

SCIENTIFIC REPORTS



OPEN

The pioneering role of *PRDM9* indel mutations in tarsier evolution

Sacha Heerschop¹, Hans Zischler¹, Stefan Merker², Dyah Perwitasari-Farajallah^{3,4} & Christine Driller¹

Received: 04 May 2016

Accepted: 09 September 2016

Published: 04 October 2016

PRDM9 is currently the sole speciation gene found in vertebrates causing hybrid sterility probably due to incompatible alleles. Its role in defining the double strand break loci during the meiotic prophase I is crucial for proper chromosome segregation. Therefore, the rapid turnover of the loci determining zinc finger array seems to be causative for incompatibilities. We here investigated the zinc finger domain-containing exon of *PRDM9* in 23 tarsiers. Tarsiers, the most basal extant haplorhine primates, exhibit two frameshifting indels at the 5'-end of the array. The first mutation event interrupts the reading frame and function while the second compensates both. The fixation of this allele variant in tarsiers led to hypothesize that de- and reactivation of the zinc finger domain drove the speciation in early haplorhine or tarsiiiform primates. Moreover, the high allelic diversity within *Tarsius* points to multiple effects of genetic drift reflecting their phylogeographic history since the Miocene.

Meiosis is a fundamental process to generate hereditary variability, thus being a driving force in evolutionary biology. Meiotic abortion is a cause for hybrid sterility which is a postzygotic barrier leading to speciation^{1,2}. The question now increasingly being asked is therefore what type of genetic modification underlies species formation. In vertebrates *PRDM9* is, at present, the top candidate gene associated with hybrid sterility^{1,3}. The gene is exclusively expressed by germ cells in the meiotic prophase I and encodes a zinc finger protein that specifies hotspots of recombination⁴⁻⁶. The zinc finger domain evolves rapidly, both as to the number and sequence of repetitive motifs resulting in altered DNA-binding specificity, which in turn may lead to genetic incompatibilities promoting species divergence³. A recently published study on the evolutionary dynamics of the primate *PRDM9* zinc finger array revealed taxon-specific alleles across 18 haplorhine species, further strengthening its role in speciation processes⁷. However, tarsiers, the sole extant representatives of non-anthropoid haplorhine primates⁸, were not included. As the deepest offshoot within haplorhini they have a long independent history, possibly covering about 80 million years of primate evolution⁹. Fossils of *Tarsius* are scarcely reported and restricted to Asia and North Africa spanning a period from the middle Eocene to the middle Miocene¹⁰⁻¹³. Assuming the fossil families of Omomyidae, Archicebidae and Afrotarsiidae as primitive tarsiiiforms, extinct ancestors of tarsiers were widespread holarctic Eocene-Oligocene species¹²⁻¹⁴ and occurred as far back as 55 Mya¹⁵. Extant tarsiers, however, are endemic to insular Southeast Asia, where they fall into the three geographically and evolutionary distinct lineages of Western, Philippine, and Sulawesi tarsiers, with the latter constituting the most species-rich clade^{16,17}. Even considering only the radiation of this ancient primate lineage in the Malay-Archipelago since the Miocene, allopatric speciation both between and within extant tarsier clades has left traceable molecular signatures of biogeographic events¹⁸⁻²¹.

Due to their independent evolution within haplorhine primates and their marked diversification, especially evident over the last 2.5 million years on the Indonesian island of Sulawesi²¹, tarsiers represent the unique opportunity for exploring the evolutionary dynamics of the *PRDM9* gene and its role in speciation processes within primates and tarsiers in particular. We therefore sequenced the *PRDM9* exon encoding the zinc finger array of 23 tarsier individuals, including 21 specimen from Sulawesi and two non-Sulawesi specimen, one each representing the Western and the Philippine tarsier clade. Based on these data we investigated the functionality and allelic diversity of the zinc finger domain in tarsiers. In addition we inferred evolutionary relationships between

¹Institute of Anthropology, Johannes Gutenberg University Mainz, Anselm-Franz-von-Bentzel-Weg 7, 55128 Mainz, Germany. ²Department of Zoology, State Museum of Natural History Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany. ³Primate Research Center, Bogor Agricultural University, Jalan Lodaya II/5, Bogor 16151, Indonesia. ⁴Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16680, Indonesia. Correspondence and requests for materials should be addressed to S.H. (email: heerschop@uni-mainz.de) or C.D. (email: christinedriller@gmx.de)

