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Population genetics of mouse lemur vomeronasal receptors: current versus past selection and demographic inference

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Abstract

Background: A major effort is underway to use population genetic approaches to identify loci involved in adaptation. One issue that has so far received limited attention is whether loci that show a phylogenetic signal of positive selection in the past also show evidence of ongoing positive selection at the population level. We address this issue using vomeronasal receptors (VRs), a diverse gene family in mammals involved in intraspecific communication and predator detection. In mouse lemurs, we previously demonstrated that both subfamilies of VRs (V1Rs and V2Rs) show a strong signal of directional selection in interspecific analyses. We predicted that ongoing sexual selection and/or co-evolution with predators may lead to current directional or balancing selection on VRs. Here, we re-sequence 17 VRs and perform a suite of selection and demographic analyses in sympatric populations of two species of mouse lemurs (*Microcebus murinus* and *M. ravelobensis*) in northwestern Madagascar.

Results: *M. ravelobensis* had consistently higher genetic diversity at VRs than *M. murinus*. In general, we find little evidence for positive selection, with most loci evolving under purifying selection and one locus even showing evidence of functional loss in *M. ravelobensis*. However, a few loci in *M. ravelobensis* show potential evidence of positive selection. Using mismatch distributions and expansion models, we infer a more recent colonisation of the habitat by *M. murinus* than by *M. ravelobensis*, which most likely speciated in this region earlier on.

Conclusions: These findings suggest that the analysis of VR variation is useful in inferring demographic and phylogeographic history of mouse lemurs. In conclusion, this study reveals a substantial heterogeneity over time in selection on VR loci, suggesting that VR evolution is episodic.

Keywords: VNO, Evolution, Madagascar, *Microcebus murinus*, *Microcebus ravelobensis*, V1R, V2R, Genetic diversity, Demography, Selection

Background

Adaptation leaves its mark in the genome. These genomic signatures of adaptation can be investigated using phylogenetic and population genetic approaches, which address the different evolutionary timescales of interspecific and intraspecific variation. A rapidly growing body of literature is documenting the loci involved in adaptation at one of these levels [1]. However, there are few studies which directly evaluate the relationship between

the two, or separate studies examining the same loci at different timescales (for example, genes involved in brain development in primates [2, 3]). Hence it is an open question whether positive selection that occurs at loci among species is generally reflected in positive selection at the same loci within species.

Different selection regimes make different predictions for the relationship between past and ongoing selection. In cases where there is an ongoing co-evolutionary dynamic, such as in host-parasite or Red Queen systems, selection on key loci may be more or less continuous and a signal of selection is expected both in the past and present. Under balancing selection, for example, multiple

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alleles may be under on-going frequency-dependent selection across speciation events, and similar signals of selection can therefore be expected to act from the past to the present. Another possibility is that directional selection during a certain evolutionary period leads to the fixation of novel and advantageous variants. After this first and potentially episodic period of adaptation, purifying selection may then follow to stabilize and maintain the acquired adaptations. Under these conditions, there will be discordance between inferences of past and ongoing selection. Many other patterns are also possible: for example, changes in the effective population size (N_e) will affect fixation of mildly deleterious alleles by drift and selection on compensatory alleles, so changes in N_e alone may lead to different signatures of selection in different timeframes [4].

In mammals, G-protein coupled chemoreceptors form one of the gene families that are most consistently found to be under positive selection in phylogenetic analyses [5]. One of the most diverse classes of chemoreceptors in terrestrial mammals is vomeronasal receptors (VRs) that function in intraspecific communication and predator detection. There is evidence for positive selection on both types of VR (V1R and V2R) in mammals [6, 7]. This is consistent with the possibility of arms races between the scent of predators and the VRs of their prey, or the action of sexual selection on VR evolution for intraspecific communication.

Mouse lemurs (*Microcebus*) are nocturnal strepsirrhine primates with an elaborated olfactory behavioural repertoire [8–10]. Olfactory stimuli play an important role in their intraspecific communication as well as in predator detection [11–14]. They have the largest repertoire of VR genes (~200 *V1Rs* and 2 *V2Rs*) of any primate. We previously showed a high level of positive selection acting on multiple families of *V1R* genes, as well as *V2R* genes, during mouse lemur evolution [15, 16]. We hypothesised that this positive selection may relate to predator detection and/or intraspecific communication. If this is the case then one would also expect current selection on mouse lemur VR loci. To the best of our knowledge, population genetics of VRs has not previously been investigated in the wild.

Functional loci have been relatively neglected in studies of demography, which favour neutral markers (e.g., [17–21]). The phylogeography of many taxa has been influenced by Pleistocene glaciation cycles, and this has been demonstrated in temperate regions as well as in the tropics (e.g., [22, 23]). For Madagascar with its highly complex ecogeography of central-eastern highlands, a dry western and a humid eastern zone and various seasonal forest types, various biogeographic hypotheses have been proposed that take into account Pleistocene climatic changes, topographic barriers such as

mountains and large rivers [24, 25]. It has been suggested that various recent endemic radiations in lemurs and other vertebrates are causally linked to these climatic fluctuations that led to cyclic vegetation changes within and across isolated centres of endemism [26].

