemur V1Rs (or branches subtending clades of dwarf and mouse lemur V1Rs). Dashed lines represent V1R lineages that are not within Cheirogaleidae.

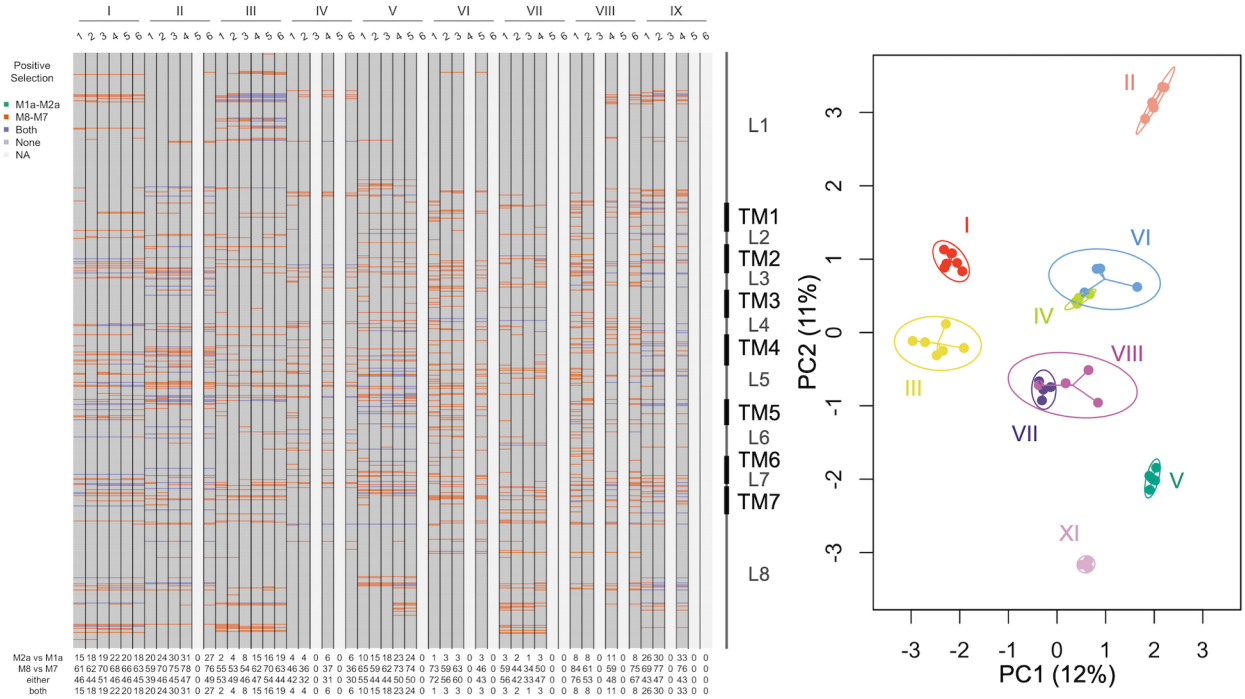


Figure 5. Sites under selection across the V1R alignment. Subfamilies are given at the top along with numbers that correspond to taxonomic filters. The first aligned codon starts at the top and the aligned codon position 588 at the bottom, with boundaries of transmembrane domains to the right. Sites under selection are colored. Missing columns means that the filter was redundant. Numbers along the bottom are counts of sites under selection detected by both model comparisons and their overlap. The boundaries of loop domains (L) and transmembrane domains (T) are shown along the aligned V1R repertoire. A PCA of sites under selection treated all codons as a binary character, determined by whether the site was under selection or not. Circle are 95% CIs for centroids of subfamily variation by taxonomic filters.

