Pervasive and Ongoing Positive Selection in the Vomeronasal-1 Receptor (V1R) Repertoire of Mouse Lemurs

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Abstract

Chemosensory genes are frequently the target of positive selection and are often present in large gene families, but little is known about heterogeneity of selection in these cases and its relation to function. Here, we use the vomeronasal-1 receptor (V1R) repertoire of mouse lemurs (Microcebus spp.) as a model system to study patterns of selection of chemosensory genes at several different levels. Mouse lemurs are small nocturnal strepsirrhine primates and have a large (~200 loci) repertoire of V1R loci that are likely important for intraspecific pheromonal communication and interspecific interactions, for example, recognition of predator cues. We investigated signals and patterns of positive selection among the 105 identified full length V1R loci in the gray mouse lemur and within 7 V1R loci amplified across multiple mouse lemur species. Phylogenetic reconstructions of published sequences revealed at least nine monophyletic clusters of V1Rs in gray mouse lemurs that have diversified since the split between lemurs and lorisoiod primates. A large majority of clusters evolved under significant positive selection. Similar results were found in V1Rs of closely related greater galagos. Comparison with function of related V1R clusters in mice suggested a potential relationship between receptor function and strength of selection. Interestingly, most codons identified as being under positive selection are located in the extracellular domains of the receptors and hence likely indicate the position of residues involved in ligand binding. Positive selection was also detected within five V1R loci (~71% of analyzed loci) sequenced from 6 to 10 mouse lemur species, indicating ongoing selection within the genus, which may be related to sexual selection and, potentially, speciation processes. Variation in strength of positive selection on V1Rs showed no simple relationship to cluster size. The diversity of V1R loci in mouse lemurs reflects their adaptive evolution and is most likely related to the fundamental relevance of olfactory communication and predator recognition in these primates. Overall, adaptive evolution is the predominant mode of evolution of V1R loci at all levels, and the substantial heterogeneity in the strength of selection may be related to receptor function.

Key words: vomeronasal organ, V1R, pheromone, olfaction, galago, Microcebus.

Introduction

The vomeronasal organ (VNO) is part of the accessory olfactory system of mammals and some other tetrapods (reviewed in Keverne 1999). It is anatomically and physiologically distinct from the main olfactory system and has traditionally been considered to be specialized for pheromone detection, that is, for intraspecific communication. Although certain pheromones are now known to be sensed by the main olfactory epithelium (Restrepo et al. 2004), recent evidence confirmed the substantial function of the VNO in pheromone detection and also showed that the VNO has a major role in the detection of kairomones (interspecific signals that benefit the receiver without benefiting the emitter), such as predator cues (Papes et al. 2010; Isogai et al. 2011).

In mice, the sensory epithelium of the VNO expresses two classes of vomeronasal receptors that are both seven-transmembrane G-protein coupled receptors: vomeronasal-1 receptors (V1Rs, Dulac and Axel 1995) and vomeronasal-2 receptors (V2Rs, Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997). The two vomeronasal receptor types are expressed in distinct regions of the VNO (Keverne 1999), and V1R responses to ligands are characterized by high sensitivity and specificity (Leinders-Zufall et al. 2000; Isogai et al. 2011), which is as expected if they have evolved to respond to specific pheromones and kairomones.

Intact V1R genes have been found in most mammalian orders examined so far, but there is enormous variation in diversity among taxa. More than 200 distinct V1R loci are estimated in mouse and platypus genomes (Grus et al. 2005, 2007; Young et al. 2010), for example, whereas only eight intact loci have been found in dogs (Grus et al. 2005). In contrast, V2R genes have only been described so far in rodents and marsupials. Primates show the most extreme variation in the V1R repertoire of any mammalian order, with more than 200 V1R loci predicted in mouse lemurs, ~80 loci in galagos, and only five apparently intact loci in humans, which are probably nonfunctional (Giorgi et al. 2000; Young et al. 2010). This variation in primates reflects the importance of the VNO and the accessory olfactory system and olfactory communication among different
groups. This ranges from a well-developed VNO in strepsirrhines (lemurs and lorisoids) to a small but functional VNO in New World monkeys to a vestigial and likely nonfunctional VNO in Old World monkeys, apes, and humans (Martin 1990), particularly because TRPC2, a cation channel protein required for transduction of V1R signals, is a pseudogene in these groups (Liman and Innan 2003; Zhang and Webb 2003). It has been proposed that pheromones may be under sexual selection in primates (Heymann 2006). Thus, primates are a very interesting group for the study of VNO and vomeronasal receptor evolution.