Mouse lemurs (*Microcebus* spp.) are a highly diverse genus with currently 24 described species [27] that are mostly confined to rather local or regional geographic ranges that are separated from each other by large rivers. Allopatric speciation has therefore been proposed as predominant mechanism of diversification within this genus [28, 29]. However, one species, the grey mouse lemur (*Microcebus murinus*), occurs from southern Madagascar up to northwestern Madagascar and can be found in partial sympatry with at least five other local or regional mouse lemur species, *M. griseorufus*, *M. berthae*, *M. myoxinus*, *M. ravelobensis* and *M. bongolavensis* (reviewed in [30]). Two recent molecular studies suggest that this large geographic range is the result of a rather recent expansion from southern to northwestern Madagascar at some time point in the late Pleistocene [21, 31]. Schneider et al. [21] inferred this expansion based on modelling and simulating the diversity and gene genealogy of mitochondrial sequence data obtained from several populations in northwestern Madagascar which also included samples from the same study site as chosen for this study. The haplotype diversity sampled in that study could be best explained by a succession of two spatial expansions that could be dated to the late Pleistocene (younger than 350,000 years old). It was hypothesised that an ancestral population of *M. murinus* colonized this region (=inter-river-system, IRS) before the last glacial maximum (LGM) and then contracted into riverine refugia during the dry period that coincided with the LGM and presumably with the preceding glacial periods in Madagascar [26]. From there it may subsequently have expanded again in parallel with forest expansion [21]. In the second study, Blair et al. [31] inferred the rather recent expansion of *M. murinus* by modelling the split between *M. murinus* and its sister species, *M. griseorufus*, in southwestern Madagascar. Coalescent methods were employed on 55–124 sequences for four molecular markers (alpha enolase intron, alpha fibrinogen intron, von Willebrand factor intron, cytochrome B concatenated with cytochrome c oxidase subunit II), respectively, stemming from localities in southern to western Madagascar. Results were concordant with allopatric speciation from a narrowly distributed common ancestor that lived in southwest Madagascar. Only *M. murinus* underwent a subsequent range expansion to the north and experienced severe population dynamics during the Pleistocene [31]. Given these scenarios, the demographic history of *M. murinus*

populations in northwestern Madagascar, the region which it last colonized, should be substantially different from those of their sympatric congeners that most likely evolved in those areas over longer timescales [28, 32].

Here we assay sequence variation in 15 *V1R* and two *V2R* loci in populations of the two sympatric mouse lemur species (*M. murinus* and *M. ravelobensis*) from northwest Madagascar. We perform various tests of neutrality and investigate the correlation between past and present selective pressures. In addition, we perform demographic analyses to assess the suitability of VR loci for making demographic inferences.

Methods

Data collection

We extracted DNA from 20 grey mouse lemurs, *M. murinus* (10 male, 10 female), and 20 golden-brown mouse lemurs, *M. ravelobensis* (10 male, 10 female, see Additional file 1 for details), using a phenol-chloroform protocol and a REPLI-g WGA kit (Qiagen). Small ear biopsies were collected between May and October 2008 in the Ankarafantsika National Park in northwestern Madagascar by S. Thorén (authorisation no. 062/08/MEEFT/SG/DGEF/DSA/SSE). All animals were live-trapped in the 30.6 ha study site JBA (46°48'E, 16°19'S; for details about the trapping procedure see [33]), and we selected individuals from different trapping locations to minimise the risk of sampling several individuals from the same sleeping group who are most likely related [34, 35]. All sampled animals of the same species are defined to belong to the same population.

We designed locus-specific primer pairs for 15 *V1R* and both *V2R* loci using the online software Primer3-Plus [36]. All loci (see Table 1 for details) are expressed in the VNO of the grey mouse lemur [37]. Previous work on these loci in mouse lemurs provided no evidence for paralogues or recent duplicates [15, 16]. We used twelve loci from seven of the nine monophyletic *V1R* clusters and three unclustered loci [16]. Clusters I and VIII were not analysed because locus-specific primers could not be successfully designed for these. The PCR amplicons of intronless *V1R* loci covered the whole locus as all primers bind outside of the coding sequence. *VN2R1* consists of six exons and because of long intron sequences between the exons we designed exon-specific external primers. In contrast to our first description of *V2Rs* [15], *VN2R2* consists of only five exons. Previously, we had used cDNA of extracted RNA without introns and sequenced a fragment spanning exon 3 to 5 according to closely related *V2Rs* of family D in mice. A short fragment (22 bp) between these exons was assigned to exon 4. However, the present study which uses external primers reveals that the 22 bp of exon 4 rather

Table 1 *V1R* and *V2R* loci analysed with corresponding gene cluster according to Hohenbrink et al. [16] and total length

Locus	Cluster	Length
<i>VN1R Mmur001</i>	IV	909 bp
<i>VN1R Mmur011</i>	VI*	930 bp
<i>VN1R Mmur031</i>	V*	909 bp
<i>VN1R Mmur033*</i>	uncl	942 bp
<i>VN1R Mmur040</i>	II*	948 bp
<i>VN1R Mmur041</i>	V*	906 bp
<i>VN1R Mmur043</i>	VI*	1005 bp
<i>VN1R Mmur048*</i>	VI*	957 bp
<i>VN1R Mmur049</i>	VII*	879 bp
<i>VN1R Mmur060*</i>	V*	906 bp
<i>VN1R Mmur065</i>	uncl	1008 bp
<i>VN1R Mmur066*</i>	uncl	897 bp
<i>VN1R Mmur067</i>	III	921 bp
<i>VN1R Mmur074*</i>	IX*	918 bp
<i>VN1R Mmur075</i>	IV	909 bp
<i>VN2R1</i>	V2R	2739 bp
<i>VN2R2*</i>	V2R	2310 of 2418 bp ^a