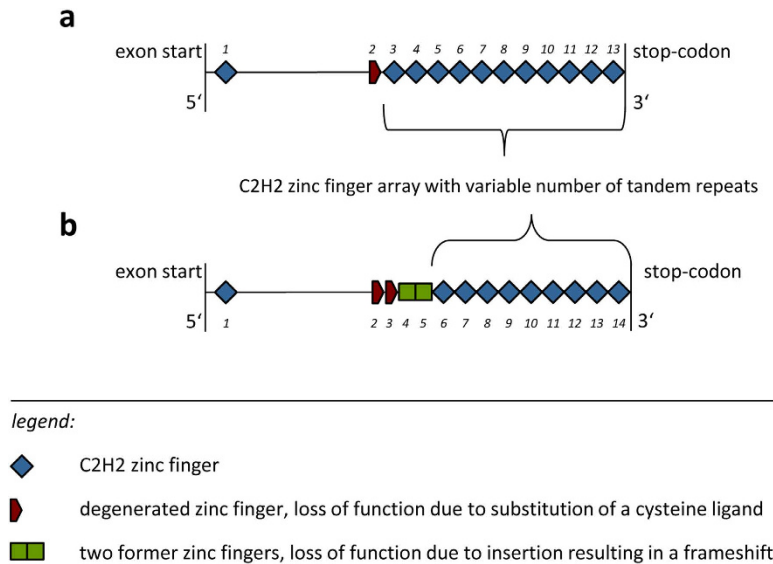


Figure 1. Schematic example of the *PRDM9* exon with the C-terminal zinc finger array of [a] human allele A as representative for anthropoid primates and of [b] allele TD1 for tarsiers. The comparison between the two exons shows the impact of the insertion and deletion on the zinc finger array, i.e. the loss of three functional C2H2 zinc fingers. Zinc finger numbers are written in italics.

CD26 ZnF3	AGCAGGGAGTGTGGGCGAGCCTTTACCCCTTAAGTCAAACCTCCTCACACACCAGAGGACACACGCACAGGGGAGAAGCCCTTTG
HSA ZnF3	S R E C G R A F T L K S N L L T H Q R T H A Q G R S P L TGCAGGGAGTGTGGGCGGGGCTTTAGCTGGAAGTCACACCTCCTCATTACCAGAGGATACACACA--GGGGAGAAGCCCTATG C R E C G R G F S W K S H L L I H Q R I H T G E K P Y
CD26 ZnF4	TCTGCAGGGAGTTCAGGCAGAGCTTTATCGGAACATCACACCTCCTCAGAAACCAGAGGACACACACAGGGGGAAGTCCTATG
HSA ZnF4	S A G S S G R A L S E H H T S S E T R G H T Q E G S P M TCTGCAGGGAGTGTGGGCGGGGCTTTAGCTGGCAGTCAGTCTCCTCACTCACCAGAGGACACACACAGGGGAGAAGCCCTATG V C R E C G R G F S W Q S V L L T H Q R T H T G E K P Y
CD26 ZnF5	TCTGCACCGAGTATGGACGGAGCTTTAGCCTTAAGTCAAACCTGCCAGACACCAGAGGA--CACACCGGGGAAAAGCCCATGTC
HSA ZnF5	S A P S M D G A L A L S Q T C P D T R G H T G E K P H V TCTGCAGGGAGTGTGGGCGGGGCTTTAGCCGGCAGTCAGTCTCCTCACTCACCAGAGGAGACACACAGGGGAGAAGCCCTATGTC V C R E C G R G F S R Q S V L L T H Q R R H T G E K P Y Y V
CD26 ZnF6	TGCAGGAAGTGTGGGTGGAGCTTTAGCCACAAGTCACGTCTCCTCAGACACCAGAGGACACACACGGGGGAGAAGCCCATGTC
HSA ZnF6	C R K C G W S F S H K S R L L R H Q R T H T G E K P H V TGCAGGGAGTGTGGGCGGGGCTTTAGCCGGCAGTCAGTCTCCTCACTCACCAGAGGAGACACACAGGGGAGAAGCCCTATGTC C R E C G R G F S R Q S V L L T H Q R R H T G E K P Y V

Figure 2. Frameshift mutations in tarsier *PRDM9*. Shown are aligned nucleic acid and peptide sequences comprising zinc fingers 3–6 (ZnF3–ZnF6) of *Homo sapiens* (HSA) and *Tarsius* (represented by a Sulawesi individual, CD26) and illustrating the frameshifting nature of insertion (blue) and deletion (green) mutations. Note the nonsynonymous substitution in the very first triplet which turns cysteine to serine (orange) and degrades ZnF3 of the tarsier. Amino acid sequences of human ZnF3–ZnF6 and ZnF6 of the tarsier show the classical C2H2 zinc finger motif (indicated by bold and underlined amino acids).

zinc fingers of anthropoid primates⁷ and tarsiers, and discussed the possible function of *PRDM9* as driving force behind the anthropoid-tarsiiform split and the divergence of the tarsiiform lineage.