Chemosensory receptor genes are one of the functional categories that are most frequently found to be under positive selection in a variety of animal taxa (Kosiol et al. 2008). For V1R loci, positive selection has been detected in a few studies. V1R genes in mice and rats showed strong evidence for positive selection in several clusters, with most evidence for selection occurring shortly after the divergence of mouse and rat lineages (Shi et al. 2005). In an early study on V1R genes in primates, evidence for positive selection was found at a small proportion of sites (Mundy and Cook 2003). However, several important issues in relation to evolution and adaptation of V1R repertoires remain unexplored. Particularly in species with a large V1R repertoire such as mouse lemurs or galagos, is positive selection acting on the whole repertoire? It might be expected that some V1R genes or clusters of genes are evolving more rapidly than others, in relation to the rate of change of their ligands. For example, pheromones signaling sex, age, or individual identity (also known as signature mixtures) would be predicted to evolve less rapidly than pheromones that were sexually selected. Similarly, kairomones that are used to identify predators may evolve more rapidly than kairomones for closely related but reproductively incompatible species. This would in turn be predicted to lead to different rates of evolution in the cognate V1R receptors. The presence of large clusters of receptors that have resulted from repeated rounds of gene duplication enables one to examine whether positive selection is stronger in relation to the size of the cluster. Another key issue is whether ongoing selection on V1R genes among closely related species can be detected, because the majority of studies have investigated deeper events in V1R evolution among distantly related species. Most studies have focused on rodents rather than other mammalian orders such as primates. Finally, there is little known about the structure–function relationship of V1Rs, including their ligand-binding sites.

Here, we use a strepsirrhine model, the mouse lemur (Microcebus), to investigate selection pressures on V1Rs in primates. Mouse lemurs are an ideal model system to study pheromonal communication in primates, because the role of odors in the social system of these strictly nocturnal primates is well established (reviewed in Perret 1995). Mouse lemurs have several scent glands that are used in different scent-marking behaviors, they perform urine washing (Glatston 1983), and they also show strong behavioral reactions to mammalian predator odors (Sündermann et al. 2008; Kappel et al. 2011). The huge importance of pheromonal communication and predator detection in mouse lemurs is associated with a complex V1R repertoire, and a previous study already identified ~100 intact individual V1R loci in the 2x gray mouse lemur (Microcebus murinus) genome with a prediction of a total repertoire of ~200 loci (Young et al. 2010). Many aspects of mouse lemur ecology, including their small body size, diet, arboreality, and strong reliance on pheromones are thought to be similar to that of ancestral primates. Additionally, mouse lemurs show a high species diversity with at least 19 different species (Mittermeier et al. 2010; Radespiel et al. 2012) that genetically and morphologically split into two clades, the gray and the red mouse lemurs, and pheromones may have played an important role in sexual selection and speciation by reproductive isolation in this group. They are therefore ideal models to investigate interspecies evolution of V1Rs.

We hypothesize that there will be a variation in the rate of V1R evolution in mouse lemurs. At least some V1R loci are expected to have evolved rapidly in mouse lemurs, such as those that bind species-specific pheromones that are probably important in reproductive biology. Because large numbers of predator-specific V1Rs are present in mice (Isogai et al. 2011) and mouse lemurs suffer from a high predation pressure (Schumann et al. 2007), we also predict high numbers of kairomone receptors in the V1R repertoire of mouse lemurs. Such receptors have probably evolved rapidly as well. On the other hand, we expect some V1Rs to evolve slowly, for example, receptors for gender detection which probably bind sulfated steroids similar to mice (He et al. 2008; Nodari et al. 2008). In this study, we test for positive Darwinian selection in the V1R repertoire of mouse lemurs. First, we identify monophyletic gene clusters in the V1R repertoire of the gray mouse lemur (M. murinus) and test whether all clusters evolved under positive selection and whether there is heterogeneity in strength of selection among V1R protein domains. Second, we investigate selection on individual V1R loci from different parts of the repertoire in multiple mouse lemur species to investigate evolutionary processes during the diversification of the genus. Third, we test for differences in selection pressures between the two mouse lemur clades. Finally, we analyze selection pressures on V1Rs in the greater galago (Otolemur garnettii) to compare the evolution of V1R genes in two clades of strepsirrhine primates.