^a = locus 3 bp longer in *M. ravelobensis*, *: Loci and clusters under significant positive selection [15, 16], uncl unclustered

belongs to exon 3 which is longer in mouse lemurs than in mice. The analogous exon 4 of mice is missing in mouse lemurs, which is in concordance with genomic data of the two strepsirrhines *Daubentonia madagascariensis* and *Otolemur garnettii* where no “exon 4” was detected previously (see [15]), but a similar 22 bp insertion at the end of exon 3 was in fact present. In the present study we could not design external primers for exon 2 of *VN2R2* because of missing genomic data. Due to internal primers we are missing 108 bp (=13.2%) of the sequence of exon 2 and therefore results on *VN2R2* are based on 95.7% of the whole coding sequence.

We used MyTaq DNA polymerase for amplification (Bioline; 25 µl total volume containing 5.0 µl MyTaq Reaction Buffer, 1 µl of each primer [10 µM stock concentration], 0.1 µl Taq DNA polymerase [5 U/µl] and 1 µl of DNA) with the following PCR conditions: 94 °C for 2 min, 40 times (94 °C for 30 s, 60 °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 capillary sequencing machine. Consensus sequences were built with SeqMan 5.05 (DNASTAR Inc., Madison, WI, USA). Sequences were aligned and analysed using MEGA 5 [38]. Before data analyses we concatenated the exons of the *V2Rs*.

Data analyses

We used DnaSP 5.10 [39] to unphase the two alleles of each diploid sequence and to identify the different haplotypes. Nucleotide diversity and haplotype diversity (expected heterozygosity, or gene diversity, [40]) were calculated with DnaSP 5.10 to estimate the genetic variation within the population. DnaSP 5.10 was used to estimate the number of polymorphic synonymous (p_S) and nonsynonymous substitutions (p_N) per site [41, 42] and p_N/p_S ratios were calculated. McDonald-Kreitman tests (= MKT, [43]) were conducted online [44] to calculate the ratio of fixed differences to polymorphic differences for synonymous and nonsynonymous SNPs. Here, all haplotypes of *M. murinus* and the most closely related haplotype sequence of *M. ravelobensis* were entered to test for positive selection in *M. murinus*, and vice versa to test in *M. ravelobensis*.

Arlequin 3.5 [45] was used to calculate Tajima's D and Fu's F_s with 1000 simulated samples. Population contractions or balancing selection can result in significant positive values of Tajima's D , whereas negative values indicate population growth [46, 47]. Fu's F_s show negative values if the data contains an excess of rare haplotypes also indicating population growth and/or positive selection [48]. In combination, positive D and positive F_s values indicate an excess of intermediate-frequency alleles after population subdivision or balancing selection, whereas negative D and negative F_s values reflect relative excess of rare variants and reveal population growth [49]. We used False Discovery Rate (FDR) to correct for multiple testing across both species for each analysis, setting FDR to 0.05 [50].

We analysed the distribution of nonsynonymous SNPs along the *VIR* protein [transmembrane, extra- or intracellular region; for details see 16]. Observed vs. expected χ^2 -tests were used to compare the observed distribution of nonsynonymous SNPs in the *VIR* protein with the expected distribution using Statistica 6.1 (StatSoft, Inc., Tulsa, OK). A previous study on *VIRs* in strepsirrhines reported that the ligand binding site of the *VIR* protein is potentially formed by about half of the 4 and 5th transmembrane region and the in-between 2nd extracellular loop (= 3rd extracellular region) [51]. This estimation was based on VR sequence data of cluster I only, but assuming structural similarities between clusters, we also tested if nonsynonymous substitutions were concentrated on the binding site proposed by Yoder et al. [51]. All statistical comparisons of dependent data between the two species were conducted with the Wilcoxon Matched Pairs test in Statistica. Here, the sample size was large enough to ignore p -value corrections that would have been necessary for smaller sample sizes [52]. No such analyses were performed for *V2Rs* since there is relatively little structural information available for them.

Arlequin 3.5 was also used to test two expansion models (demographic and spatial expansion) on the mismatch distributions of the haplotypes within this single population of each species that span the same spatial scale. A mismatch distribution is a distribution of the number of nucleotide mismatches between all pairs of nucleotide sequences of one locus within a given sample. For this study each individual (homozygous or heterozygous) always entered two sequences into the data pool. Mismatch distributions can be directly compared between loci or species in this study, because of the same sampling regime (see above), same spatial spread of the samples, and the same sample size of 40 nucleotide sequences per locus and species. The shape of a mismatch distribution has been shown to be influenced by demographic events like past expansions or population bottlenecks [53]. Mismatch distributions are bell-shape (= unimodal) in populations having increased in the past as a consequence of one demographic [53] or spatial expansion [54, 55] or L-shaped in case of a very recent size reduction [53]. A previous modelling approach [21] revealed that two successive expansions can generate a tri-modal mismatch distribution and the position of these modes corresponds to the time of the expansion. In contrast, populations at demographic equilibrium show a more ragged distribution [53] (= multimodal). Demographic expansions usually result from past genetic bottlenecks, whereas spatial expansions usually follow a colonisation event by relatively few founder individuals. We tested the expansion models available in Arlequin 3.5 to evaluate the evidence for a preceding colonisation event [54, 56]. The models also calculate τ -(*Tau*-) values that reflect the time of the expansion (in mutation units, $\tau = 2T\mu$), although exact time points are difficult to infer as reliable mutation rates are often not known. However, higher values of τ indicate that the expansion happened further in the past.