Results

Tarsier *PRDM9* sequence characterization. The examined exon of Western, Philippine and Sulawesi tarsiers was homologous to exon 11 of the human *PRDM9* gene and was about 1037–1793 bp long depending on the number of zinc finger repeats. It could be divided into two parts, the 5' sequence and the 3' C2H2 zinc finger array. Structural and functional properties of the zinc finger protein encoding exon are shown in Fig. 1.

In contrast to the variable C2H2 zinc finger domain which was used for the analyses, the 5' sequence of the exon was more conserved. This part of the sequence contained a C2H2 zinc finger (ZnF1) at the beginning of the exon and four presumably former functional zinc fingers. The second and third zinc fingers were both degenerated by the loss of a zinc ion binding cysteine ligand, see Fig. 2, which is in principal not uncommon in zinc finger

Individuals		Origin		Alleles (Number of C2H2-ZnFs)	
Species	Sample ID	Biogeographic Region	Sample Site	1	2
<i>Tarsius syrichta</i>	TSY	Philippines	N/A	TS1 (3)	
<i>Tarsius bancanus</i>	TBA	Sundaland	N/A	TB1 (8)	TB2 (7)
<i>Tarsius dentatus</i>	T111	Wallacea	Laone, Central Sulawesi	TD1 (9)	TD2 (8)
<i>Tarsius lariang</i>	T47	Wallacea	Koja, Central Sulawesi	TL1 (10)	TL2 (7)
<i>Tarsius sp.</i>	CD02	Wallacea	Ogatemuku, North-Sulawesi	TSP1 (9)	TSP2 (10)
<i>Tarsius sp.</i>	CD05			TSP3 (9)	TSP4 (9)
<i>Tarsius sp.</i>	CD10			TSP1 (9)	TSP4 (9)
<i>Tarsius dentatus</i>	CD14	Wallacea	Korosule, Central Sulawesi	TD3 (6)	TD4 (7)
<i>Tarsius dentatus</i>	CD16			TD5 (8)	TD6 (9)
<i>Tarsius dentatus</i>	CD17			TD3 (6)	TD7 (6)
<i>Tarsius dentatus</i>	CD19	Wallacea	Luwuk, Central Sulawesi	TD1 (9)	
<i>Tarsius dentatus</i>	CD24			TD1 (9)	
<i>Tarsius dentatus</i>	CD26			TD1 (9)	
<i>Tarsius dentatus</i>	CD27			TD1 (9)	
<i>Tarsius sp.</i>	CD34	Wallacea	Labanu, North Sulawesi	TSP5 (6)	
<i>Tarsius sp.</i>	CD36			TSP6 (10)	TSP7 (10)
<i>Tarsius sp.</i>	CD41	Wallacea	Kendari, South Sulawesi	TSP8 (7)	TSP9 (12)
<i>Tarsius sp.</i>	CD43			TSP10 (10)	TSP11 (7)
<i>Tarsius sp.</i>	CD46	Wallacea	Duasaudara, North Sulawesi	TSP12 (10)	
<i>Tarsius sp.</i>	CD48			TSP13 (10)	
<i>Tarsius sp.</i>	CD51			TSP14 (10)	TSP12 (10)
<i>Tarsius fuscus</i>	CD64	Wallacea	Bantimurung, South Sulawesi	TF1 (6)	TF2 (11)
<i>Tarsius fuscus</i>	CD65			TF1 (6)	TF2 (11)

Table 1. Allelic diversity of the zinc finger array in tarsiers. Each individual is listed with its species, sample ID, origin, allele identification and the number of C2H2 zinc fingers per allele. TSY: *Tarsius syrichta*; TBA: *Tarsius bancanus*; *Tarsius sp.*: Taxon yet unclassified and currently affiliated with the *Tarsius tarsier*-population¹⁷.

arrays²². The sequence of the third zinc finger was also interrupted by a 2 bp-insertion that caused a framing error affecting the next two zinc fingers which we refer to as former zinc fingers. A deletion in the second altered zinc finger, which would be the fifth overall, restored the reading frame. The array of classical 84 bp C2H2 zinc fingers started with the end of this fifth zinc finger (Figs 1 and 2).