Materials and Methods

Data Collection

We used 105 of the 107 previously published V1R sequences from the gray mouse lemur (M. murinus, supplementary material from Young et al. 2010). For our nomenclature, we took the last three digits of the listing of the sequences published by Young et al. Instead of “micMurV1R6054_TIs: 1562366684...”, we are using the more convenient name “Mmur054” (= > Mmur000 to Mmur106; V1NR-Mmur054 on Genbank following human nomenclature). We removed two sequences—Mmur102, which has the same amino acid sequence as Mmur101, and Mmur073, which is not a full length sequence (it is too short to form the first transmembrane helix and it is missing an ATG start codon). The intact
sequences were 864–1,008-bp long. We also used published V1R sequences of other Euarchontoglires (nine primates, one tree shrew, two lagomorph, and five rodent species from Young et al. 2010) to identify monophyletic clusters in the gray mouse lemur. Furthermore, we compared the selection signals on V1Rs in mouse lemur with those on V1Rs of the greater galago (O. garnetti). This species is another nocturnal strepsirrhine primate but a member of the sister clade (Lorisiformes) to lemurs. There are 61 intact galago V1R loci published, out of an estimated 78 (Young et al. 2010).

For locus-specific analyses, we sampled up to 10 different mouse lemur species (M. bongolavensis, M. danfossi, M. lehilahytsara, M. macarthuri, M. mammiratra, M. mittermeieri, M. murinus, M. myoxinus, M. ravelobensis, and M. sambiranensis, supplementary material S1, Supplementary Material online, for capture site information). DNA was extracted from ear tissue using a DNeasy Tissue Kit (Qiagen) and a REPLI-g WGA kit (Qiagen). The V1R loci for resequencing were randomly selected from the published V1R repertoire (Young et al. 2010), except for locus Mmur033, which was selected because it is known to be expressed in the VNO tissue of mouse lemurs (Talarico M, personal communication). We selected five loci in gene clusters and two nonclustered loci. Corresponding regions were searched in the mouse lemur genome using basic local alignment search tool. Flanking regions were used to design locus-specific external primers (supplementary material S2, Supplementary Material online, for primer sequences). All V1R genes were amplified with Bioline Taq (25 μJ total volume containing 0.75 μM MgCl2 [50 mM], 0.05 μM of each dNTP [25 mM], 2.5 μM 10 x NH4 reaction buffer IV, 1 μM of each primer [10 μM], 0.1 μM Taq DNA polymerase [5 U/μJ] and 1 μM of DNA) with the following polymerase chain reaction (PCR) conditions: 94°C for 2 min, 40 times (94°C for 30 s, primer-specific annealing temperature [60–63°C for 45 s, 72°C for 90 s], 72°C for 5 min. PCR products were sequenced on both strands using BigDye Terminator 3.1 (Applied Biosystems) under standard conditions and run on an Applied Biosystems 3500 or 3730xl 96 capillary sequencing machine.

**Data Analysis**

The consensus sequence of single genes was built with the software SeqMan 5.05 (DNASTAR Inc., Madison, WI, USA). These genes and the 105 intact full length V1R sequences were aligned and analyzed with MEGA 5 (Tamura et al. 2007). Phylogenetic reconstructions were performed using the neighbor joining method with maximum composite likelihood model, pairwise deletions, and 500 bootstrap replications in MEGA.

Codon-based site-specific substitution models were used to estimate dN/dS ratios (ω) with codeml in phylogenetic analysis by maximum likelihood (PAML) 4.4 (Yang and Bielawski 2000; Yang 2007): Model M0 (“null” model—one single average dN/dS ratio among all sites), M1a (“nearly neutral” model—two classes of sites: one with dN/dS < 1 and one with a fixed ratio of 1), M2a (“positive selection” model—three classes of sites: one with dN/dS < 1, one with a fixed ratio of 1, the third with a ratio > 1) (Wong et al. 2004), M8a (“modified null” model—eight classes of sites: eight dN/dS ratios ranging from 0 to 1, taken from a discrete approximation of the beta distribution plus one class of sites with dN/dS = 1) (Swanson et al. 2003), and M8 (“beta plus omega” model—eight classes of sites from a beta distribution like in M8a plus additional class of sites with a dN/dS ratio ≥ 1).