Results

Comparison of genetic diversity

For *VIR*, almost all comparisons showed substantially higher genetic diversity in *M. ravelobensis* than *M. murinus* (Table 2). Across all loci, *M. ravelobensis* possessed significantly more polymorphic sites than *M. murinus* (Wilcoxon-Test, $n = 15$, $Z = 3.04$, $p < 0.01$), more haplotypes (Wilcoxon-Test, $n = 15$, $Z = 3.15$, $p = 0.001$), higher nucleotide diversity (Wilcoxon-Test, $n = 15$, $Z = 2.78$, $p = 0.005$), higher haplotype diversity (Wilcoxon-Test, $n = 15$, $Z = 2.44$, $p = 0.015$) and higher numbers of protein alleles (Wilcoxon-Test, $n = 15$, $Z = 2.76$, $p = 0.006$, Table 2). All alleles at all loci had open reading frames of the expected length except locus *Mmur040* in *M. ravelobensis*, where an allele with an internal stop codon,

Table 2 Measures of genetic diversity for each locus and species

Locus	Number of haplotypes		No. diff. AA sequences		Nucleotide diversity		Haplotype diversity		No. of poly-morphic sites	
	Mmur	Mrav	Mmur	Mrav	Mmur	Mrav	Mmur	Mrav	Mmur	Mrav
001	7	10	3	8	.00073	.00493	.528	.787	4	13
011	3	4	3	3	.00194	.00061	.600	.442	4	3
031	5	9	5	5	.00141	.00194	.686	.708	6	9
033	3	9	2	9	.00089	.00445	.472	.877	3	15
040	6	10	5	10	.00074	.00420	.592	.622	5	21
041	4	9	2	4	.00141	.00202	.727	.745	4	10
043	4	5	3	3	.00048	.00070	.377	.503	4	4
048	11	9	6	3	.00170	.00072	.785	.474	10	9
049	2	9	2	8	.00033	.00180	.296	.819	1	9
060	3	9	2	6	.00016	.00265	.145	.732	2	14
065	1	2	1	1	.00000	.00014	.000	.142	0	1
066	4	17	3	14	.00139	.00560	.558	.954	5	28
067	2	14	1	9	.00015	.00417	.142	.859	1	15
074	6	12	6	10	.00093	.00475	.487	.777	8	25
075	7	9	1	6	.00098	.00506	.668	.709	7	21
∅ VIR	4.5	9.1	3.0	6.6	.00088	.00292	.471	.677	4.3	13.1
VN2R1	25	28	24	25	.00332	.00268	.973	.976	29	32
VN2R2	14	27	8	20	.00053	.00220	.838	.967	16	28
∅ V2R	19.5	27.5	16	22.5	.00193	0.0024	.906	.972	22.5	30

diff. AA sequences: number of different amino acid sequences, Mmur: *M. murinus*, Mrav: *M. ravelobensis*, ∅ VIR/V2R: mean

indicating pseudogenization, occurred at a frequency of $6/40 = 0.15$.

For both species, there were more haplotypes at V2R loci than VIR loci (Table 2). However, the number of polymorphic sites in V2Rs was increased more dramatically in *M. murinus* than in *M. ravelobensis*. The number of unique amino acid sequences was considerably lower than the number of haplotypes in VN2R2, but this was not the case in VN2R1 indicating that here most haplotypes differed by at least one nonsynonymous substitution. Overall, the genetic diversity in VIRs and VN2R2 differs between the species, whereas it was equally high in VN2R1 of both species.

Tests of neutrality

Several loci in both species had significantly negative neutrality tests using uncorrected p-values (Table 3). However, using FDR, only three loci remained significant, all for Fu's F_s in *M. ravelobensis* (Mmur048, VN2R1 and VN2R2). No significantly positive values were found and test values did not differ significantly between the species (Tajima's D : $n = 15$, $Z = 0$, $p = 1.000$; Fu's F_s : $n = 15$, $Z = 0.68$, $p = 0.500$).

Tests for selection and distribution of mutations across VR proteins

The p_S of VIRs was significantly higher than p_N in both species (*M. murinus*: $n = 15$, $Z = 2.92$, $p = 0.004$; *M.*

ravelobensis: $n = 15$, $Z = 2.39$, $p = 0.017$, Table 3). The p_N/p_S ratios of VIRs did not differ significantly between species (Table 3; $n = 13$, $Z = 1.01$, $p = 0.311$). Using uncorrected p-values, McDonald-Kreitman Tests (MKT) were only significant for VN1R Mmur066 in *M. ravelobensis* ($p = 0.026$, Table 4), but this result was not robust to multiple testing correction using FDR. There was no significant correlation between the d_N/d_S of loci estimated across *Microcebus* species [16] and the p_N/p_S ratios determined intraspecifically in the current study (*M. murinus*: $n = 5$ loci, $r_s = 0.5$, n.s.; *M. ravelobensis*: $n = 5$ loci, $r_s = 0.0$, n.s.).