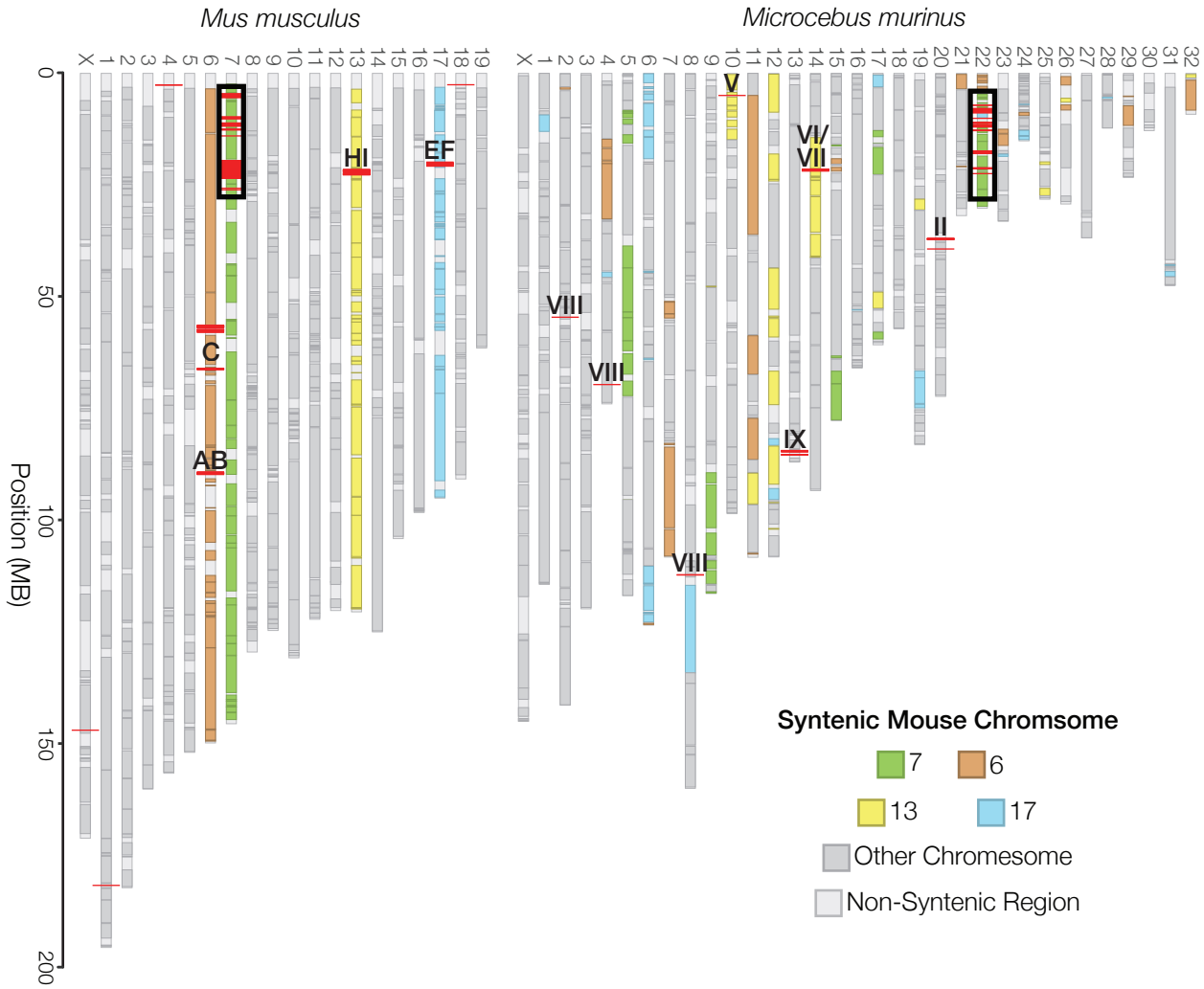


Figure 6. Chromosomal synteny between mouse and mouse lemur V1R-containing regions. Synteny between *Mus musculus* and *Microcebus murinus* was estimated using the SynChro software. Chromosomes are colored relative to V1R-containing mouse chromosomes. V1R loci are indicated with red lines and are labelled by subfamily identity. Regions outlined in black are enriched for V1R loci and are examined in further detail in Figure 7B.