PAML also estimates the corresponding proportions of sites under these models. Gaps and ambiguous sites were not removed (“cleandata = 0”). Likelihood ratio tests were used to determine whether nested models are significantly more likely (α = 0.05) than models that do not allow sites under positive selection. The test statistic (−2 [log likelihood1 – log likelihood2]) was calculated to compare models M1a versus M2a and M8a versus M8. The Bayes Empirical Bayes (BEB) analysis in model M2a was used to identify codons under significant positive selection. The locations of codons under positive selection were estimated by using the published structure of human VN1R1 (after Saito et al. 1998; Rodriguez et al. 2000) and placed in three categories (extracellular, transmembrane, and intracellular domains). A transmembrane hidden Markov model analysis (results not shown, Krogh et al. 2001) used to estimate the position of transmembrane helices revealed a strong congruence between the estimated transmembrane domains and the domains detected by the VN1R1 alignment. It can therefore be concluded that the location of the transmembrane domains is highly conserved across the V1R repertoire. Because gene conversion can potentially lead to moderate false detection of positive selection among paralogs using PAML (Casola and Hahn 2009), we tested for gene conversion using the program GENECONV 1.81 (Sawyer 1989).

Site models in PAML were used to detect signals of positive selection in the V1R repertoire on several levels: 1) the full repertoire, 2) all monophyletic clusters of V1R loci, and 3) resequenced single loci. We used the following parameters to compare different clusters: overall ω value of the null model (ω M0), the ω value of the third class in the positive selection model (ω3 M2a), the corresponding proportion of sites in the positive selection model (p3 M2a), and the product of the last two parameters (ω3 p3 M2a), as an estimate of the overall “strength” of positive selection. For locus-specific analyses, we used sequence data from seven single loci for 6–10 mouse lemur species. Each analysis included one sequence per mouse lemur species except for the gray mouse lemur (M. murinus), the most widespread species, where we used sequences from two individuals, one southern (near Tolagnaro) and one northern (in Ankarafantsika National Park), because of their relatively high nucleotide sequence difference. If heterozygous sites were present (1–6 heterozygous positions per sequence), we used DnaSP 5.10 (Librado and Rozas 2009) to reconstruct the different alleles, built a haplotype network with the software Network 4.6 (Bandelt et al. 1999), and conservatively used the allele most closely related to the alleles of the other mouse lemur species for the subsequent analyses. Sequences have been submitted to Genbank under accession numbers
Models in PAML as described earlier. Sister gene clusters to 29% extracellular, /C24 on the proportion of amino acid residues in each category: significant positive selection in the receptor protein, based significant differences in the distribution of codons under V1R repertoire (fig. 1, see also Young et al. 2010). Most of the published V1R loci formed nine different clusters (I–IX) that remain monophyletic after including published V1R sequences of other Euarchontoglires species (notably from O. garnettii; phylogenetic tree with V1R sequences of primates, tree shrew, lagomorphs, and rodents in supplementary material S3, Supplementary Material online; the gray mouse lemur was the only available lemur species). The clusters varied in size from 4 to 29 loci. Ten loci were not clustered and evolved from nine different origins according to the Euarchontoglires tree.

A proportion of gray mouse lemur V1R loci (~16%) showed significant signals for gene conversion (see details in supplementary material SSB, Supplementary Material online), with more than half of putative conversion events occurring in cluster I. The length of the putative converted tracts was generally short (mean: ~15% of the total gene length (~929 bp), range: 67–300 bp). Importantly, PAML analyses run when these conversion tracts were excluded were similar to those before exclusion (supplementary material SSC, Supplementary Material online), and, in particular, in no case was significant positive selection lost. In simulated data with gene conversion, data sets with parameters similar to our data (in terms of dS) showed only a low percentage of false-positive results in site-model tests in PAML (below 5%, Casola and Hahn 2009). We, therefore, conclude that effects of gene conversion on our analysis of selection are minimal and present result without excluding data.

We analyzed selection across the mouse lemur V1R repertoire using a 939 bp alignment (part of the intracellular tail could not be analyzed due to alignment difficulties). The dN/dS ratio of model M0 over all loci was 0.63, and there was significant evidence for a proportion of sites under positive selection over the whole tree in both selection model comparisons (14.6%, table 1). After separate analyses of each monophyletic cluster, seven of nine clusters showed significant evidence for sites under positive selection with 2.2–16.9% of sites selected with ω from 2.75 to 10.26 (table 1). Only in clusters III and IV was no significant evidence for positive selection found. The number of loci per cluster did not correlate with the overall ω value of model M0, ω3 M2a, ω3 M2a, or ω3* M2a (Spearman rank correlation, n = 9, -0.31 < R < 0.16, P > 0.424).