The distribution of nonsynonymous SNPs in the domains of the VIR protein did not differ significantly from the expected distribution in any species when looking at the data of all VIR loci combined (*M. murinus*: $\chi^2 = 3.2$, $df = 2$, $p = 0.200$; *M. ravelobensis*: $\chi^2 = 1.0$, $df = 2$, $p = 0.606$). Similarly, no single locus showed a significant deviation from the expected distributions (for example, VN1R Mmur066, the only locus with significant MKT: $\chi^2 = 2.6$, $df = 2$, $p = 0.268$; all other loci also with $p > 0.05$). A proposed odorant binding site [51] comprised about 20% of the VIR protein. There was no evidence for concentration of NS mutations in this region: across all loci, 21.9% of *M. murinus* NS substitutions and 19.7% of *M. ravelobensis* NS substitutions occurred here, and no single

Table 3 Results of the neutrality tests, and p_N/p_S ratios for each locus and species

Locus	Tajima's D				Fu's F_s				p_N/p_S	
	Mmur	p	Mrav	p	Mmur	p	Mrav	p	Mmur	Mrav
001	-0.70	0.287	1.46	0.939	-3.83	0.005	0.78	0.649	0.19	0.18
011	2.18	0.978	-0.42	0.398	3.96	0.941	-0.70	0.286	0.32	0.87
031	-0.24	0.469	-0.48	0.341	0.36	0.605	-2.05	0.147	0.91	0.11
033	0.42	0.700	0.60	0.763	1.42	0.806	1.21	0.743	0.20	1.13
040	-1.02	0.170	0.76	0.828	-2.38	0.055	2.13	0.815	0.29	2.53
041	0.86	0.804	-0.66	0.294	1.37	0.794	-1.91	0.160	0.11	0.35
043	-1.25	0.107	-0.74	0.272	-1.25	0.167	-1.49	0.134	2.43	0.05
048	-1.13	0.137	-1.98	0.007	-4.48	0.011	6.69	*0.000	0.39	0.08
049	0.37	0.807	-0.70	0.283	0.84	0.497	-2.44	0.103	—	0.53
060	-1.30	0.072	-0.86	0.222	-2.03	0.012	-0.89	0.362	0.15	0.14
065	0.00	1.000	-0.56	0.252	0.00	1.000	-0.22	0.209	—	0.00
066	0.16	0.608	-0.50	0.358	1.29	0.755	-2.34	0.218	0.31	1.62
067	-0.56	0.252	0.28	0.673	-0.22	0.218	-2.63	0.152	0.00	0.59
074	-1.56	0.024	-0.87	0.226	-1.77	0.102	-0.63	0.443	0.17	0.45
075	-1.27	0.094	-0.23	0.471	-2.69	0.051	1.61	0.776	0.00	0.24
VN2R1	1.15	0.914	-0.08	0.526	-6.78	0.022	-13.58	*0.000	0.19	0.49
VN2R2	-1.39	0.072	-0.78	0.249	-6.33	0.004	-17.25	*0.000	0.28	0.36

*: $p < 0.05$ with FDR of $q = 0.05$; Mmur: *M. murinus*, Mrav: *M. ravelobensis*, —: p_S was zero

Table 4 Results of MK tests and mismatch distributions for each locus and species

Locus	MK		T_{demo}				T_{spat}			
	Mmur p	Mrav p	Mmur	p	Mrav	p	Mmur	p	Mrav	p
001	0.538	0.379	0.75	0.47	8.63	0.09	0.75	0.35	6.28	0.33
011	0.599	0.998	5.02	0.11	0.58	0.84	3.85	0.13	0.56	0.68
031	0.272	0.597	1.88	0.69	3.82	0.88	1.65	0.81	0.18	0.79
033	0.051	0.821	0.00	*0.00	6.47	0.20	1.77	0.33	4.86	0.38
040	0.140	0.393	0.88	0.10	11.69	0.01	0.87	0.08	9.11	0.43
041	0.711	0.873	1.50	0.82	2.61	0.02	1.51	0.82	2.49	0.12
043	0.090	0.397	0.48	0.70	0.74	0.84	0.48	0.47	0.73	0.69
048	0.135	0.779	1.77	0.86	0.98	0.91	1.01	0.84	0.83	0.86
049	0.531	0.731	2.98	0.18	1.48	0.19	0.38	0.08	1.49	0.10
060	0.995	0.869	3.00	0.36	3.95	0.27	0.10	0.33	3.06	0.85
065	-	0.334	0.00	-	0.21	0.39	0.00	-	0.16	0.35
066	0.704	0.026	2.82	0.11	8.28	0.57	2.24	0.10	5.69	0.18
067	0.493	0.107	0.21	0.52	7.27	0.61	0.16	0.33	3.80	0.39
074	0.449	0.142	3.73	0.49	6.63	0.05	0.05	0.41	5.37	0.22
075	0.263	0.399	1.00	0.10	8.00	0.02	1.01	0.04	6.80	0.53
VN2R1	0.935	0.225	0.48	0.27	0.74	0.69	0.48	0.16	0.73	0.39
VN2R2	0.887	0.344	2.54	0.36	4.46	0.74	2.46	0.56	3.83	0.81

*: $p < 0.05$ with FDR of $q = 0.05$; Mmur: *M. murinus*, Mrav: *M. ravelobensis*, T_{demo} : t -values for demographic expansion model, T_{spat} : t -values for spatial expansion model, —: incalculable because of absence of variation

locus showed an excess of NS substitutions in this region.