Indel events are quite rare in coding regions due to purifying selection^{23,24}, especially if their length is not a multiple of three²⁵. While the insertion results in a different amino acid sequence and may alter protein function, the deletion alone generates premature stop codons which lead to a faster pseudogenization respectively gene loss²⁶. We therefore consider it likely that the insertion predates the deletion mutation which then in turn acted as a compensatory mutation. Compensatory mutations are twice as common as reversions to the original state and are often found in close proximity of the initial mutation^{27,28} being consistent with our findings. As only both indels jointly restore protein integrity we further assume that the temporal offset between indel mutations resulted in a temporary loss or limited protein function.

Allelic variation of the zinc finger array in tarsiers. The alleles are defined on amino acid level, i.e. synonymous nucleotide changes are excluded, and are restricted to the C2H2 array. We found 28 alleles in 23 individuals with 15 individuals being heterozygous, (Table 1, Fig. 3).

The 25 alleles exclusively found in Sulawesi tarsiers show similarities, most often within populations (see Materials and Methods for sample sites). They mainly differ in the number of zinc fingers but hardly in their sequence like the alleles 9 and 10. Alleles 4, 5 and 6 have each ten zinc fingers with minor changes in the amino acid sequence (Fig. 3). Only allele 14 is shared between two Sulawesi populations (Luwuk and Laone), both belonging to *Tarsius dentatus*.

There are two types of 5'-most C2H2 zinc fingers differing in one binding amino acid (HHR, HRR). One variant can be found on the northern peninsula (Duasaudara, Labanu, Ogatemuku) and in Korosule. The other is specific to populations inhabiting the central, eastern and southern parts of Sulawesi, with the exception of one copy also occurring in north-eastern Sulawesi. The 5'-most C2H2 zinc fingers accord to some extent with the two lineages found in Driller *et al.*²¹. Lineage 1 comprises the populations on the northern peninsula and Kendari, lineage 2 all other populations.

The 3'-most C2H2 zinc fingers are, with one exception, identical regarding the three binding amino acids RHT (Fig. 3). Five of them show a nonsynonymous substitution which, however, does not affect the DNA-binding sites. They were observed in the populations of Labanu, Korosule and Koja. Three out of 19 zinc fingers with key codon positions QSR, LSR, and RNT are unique. Some are only found in few populations like the motifs specifying the binding triplet QNI or RNR and being restricted to Laone and Kendari, or Labanu and Ogatemuku, respectively.