Distribution of Sites under Positive Selection across the V1R Protein
Across the whole V1R repertoire of mouse lemurs, 11 codons were under significant positive selection (BEB P > 0.05), and these occurred in just three parts of the protein: the second and third extracellular loops and the seventh transmembrane domain. There was significant over-representation in the extracellular domains (nine codons, fig. 2) compared with transmembrane (two codons) and intracellular domains (no codon) (χ² = 8.7, df = 2, P = 0.013). The number of positively selected codons in the extracellular domains was significantly higher than expected (χ² = 4.3, df = 1, P = 0.038), whereas the transmembrane domain contained significantly fewer codons under positive selection than expected (χ² = 4.4, df = 1, P = 0.037).

In the separate analyses on monophyletic clusters, only two clusters had sufficient codons under significant positive selection to test for domain distribution (I and IX), and neither were significant (P > 0.538; details about the distribution of codons under significant positive selection in the supplementary material S4, Supplementary Material online).

Selection on Individual Loci
Using locus-specific primers, we amplified seven individual V1R loci in multiple mouse lemur species, and as there was no indication that more than one locus was amplified per primer pair, we assume we have successfully isolated orthologous loci. Analysis in PAML showed significant evidence for a proportion of sites under positive selection in five of the seven analyzed loci (Mmur033, Mmur048, Mmur060, Mmur066, and Mmur074, table 2). Loci Mmur060 (cluster V), Mmur048 (cluster VI), and Mmur074 (cluster IX) evolved under significant positive selection as did the whole clusters they belong to. However, the selection pressures during the evolution of the single loci were not always consistent with the selection pressures within the corresponding cluster in the gray mouse lemur. Cluster VII showed strong signals for positive selection, but no such
Fig. 1. V1R repertoire of gray mouse lemurs (neighbor joining with maximum composite likelihood method, 500 bootstrap replications consensus tree, and bootstrap support within clusters not shown); the roman numbers indicate monophyletic clusters of loci specific to mouse lemurs.
evidence was found for locus Mmur045 or Mmur054. The nonclustered single origin loci Mmur033 and Mmur066 evolved under significant positive selection. Individual loci and the corresponding clusters were uncorrelated for $\omega$, $\omega_3$, M2a, $p_3$ M2a, or $\omega_3$ $p_3$ M2a (Spearman rank correlation, $n = 5$, $-0.88 < R < -0.05$, $P > 0.053$). Only 1–3 codons under significant positive selection significant sites were found in the other five loci, and because of the small sample size, no statistical analyses were conducted (details about the distribution of codons under significant positive selection in the supplementary material S4, Supplementary Material online).

Table 1. Output of the PAML Analysis across the Whole V1R Repertoire (105 Loci) of Microcebus murinus and across the Separate Monophyletic Clusters with Likelihood Ratio Statistics.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>No. of Loci</th>
<th>Potential Function</th>
<th>$\omega$ M0</th>
<th>$\omega_3$ M2a</th>
<th>$p_3$ M2a (%)</th>
<th>M1a vs. M2a</th>
<th>M8a vs. M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole repertoire</td>
<td>105</td>
<td>—</td>
<td>0.63</td>
<td>1.89</td>
<td>14.6</td>
<td>106.7***</td>
<td>91.0***</td>
</tr>
<tr>
<td>I</td>
<td>29</td>
<td>f h</td>
<td>0.69</td>
<td>3.48</td>
<td>14.0</td>
<td>81.5***</td>
<td>79.4***</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>f h</td>
<td>0.84</td>
<td>3.09</td>
<td>14.7</td>
<td>21.9***</td>
<td>21.8***</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>f h</td>
<td>0.79</td>
<td>1.37</td>
<td>62.3</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>f</td>
<td>0.59</td>
<td>2.61</td>
<td>15.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>V</td>
<td>14</td>
<td>—</td>
<td>0.81</td>
<td>2.75</td>
<td>16.9</td>
<td>41.4***</td>
<td>41.0***</td>
</tr>
<tr>
<td>VI</td>
<td>9</td>
<td>m f h</td>
<td>0.54</td>
<td>6.51</td>
<td>2.2</td>
<td>12.9***</td>
<td>13.3***</td>
</tr>
<tr>
<td>VII</td>
<td>4</td>
<td>f h</td>
<td>0.99</td>
<td>9.68</td>
<td>8.3</td>
<td>23.9***</td>
<td>23.9***</td>
</tr>
<tr>
<td>VIII</td>
<td>5</td>
<td>f h</td>
<td>0.98</td>
<td>10.26</td>
<td>5.6</td>
<td>18.1***</td>
<td>18.0***</td>
</tr>
<tr>
<td>IX</td>
<td>19</td>
<td>f h</td>
<td>0.64</td>
<td>4.06</td>
<td>11.6</td>
<td>73.7***</td>
<td>73.5***</td>
</tr>
</tbody>
</table>

Note.—$\omega = d_N/d_S$, $\omega_3 = d_N/d_S$ of the third class in M2a, and $p_3$ = proportion of sites in third class of M2a.