Mismatch distributions

The mismatch distributions showed huge variation between loci and species (Fig. 1 and Additional file 2). In *M. murinus* most *V1R* loci ($n = 11$) had half-bell shaped distributions close to zero pairwise differences or the peak was at zero (Fig. 1a, Table 5, Additional file 2). Furthermore, *M. murinus* had four loci with unimodal distributions (*VN1R Mmur011, 033, 066* and *074*) but no locus with multimodal or ragged distributions (Fig. 1c). In contrast, three loci in *M. ravelobensis* showed ragged mismatch distributions (*VN1R Mmur001, 040,* and *067,* see Fig. 1b) and six had mostly broad unimodal distributions (*VN1R Mmur033, 041, 060, 066, 074* and *075,* see Fig. 1d). The remaining six *V1R*s showed half-bell shaped distributions similar to *M. murinus*. Notably, unimodal distributions in *M. murinus* were still close to zero pairwise differences with a high peak, whereas they were generally broader and flat in *M. ravelobensis* (compare Fig. 1c + d).

The patterns of occurrence of half-bell shaped, unimodal and ragged distributions in the two species are summarized in Table 5. About half of the loci (8 of 15) had similar types of distributions in both species, but six of the remaining mismatch distributions showed higher variation in *M. ravelobensis* than in *M. murinus*. In *V2R*s

Table 5 Pairwise occurrence of the three types of observed mismatch distributions for the *V1R* loci in both species

		<i>M. murinus</i>			
		HB	UM	RG	Σ
<i>M. ravelobensis</i>	HB	5	1	0	6
	UM	3	3	0	6
	RG	3	0	0	3
	Σ	11	4	0	15

HB: half-bell (L-) shape distribution, UM: unimodal distribution, RG: ragged distribution, Σ: Sum

(Additional file 2), the mismatch distributions in *M. murinus* showed a ragged distribution for *VN2R1* and a unimodal distribution for *VN2R2*. The distributions in *M. ravelobensis* were unimodal for both *V2R*s.

Using uncorrected p-values, tests of demographic expansion were significant in one locus of *M. murinus* (*VN1R Mmur033, p = 0.00*) and three *V1R* loci of *M. ravelobensis* (*VN1R Mmur040: p = 0.01; VN1R Mmur041: p = 0.02; Mmur075: p = 0.02*), and tests of spatial expansion were significant in one locus of *M. murinus* (*Mmur075: p = 0.04*, Table 4). However, only one of these results remained significant after FDR correction – demographic expansion for *Mmur033* in *M. murinus*. The τ -values were significantly larger in *M. ravelobensis* compared to *M. murinus* using both models (Table 4; demographic expansion: $n = 15, Z = 2.38, p =$

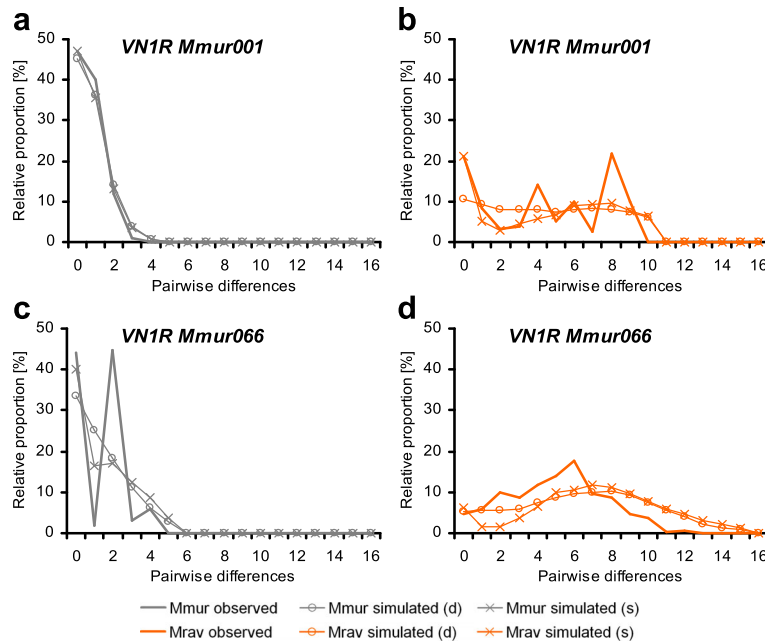


Fig. 1 Observed and simulated mismatch distributions of *VN1R Mmur001* and *066* in *M. murinus* (Mmur, left side, grey) and *M. ravelobensis* (Mrav, right side, orange); the two loci were selected to show the three observed types of distributions: half-bell shaped (a), unimodal (c + d) and ragged (b); mismatch distributions of the remaining loci are shown in Additional file 2; simulated (d) = simulated under demographic expansion model (line with circles), simulated (s) = simulated under spatial expansion model (line with crosses)

0.017; spatial expansion: $n = 15$, $Z = 2.44$, $p = 0.015$) indicating an older starting point of the putative expansion in *M. ravelobensis* compared to *M. murinus*.