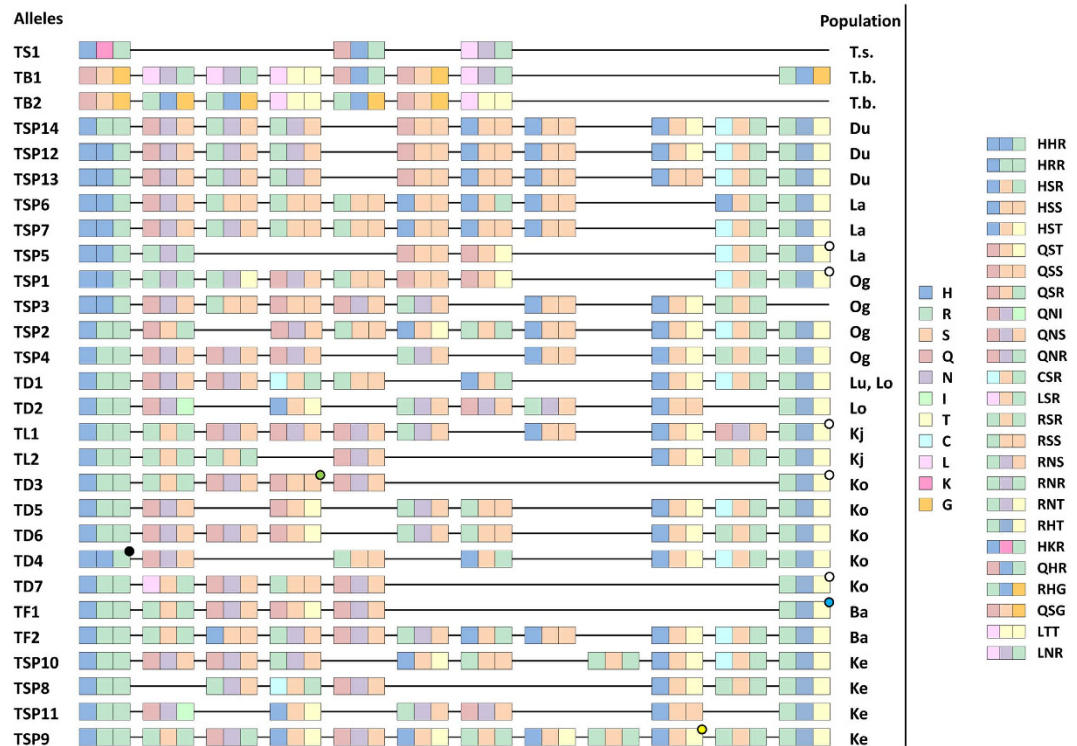


Figure 3. Alignment of C2H2 zinc finger alleles. The numbers identifying the alleles are identical to those in Table 1. Each zinc finger is depicted with a triplet composed of its binding amino acids (–1, 3, 6). Each amino acid is marked with a distinct colour. Dots hint towards one or more nonsynonymous substitutions apart from the shown amino acids. Abbreviations: T.s., *Tarsius syrichta*; T.b., *Tarsius bancanus*; Du, Duasudara; La, Labanu; Og, Oгатemuku; Lu, Luwuk; Lo, Laone; Kj, Koja; Ko, Korosule; Ba, Bantimuring; Ke, Kendari.

Especially the hints on the relationship of the populations on the northern peninsula among each other and between Laone and Luwuk are comparable with results of Driller *et al.*²¹. The relatively conserved 5'-most and 3'-most C2H2 zinc finger, the more variable ZnFs in between and the appearance of similar ZnFs in different species are also seen in mice²⁹.

Alleles of *T. bancanus* and *T. syrichta* are species-specific encoding zinc finger motifs not present in Sulawesi tarsiers. Despite their long geographic isolation they still share two zinc fingers (Fig. 3). Both alleles of the Western tarsier end with a zinc finger degenerated by the exchange of the second zinc binding histidine ligand with asparagine. With only three repeats the zinc finger domain of the homozygous Philippine specimen is unusually short, but according to Stubbs *et al.*³⁰ capable to bind DNA. However, more individuals or rather more alleles of both species could reveal further and better insights regarding the relationship of *PRDM9* zinc fingers between the three main tarsier groups.

Phylogenetic analysis. To estimate the phylogenetic affiliations of the sequenced tarsier zinc fingers within the haplorhine divergence we reconstructed a phylogeny with *Microcebus murinus* as strepsirhine outgroup. Including all first degenerated and C2H2 zinc fingers (Fig. 4a) it completes the results of Schwartz *et al.*⁷ from a haplorhine perspective. The basal trifurcation separates degenerated zinc fingers of tarsiers, degenerated zinc fingers of anthropoid primates and all C2H2 zinc fingers with node support values above 0.9 (Fig. 4a). Within the C2H2 cluster tarsier zinc fingers form a well-supported monophyletic group. Within this group 5' most and 3' most C2H2 zinc fingers are clustered reflecting the biogeography of the individuals tested. Apart from these findings our tree topologies (Fig. 4) are congruent with those obtained for anthropoids as presented by Schwartz *et al.*⁷.

The second phylogeny (Fig. 4b) is based exclusively on first degenerated zinc fingers. Here again tarsiers constitute a monophyletic group basal to the anthropoid clade. Within *Tarsius* Sulawesi tarsiers are monophyletic with respect to Western and Philippine tarsiers.

Positive and negative selected sites. Zinc finger genes are known to alter quickly in their zinc finger array and in particular at the DNA-binding codons (positions –1, 3 and 6 relative to the α -helix) which show signatures of positive selection^{31–33}. The *PRDM9* zinc finger domain is no exception as shown by positively selected DNA-binding amino acids in rodents and primates^{3,34,35}. The high mutation rates at codon positions directing the interaction of *PRDM9* with DNA is deemed responsible for the formation of new hotspots and thus antagonizing hotspot erosion^{5,36,37}.