According to mouse orthologs (m, male cues; f, female cues; h, heterospecific cues; Isogai et al. 2011).

Likelihood ratio test (LRT) (df = 2).

LRT (df = 1)

**$P < 0.01$.

***$P < 0.001$.

Fig. 2. The distribution of positively selected codons in V1R domains. The amino acid sequence of V1R Mmur101 is given as a representative for the whole V1R repertoire; residues under significant positive selection under PAML model M2a are shown in white on black background. The part in brackets was not analyzed across the repertoire due to alignment difficulties. The transmembrane region (TM) is shown in gray; ext, extracellular; int, intracellular.
The branch models showed that the $d_{s}/d_{i}$ ratios were higher in the gray than in the red mouse lemur clade for all seven loci. At the level of individual loci, one locus was significantly different—$Mmur074$ ($\chi^2 = 3.9$, df = 1, $P = 0.048$; all other loci with $\chi^2 < 1.2$, $P > 0.290$).

### Comparison with Mouse V1R Clades and Potential Function of Mouse Lemur V1R Gene Clusters

The phylogenetic reconstruction of V1R evolution in Euarctontoglires (supplementary material S3, Supplementary Material online) revealed the relationship between clades of V1Rs in mice (V1Ra–V1Rk) and mouse lemurs. Cluster I of mouse lemurs was distinct, whereas clusters II and III lay within the mouse V1Ref clade. Clusters IV, V, VI, and VII were most closely related to mouse clades V1Rd, V1Rk, V1Ri, and V1Rh, respectively. Clusters VIII and IX were both most closely related to clade V1Rc. Some mouse V1R clades are specific to one class of odor cue (Isogai et al. 2011). If the general function of V1R clades is conserved, we could use the function of mouse clades to tentatively assign specific functions to closely related mouse lemur clusters (table 1, Isogai et al. 2011). Thus, according to mouse V1R function, mouse lemur V1Rs in cluster IV would detect female cues, cluster V would detect heterospecific cues, and detection of male cues would be coded by cluster VI genes. Interestingly, cluster IV showed no evidence of positive selection, whereas clusters V and VI showed strong evidence of positive selection (table 1). The remaining clusters (instead of cluster I that seems to be mouse lemur specific) would detect female and heterospecific cues.

### Comparison with Another Strepsirrhine

The known functional V1R repertoire of the galago (O. garnettii) consists of 61 different loci, with a further 17 loci predicted (Young et al. 2010), less than half the number found in mouse lemurs. The overall repertoire evolved under significant positive selection ($\omega M0 = 0.54$, $\omega M2a = 1.74$, $p_3 M2a = 11.5\%$, likelihood ratio tests: $M1a$ vs. $M2a$, df = 2, $\chi^2 = 49.3$, $P \leq 0.001$; $M8a$ vs. $M8$, df = 1, $\chi^2 = 43.1$, $P \leq 0.001$).

In total, six monophyletic clusters were found in the galago, of which five were sister clusters to the mouse lemur clusters I, III, V, VI, and VIII, and hence presumably diverged at the time of the lemur–lorisoid split. Galagos had a further specific cluster closely related to the single locus $Mmur066$ in mouse lemurs. Cluster sizes were smaller in galagos than in mouse lemurs (maximum of 10 loci per cluster compared with 29 in mouse lemurs). The clusters IV and VII of mouse lemurs had no direct galago sister sequence or cluster, whereas clusters II and IX were closely related to a single galago locus. Comparisons of pairs of closely related sister clusters in galagos and mouse lemurs showed similar results, that is, cluster III and the corresponding cluster in the galago V1R repertoire showed no significant signals of positive selection, whereas the four other mouse lemur clusters and their corresponding galago clusters all evolved under significant positive selection (detailed results are shown in the supplementary material S5A, Supplementary Material online). There was no correlation between sister clusters in the overall $\omega$ of $M0$, $\omega M2a$, $p_3 M2a$, or $\omega^*_3 p_3 M2a$ (Spearman rank correlation, $n = 5$, $-0.8 \leq R \leq 0.6$, $P > 0.104$).