Discussion

Selection in the recent evolutionary history of vomeronasal receptor genes

It was previously shown that the majority of *VIR* gene clusters in mouse lemurs evolved under strong positive selection and repeated gene duplication led to the evolution of a large *VIR* repertoire [16]. Positive selection still acted on *VIRs* during the diversification of mouse lemurs as indicated by analyses of single *VIR* loci across different mouse lemur species [16] or of a *VIR* subfamily across different lemur species [51]. Positive selection, which was probably involved in generating this high *VIR* diversity in evolutionary timescales, could in principle be ongoing in present-day populations. In contrast to this expectation, the *VRs* we studied seem to be mostly evolving under purifying selection. Several results support the presence of purifying selection at the population level: 1) McDonald-Kreitman tests were mostly non-significant (with one possible exception, see below). 2) Neutrality tests were non-significant in most cases (see below for further discussion). 3) The p_N/p_S ratio was less than one in most loci of both species. 4) Nonsynonymous substitutions were randomly distributed within the *VIR* protein indicating neutral evolution rather than positive selection. This contrasts with our previous phylogenetic study where replacement substitutions were significantly concentrated at particular parts of the protein, consistent with odorant binding sites [16]. For one locus, *Mmur040* in *M. ravelobensis*, there is even evidence for ongoing loss of function, with an allele encoding a pseudogene segregating at appreciable frequency.

Only few loci showed patterns consistent with current positive selection. *VNIR Mmur066* in *M. ravelobensis* was the only example of a significant MK test with an excess of non-synonymous SNPs segregating in the population, although this was not robust to FDR correction. However, the high haplotype diversity and p_N/p_S ratio greater than one suggest that the possibility of balancing selection at this locus warrants further investigation. Three loci in *M. ravelobensis* showed significant evidence for departure from neutrality, with a negative Fu's F_s that was robust to FDR correction. The cause of this non-neutrality however is unclear – it may be due to directional selection or population expansion, which was not rejected for these loci from the mismatch distribution tests.

In a previous study we argued that diversification and positive selection on *VR* loci may be involved in reproductive isolation and speciation of mouse lemurs [16]. If

indeed functionally linked to reproduction, it would also be possible that the loci that are potentially under positive selection in the present study could be involved in olfactory mate choice or pheromonal communication in the context of finding a suitable mate (reviewed in [57]). Selection may thus act upon the *VRs* in the VNO and partly the main olfactory epithelium (MOE) [37]. Although some information is available on the function of certain *VIR* clusters in mice (e.g. [58, 59]), knowledge of the biological function of certain *VIRs* or *V2Rs* are lacking completely for primates [16, 51]. In view of the rich and functional *VIR* repertoires of nocturnal strepsirrhines, future studies are urgently needed that shed light on their biological functions. Fruitful future experimental approaches may include behavioural assays involving individuals with polymorphisms at individual loci, and tests of the effect of odorants on the activity of *VRs* expressed *in vitro*. By contrast, the use of VNO tissue slices for immunohistochemistry or of anaesthetised individuals for electrophysiological recordings, which has been a useful method in rodents [48], is not a viable option for these endangered species.

The higher haplotype diversity for *V2R* genes than *VIR* loci is interesting. The repertoire of *VIR* genes is much more diverse in mouse lemurs than their repertoire of *V2R* genes, with ~200 *VIR* loci [60] and only 2 *V2R* loci currently known in mouse lemurs [15]. It is possible that the paucity of *V2R* genes in the VNO is partly compensated by a high allelic diversity which also translates into a relatively high number of amino acid sequences and could therefore lead to a further increase of the olfactory sensory resolution in the VNO.

The presence of only weak evidence for ongoing selection at *VR* loci in these two mouse lemur species is in strong contrast to the prevalence of positive selection at the same loci in a comparative study [12]. This is one of the first studies to explicitly compare patterns of positive selection at these two levels, although there have been a few studies in humans [2, 3]. This obvious discordance between the results of the two studies suggests that fixation of beneficial mutations at *VRs* during mouse lemur evolution may have been highly episodic, i.e. occurring over short periods of time. However, one issue for further consideration is whether the power to detect positive selection is the same in the two approaches. Future studies would be needed to address this issue, for example using simulations.

Demographic history of two sympatric mouse lemur species

Under the assumption of a divergent phylogeographic history of both mouse lemur species in northwestern Madagascar, we predicted to find differences in the genetic diversity of the *VRs* between the two species. This prediction was confirmed by several datasets. The

species with the longer phylogeographic history in the region, *M. ravelobensis*, possessed significantly more polymorphic sites, a higher number of haplotypes, a higher haplotype and nucleotide diversity, as well as a higher number of different amino acid sequences in its *VIR* loci than its congener with the supposedly shorter phylogeographic history in the region, *M. murinus*. Despite some degree of variability between loci, the results from these functional loci therefore support the conclusion of previous studies about the relatively recent expansion of *M. murinus* into northwestern Madagascar [21, 31] and suggest a distinct founder effect in this species. A similar interspecific difference was visible in the diversity of the two *V2R* loci, although these were generally more diverse in *M. murinus* than the *VIRs*.

Based on the scenario that *M. murinus* colonized northwestern Madagascar only sometime in the late Pleistocene [21, 31], we predicted to find signals of a stronger and/or more recent bottleneck in *M. murinus* than in *M. ravelobensis* which most likely evolved somewhere in this region earlier on [28] and may have maintained a larger ancient effective population size than the expanding founder population of *M. murinus*. As already noted above, although the majority of loci showed negative values in the summary statistics (Tajima's *D*, Fu's *F_s*) of both species, which would indicate population expansion under neutrality and/or positive selection, only very few loci deviated significantly from mutation-drift equilibrium. On the other hand, only *M. ravelobensis* showed significantly negative values of Fu's *F_s* in both *V2R* loci. However, the overall similarity in the patterns of these summary statistics does not support the hypotheses of a largely different demographic history of both species. There may be several reasons for these findings: first, it is possible that these results mostly reflect the most recent demographic history of both species that may have been rather similar in the late Pleistocene forests of northwestern Madagascar. The extent of the forest surface most likely contracted in all western lowland areas of Madagascar towards the last glacial maximum (LGM) and expanded again only afterwards [24–26]. During the LGM, all species, independent on whether they had a long or short phylogeographic history in these lowland forests, probably underwent population contractions and expanded again afterwards together with the forests [21, 26]. In addition, both tested mouse lemur species have most likely been equally affected by the anthropogenic habitat loss that started in large parts of Madagascar after the arrival of man within the last few thousand years and continues until today [61–63]. Second, it is possible that similar selection regimes in the two species acting across multiple loci could affect the allelic

diversity of VRs. However, this is unlikely, since there was little evidence for widespread directional selection across VR loci (see above).