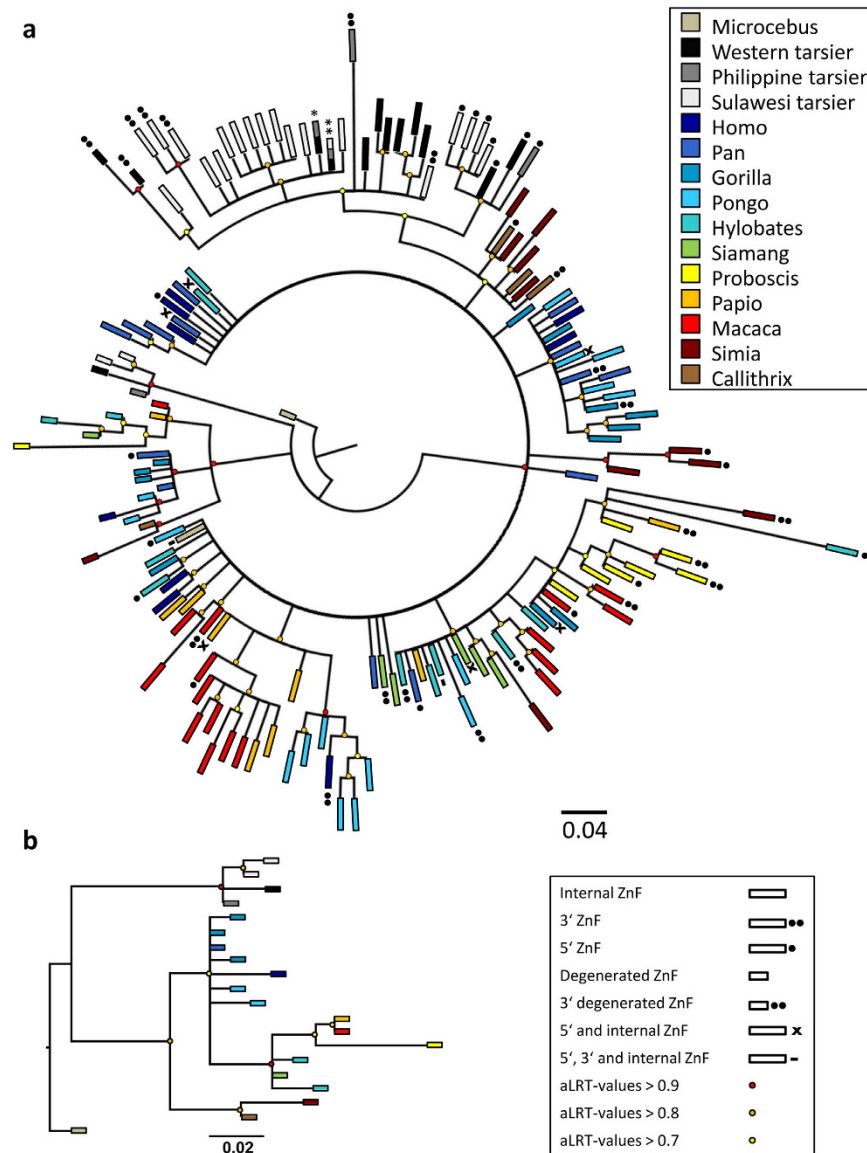


Figure 4. Phylogeny reconstructions based on Schwartz *et al.*⁷, including tarsier zinc fingers. [a] Shows a tree topology including the first degenerated zinc finger and all C2H2 zinc fingers of the array. [b] Depicts only the first degenerated zinc finger. The genera can be identified by colour, different kinds of or particular zinc fingers can be distinguished by their form, see key. Branches with aLRT-values above 0.9, 0.8 and 0.7 are indicated with a red, orange and yellow dot, respectively. The three main branches towards tarsier degenerated zinc fingers, anthropoid degenerated zinc fingers and towards C2H2 zinc finger show the highest aLRT-values of 0.992, 0.966 and 0.98, respectively. One asterisk indicates identical zinc fingers of Western and Philippine tarsier. Two asterisks denote ZnFs shared by Western, Philippine and Sulawesi tarsier including the 3'-most C2H2 ZnFs of the Western tarsier.

We found positive selection at the three DNA-binding codons, i.e. positions -1 , 3 , 6 relative to the α -helix (see Fig. 5). Structural important sites like the zinc ion ligands are conserved by negative selection. We consider the ongoing selective pressure – positive as well as negative – acting on tarsier *PRDM9* as relevant proof for gene integrity being important in the context of a probable temporary loss of function which was subsequently abolished by a compensatory mutation.