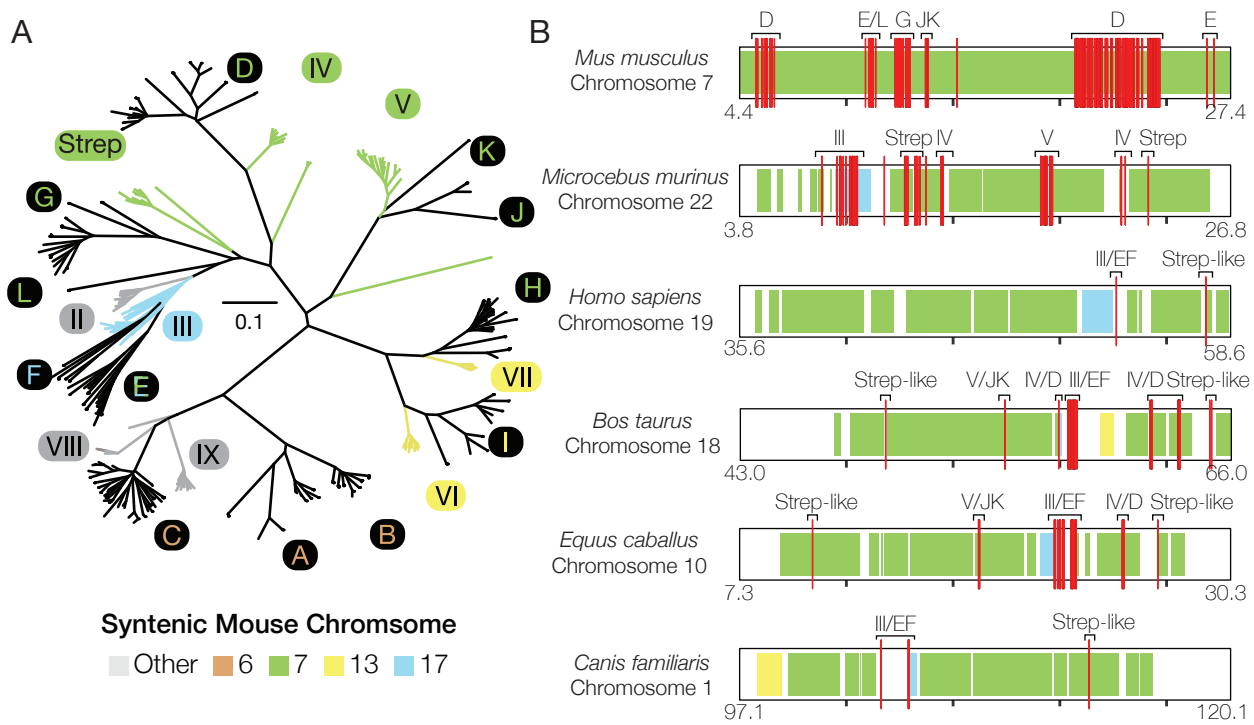


Figure 7. Highly orthologous loci on "hotspot" V1R chromosome. (A) RAxML tree of *Mus musculus* V1R cDNA sequences with intact V1R sequences from the *Microcebus murinus* genome. Mouse subfamilies are encircled in black and labelled by chromosomal location. Mouse lemur subfamilies are labelled in black and encircled in the color corresponding to the syntenic mouse chromosomal location. (B) Chromosomal "hotspot" regions enriched in V1R loci from several mammalian taxa. Orthologous regions are shaded by syntenic mouse chromosome. V1Rs loci are labelled by phylogenetic relationship to mouse/lemur subfamilies. Starting and end genomic positions are given for each species, and all regions are 23Mb long with tick marks representing 5 Mb intervals.

986 **Tables**

987 **Table 1. Inference of V1R birth-death process within primates.**

Node/lineage	Clade	Gains - Losses	Subfamilies Retained	Subfamilies Gained	Subfamilies Lost
Node 1	Euarchonta	NA	I, II, III, IV, VIII	NA	NA
Node 2	Primates plus Dermoptera	0 - 1	I, II, III, VIII	-	IV
Node 3	Euprimates	1 - 0	I, II, III, VIII	V	-
Node 4	Haplorrhini	0 - 2	I, III, V	-	II, VIII
Node 5	Anthropoidea	0 - 1	III, V	-	I
Node 6	Hominoidea	0 - 1	III	-	V
N. leucogenys	white-cheeked gibbon	1 - 1	-	VII	V
Node 7	Strepsirrhini	1 - 0	I, II, III, V, VIII	VI	-
Node 8	Lemuriformes	1 - 0	I, II, III, V, VI, VIII	IV	-
D. madagascariensis	aye-aye	1 - 3	III, VI, VIII	IX	I, II, V
Node 9	Lemuridae	0 - 1	I, II, III, V, VI	-	VIII
Node 10	*Eulemur*	1 - 0	I, II, III, V, VI	VII	-
Node 11	Cheirogaleidae	3 - 0	I, II, III, V, VI, VIII	IV, VII, IX	-

988
989 Note - Node numbers correspond to Figure 1. Gains and losses cannot be evaluated for Node 1
990 because it is the root node of the species tree.
991