In addition to the summary statistics discussed above, mismatch distributions were used to compare the distribution of haplotype diversity within both species. Three types of mismatch distributions were identified in both mouse lemur species, which differed in relative frequency: 1) Half-bell shaped or L-shaped distributions showing a lack of variation which indicates purifying selection or recent bottlenecks [53]; 2) Unimodal distributions that are seen after one demographic or spatial expansion [53]; 3) Ragged distributions that are typical for populations at demographic equilibrium when colonisation events are very old and diversification is not constrained. Although the two species shared the half-bell shape distribution in five loci, *M. ravelobensis* showed the more diverse types of distributions ($n = 9$) more often than *M. murinus* ($n = 4$). In accordance with the results on genetic diversity presented above, these findings are in agreement with the hypothesis of a larger ancient effective population size in *M. ravelobensis* that was able to maintain a larger degree of genetic variability across time than its congener *M. murinus*. The mismatch distributions of both species did not differ significantly from the simulated distribution after one demographic or spatial expansion in most loci (exception: one locus in *M. murinus*). However, the τ -values were significantly higher in *M. ravelobensis* indicating that the putative expansion of *M. ravelobensis* is older than that of *M. murinus*. A more precise estimation of the time since expansion is not possible, since reasonable mutation rates for VR loci are not available and evolutionary rates were shown to vary across the entire gene family [16]. Therefore, these species differences cannot be easily reconciled with the present knowledge on certain historic vegetation changes.

Conclusions

The current VR diversity of *M. murinus* and *M. ravelobensis* in northwestern Madagascar appears to be shaped by various processes such as divergent scenarios of population expansions, purifying selection, loss of function and a potential contribution from positive selection. Whereas strong positive selection, found in the whole VR repertoire (both *VIRs* and *V2Rs*) and within individual gene clusters occurred in the past [16], ongoing selection may have shifted towards purifying selection in the majority of *VIR* loci to maintain the adaptive function of individual receptors, e.g. in the context of olfactory reproductive isolation between species as well as sex or kairomone recognition. This study only analysed a small subset of the large VR repertoire of mouse lemurs but gives important insights into the recent

evolution of VRs and suggests a previously unknown shift in selection pressures acting on these functional genes that are probably of highest relevance for nocturnal solitary foragers that rely heavily on olfactory communication. Functional VR loci may not be best-suited for demographic modelling considering the difficulty of differentiating between signals of purifying selection and recent population bottlenecks. However, the simultaneous analysis of synonymous and nonsynonymous substitutions can help to disentangle these different processes. Future studies on the functional diversity and molecular evolution of olfactory receptor genes will certainly add substantially to our understanding of adaptive radiations, local adaptation and reproductive strategies in various mammalian clades whose life styles, social systems and reproductive strategies rely on pheromonal communication.

Additional files

Additional file 1: IDs of the sampled individuals; the table contains the IDs of all males and females that were used for sequencing 17 VR loci in the two mouse lemur species *Microcebus murinus* ($n = 20$) and *M. ravelobensis* ($n = 20$) in the study site JBA. (DOCX 14 kb)

Additional file 2: Observed and simulated mismatch distributions of *M. murinus* and *M. ravelobensis* under the demographic and the spatial expansion model; Graphic representation of the 15 mismatch distributions per species that were not included as examples in the main manuscript. The two species-specific distributions for each locus are displayed side by side (Mmur, left side, grey; Mrav, right side, orange). simulated (d) = simulated after demographic expansion model (line with circles), simulated (s) = simulated after spatial expansion model (line with crosses). (PDF 90 kb)

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Availability of data and material

All sequences generated during the current study are deposited in GenBank (accession no. KY386903 – KY387582).

Authors' contributions

Conception and design of the study: UR, PH, NIM. Genetic data collection: PH. Data analysis: PH, NIM, UR. Manuscript drafting: UR, NIM, PH. All authors contributed to the finalization of the manuscript and approved the final version.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The trapping of mouse lemurs in the Ankarafantsika National Park, Madagascar, and the collection of individual tissue samples for the purpose

of genetic analyses adhered to the legal requirements of Madagascar. All procedures were officially approved by the Malagasy Ministry of Environment, Water and Forests (authorisation no. 062/08/MEEFT/ SG/DGEF/ DSA/SSE). We have complied with the international ethical standards for the treatment of primates and with national laws and research rules formulated by the Malagasy authorities. The University of Veterinary Medicine Hannover and the German law do not request the approval of field work that is conducted outside Germany by an ethics committee. However, the general sampling routines correspond to those that have been approved by the Bezirksregierung Hannover (reference number: AZ 33.9-42502-05-10A080) for mouse lemurs that are housed and bred for scientific purposes at the Institute of Zoology in Hanover, Germany.

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