Discussion

With four non-functional zinc fingers at the 5' end of the tarsier zinc finger array (Fig. 1), degenerated by missing cysteine ligands and frameshifting indels, we detected a completely new *PRDM9* allelic variant in primates and mammals in general. This sequence singularity is also reflected in our inferred phylogenies including first degenerated and all functional C2H2 zinc fingers of the array and clearly designating tarsiers as monophyletic group within haplorhine primates (Fig. 4a,b).

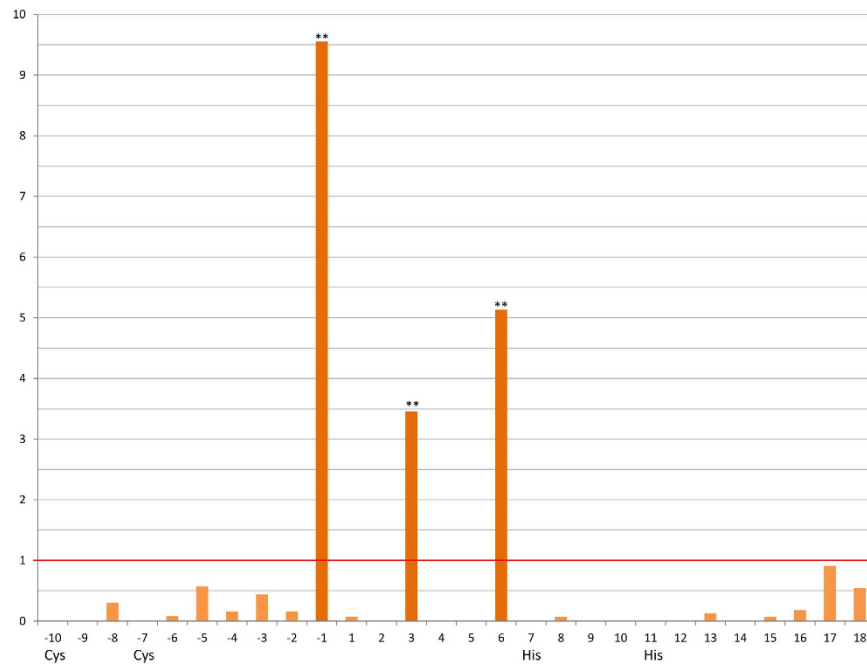


Figure 5. ω -values of C2H2 zinc finger sites in tarsiers. Sites on the x-axis are indicated with their position relative to the beginning of the α -helix. Ligands - cysteine and histidine - are also labeled. The y-axis shows the ω -values with one being the value for neutral selection highlighted by a red line. The three DNA-binding sites (-1, 3, 6) are positively selected with a p-value < 0.01 after Benjamini-Hochberg correction (**). The other sites show ω -values revealing negative selection with p-values < 0.05 after Benjamini-Hochberg correction (except for the sites -5 to -2, 2, 17 and 18).

All these sequence autapomorphies, but especially those associated with a temporary loss of allelic integrity (see results) might possibly indicate an active role of *PRDM9* in the divergence process between anthropoid and tarsiiform primates or alternatively, along the tarsiid lineage. A possible speciation scenario could be as follows:

In a population of haplorhine or tarsiid ancestors a damaging, frameshifting mutation occurs in one allele (Figs 2 and 6). Heterozygous individuals may be subfertile because gene function is reduced due to one non-functional allele. However, Brick *et al.*⁶ showed that one allele can determine 75% of the hotspots while the other is responsible for 22% [1% of all hotspots are shared and 2% are new in comparison to parental homozygote constellations]. Furthermore, male mice which are heterozygote null for *Prdm9* are not sterile but reproduce at a later stage for the first time and father fewer offspring, both in comparison to the wild-type³⁸. Individuals homozygous for the damaged allele are probably infertile due to a considerably reduced or loss of gene function and comparable to *Prdm9* knockout mice where a meiotic arrest at pachytene stage leads to sterility⁴. The second frameshifting mutation restores the functionality of the former erroneous allele (Figs 2 and 6) and is therefore positively selected and fixed^{39,40}.