### Discussion

We analyzed patterns of selection on mouse lemur V1R loci at several different levels. We found evidence for pervasive positive selection in the whole V1R repertoire and in multiple monophyletic V1R clusters, as well as evidence for ongoing selection on individual V1R loci within mouse lemurs. In addition, there was some evidence for variable selection pressures on different loci. As now discussed, these results have important consequences for models of V1R evolution. It has to be kept in mind that the analyzed V1R repertoire contains ~50% of the total estimated number. Nevertheless, we have no reason to expect any sampling bias.

### Patterns of Selection Pressures on V1R Genes

There was strong evidence for positive selection across the whole mouse lemur V1R repertoire, which echoes the results of studies on V1R selection in rodents (mouse/rat: Shi et al.

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**Table 2. V1R Loci Analyzed in Multiple Mouse Lemur Species, with Length of the Analyzed Region (Full Coding Sequence Length in Brackets), Number of Sampled Species (supplementary material S1, Supplementary Material online), and Output of PAML Analyses with Likelihood Ratio Statistics.**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Cluster</th>
<th>Analyzed Region (bp)</th>
<th>No. of Species</th>
<th>$\omega$ M0</th>
<th>$\omega_3$ M2a</th>
<th>$p_3$ M2a (%)</th>
<th>$M1a$ vs. $M2a$</th>
<th>$M8a$ vs. $M8b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Mmur033$</td>
<td>—</td>
<td>942 (942)</td>
<td>10</td>
<td>0.69</td>
<td>7.51</td>
<td>5.2</td>
<td>9.2*</td>
<td>9.2*</td>
</tr>
<tr>
<td>$Mmur045$</td>
<td>VII</td>
<td>915 (945)</td>
<td>7</td>
<td>0.65</td>
<td>1.67</td>
<td>40.3</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>$Mmur048$</td>
<td>VI</td>
<td>957 (957)</td>
<td>6</td>
<td>0.58</td>
<td>5.29</td>
<td>13.7</td>
<td>14.6**</td>
<td>14.6**</td>
</tr>
<tr>
<td>$Mmur054$</td>
<td>VII</td>
<td>897 (897)</td>
<td>6</td>
<td>0.24</td>
<td>1.00</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>$Mmur060$</td>
<td>V</td>
<td>906 (906)</td>
<td>9</td>
<td>0.29</td>
<td>5.62</td>
<td>4.2</td>
<td>7.3***</td>
<td>7.3*</td>
</tr>
<tr>
<td>$Mmur066$</td>
<td>—</td>
<td>897 (897)</td>
<td>9</td>
<td>0.48</td>
<td>6.92</td>
<td>5.7</td>
<td>13.0*</td>
<td>13.0**</td>
</tr>
<tr>
<td>$Mmur074$</td>
<td>IX</td>
<td>918 (918)</td>
<td>8</td>
<td>0.56</td>
<td>4.18</td>
<td>7.9</td>
<td>7.3***</td>
<td>7.3*</td>
</tr>
</tbody>
</table>

*Note:* $\omega = d_{s}/d_{i}$, $\omega_3 = d_{s}/d_{i}$ of the third class in $M2a$, and $p_3 =$ proportion of sites in third class of $M2a$.

*LRT (df = 2).*

*P < 0.01.*

**P < 0.001.*

***P < 0.001.*
We also investigated patterns of selection in monophyletic clades of V1Rs in the gray mouse lemur. Remarkably, seven of nine monophyletic V1R clusters showed evidence of positive selection in site tests, showing that this is the dominant mode of V1R evolution over all clusters. The lack of signals in two clusters (III and IV) could be related to low statistical power relating to the small number of genes per cluster (4 in both cases). However, clusters VII (four loci) and VIII (five loci) showed highly significant signals of selection despite their low sample size, which suggests that clusters III and IV may have rather evolved under purifying selection. The results, therefore, provide some support for our prediction that some V1R loci are evolving more rapidly than others. However, there was no evidence that the strength of selection in a particular cluster was correlated with the size of the cluster, which would be predicted if the rate of gene duplication is related to the strength of selection. Although only sequences from the gray mouse lemur were used for the analyses of the whole V1R repertoire and monophyletic clusters, patterns most likely contain evolutionary information from the whole genus Microcebus and potentially other lemurs.

The comparison with V1R clades of mice revealed the potential function of the gene clusters in mouse lemurs, and several patterns show good concordance with these functions. Mouse lemur cluster IV is a small cluster with no significant evidence of positive selection, and genes within this cluster should bind female cues. On the other hand, clusters V and VI and two of their individual loci (Mmur060 and Mmur048, respectively) should detect heterospecific or male cues and showed strong evidence for positive selection. Female cues include sulfated steroids (Nodari et al. 2008; Isogai et al. 2011), which are strongly conserved over evolutionary time, whereas some male cues may rapidly evolve by sexual selection (either by male–male competition or female choice), and interspecific predator cues (kairomones) may be evolving in arms races in some circumstances.

We compared the selection on V1Rs between the gray mouse lemur and greater galago, which is a member of the sister group (Lorisiformes) to the lemurs. The two species share strong ecological similarities, being small, nocturnal, and arboreal (Bearder 1987). As in mouse lemurs, the majority of species-specific V1R clusters in the greater galago were under positive selection. The data suggest some conservation of the rates and possibly function of molecular evolution among the two groups, because the selection on sister V1R clusters was broadly similar suggesting some conservation of function among related V1R clusters in different groups, which is consistent with the discussion of mouse lemur V1R clusters above. However, the gray mouse lemur is predicted to have more than twice as many functional V1R loci as the galago, and it is interesting to note this is related to a higher number of V1R loci per cluster in the mouse lemur, rather than an increase in number of clusters.

To investigate the more recent evolution of V1R loci in mouse lemurs, we resequenced a set of single loci from up to 10 divergent mouse lemur species. We found significant signals for positive selection in five out of seven analyzed loci, showing frequent ongoing positive selection in the V1R repertoire in the genus Microcebus. This is one of the first studies to demonstrate positive selection on single V1R loci in a phylogenetic context and shows that selection in V1Rs is not confined to deeper evolutionary events. We found no correlation between the strength of positive selection in a V1R cluster in the gray mouse lemur and the strength of selection on a particular locus in the cluster among Microcebus species but demonstrated heterogeneity in the rate of molecular evolution of V1R loci within clusters. Indeed, the two loci that showed no evidence for positive selection among Microcebus species were members of cluster VII, which has been shown to be under positive selection. So either different V1R loci in the same cluster are evolving differently in mouse lemurs or there are species differences in the evolution of individual loci. However, we found no significant differences between the signals of positive selection in the gray and the red clade in either of the analyzed cluster VII loci using branch models. An intriguing possibility is that ongoing positive selection in individual loci could be related to reproductive isolation and potentially speciation within the genus Microcebus. Isogai et al. (2011) showed that in mice vomeronasal receptors activated by male cues of conspecific and heterospecific mice are often closely related but mostly distinct. As mentioned earlier, Mmur048 is a member of a cluster (VI) whose sister clade in mice contains V1Rs responding to male cues, so this is a candidate locus involved in sexual selection. One other study has investigated selection on individual V1R loci using a population genetics approach in a few species of mice (Park et al. 2011), with positive selection detected in ~5% loci. However, the different experimental approach, which is assaying more recent selection than in our study, makes quantitative comparisons between the two studies difficult.

Overall, therefore, we found that positive selection has acted in many different V1R lineages in mouse lemurs and galagos, and in different Microcebus species. We have identified two loci, Mmur048 and Mmur060, under selection in mouse lemurs that are candidate male pheromone and kairomone receptors, respectively. We also found some evidence of heterogeneity in strength of positive selection among clusters and individual loci. However, the evolutionary background and function of this heterogeneity remains to be elucidated and is an important question for future research.

Selection on Protein Level

We investigated the distribution of positively selected sites within the V1R protein. For the whole V1R repertoire of mouse lemurs, amino acid residues with significant evidence for an $\omega$ above 1 were significantly biased toward the extracellular domain and specifically toward the third and fourth extracellular domain (= second and third extracellular loop). Little is known about the ligand binding domains of V1Rs, and these results strongly suggest that the second and third extracellular loops are the most important for affecting ligand binding. A study on mice and rats found only single sites under positive selection in different subsets of V1R sequences (Shi et al. 2005). One subset, however, showed a handful of
significant sites located within the third extracellular loop (positions 262, 263, and 266) or close to transmembrane regions (259 and 270) after aligning with our data.

In conclusion, V1Rs in mouse lemurs evolved under strong but variable positive selection reflecting the importance of pheromone communication and kairomone detection in these primates. As nonclustered loci were also targeted, it may be possible to find positive selection in other primate species that may not have such a diversified V1R repertoire. Future studies on V1Rs should therefore consider species with both larger and smaller repertoires. Further research is needed to understand the role of vomeronasal receptors in primates.

Supplementary Material

Supplementary materials S1–S5 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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References