Two of the now three possible allelic variants in homozygotes are functional (Fig. 6), the one with two original or wild-type alleles and the other with two alleles carrying both indel mutations. By contrast, all heterozygotes shown in Fig. 6 are possibly less fit or rather subfertile resulting from a decreased recombination activity affected by either one damaged allele or different hotspot usage among the two types of functional *PRDM9* alleles⁴¹. The novel allele finally get fixed in a precursor of tarsiiform primates but in any case no later than 20 MYA, the time to the most recent common ancestor (MRCA) of the three extant tarsiid clades^{17,19,21}, all exclusively carrying the new allelic variant. Explanations for the fixation of the mutant allele in an ancestral population of modern tarsiers may include directed and/or random evolutionary processes like biased gene conversion or genetic drift. With regard to the former it has been shown that a newly introduced *PRDM9* allele increased hotspot activity in mice through altered and haplotype-biased DNA-binding, thus counteracting fitness loss during the process of hotspot erosion by allelic gene conversion³⁶. The mutant allele could have had a similar hotspot enhancing and therewith beneficial fitness effect in a population of ancient tarsiids or their progenitors. Given that its selective advantage was sufficiently large the novel allele could also have overcome the effects of genetic drift where typically rare beneficial mutations are more likely to go extinct^{42,43}. On the other hand, we could suppose that it was this process of random change in genetic composition that maintained the newly arisen allele resulting in the structural uniformity of the *PRDM9* zinc finger domain and facilitating divergence of an ancestral lineage finally leading to modern tarsiers.

Extinct relatives of living tarsiids branched away from anthropoid primates very shortly after the haplorhine-strepsirrhine split^{9,19,44}. Depending on the data, either paleontological or molecular, first primates evolved sometime between 55–87 MYA^{9,15,19,45}. Within this period two drastic climate changes occurred and both induced mass extinctions^{44,46,47} creating conditions promoting adaptive radiation⁴⁴. Proto-tarsiiforms could have survived or

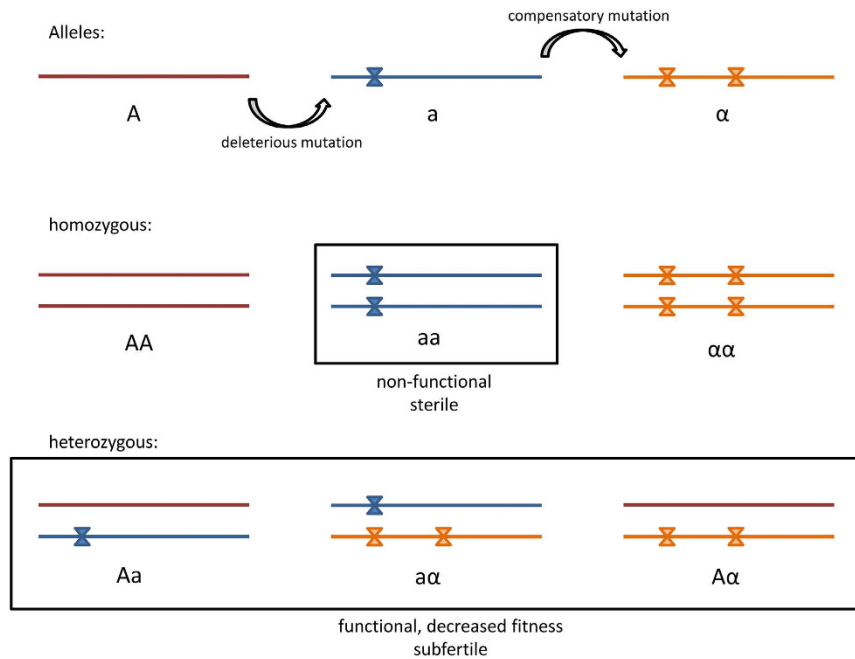


Figure 6. Schematic demonstration of the mutational events in tarsier *PRDM9* zinc finger array. The insertion and deletion create two more alleles, a and α , besides the wild-type allele A. All possible genotypes are shown where all heterozygous are possibly functional but subfertile and two out of three homozygous are functional without constraints and the third is non-functional and sterile, comparable to *Prdm9* knockout mice⁶.

emerged from these evolutionary bottlenecks possibly also because *PRDM9* produced new recombination landscapes allowing allele combinations more favourable or adaptive to the changing environmental conditions and/or newly vacant ecological niches. Another extinction event associated with Eocene-Oligocene cooling⁴⁸ largely decreased primate diversity with the disappearance of omomyids, a controversial fossil tarsiid primate^{49–51}. This event presumably caused a geographic shift in tarsier distribution from mainland to insular Southeast Asia^{52,53} exposing tarsiids of modern aspects to another population bottleneck and thus providing a further option for the fixation of the novel tarsier-specific *PRDM9* allele. A heightened relevance of genetic drift in tarsier molecular evolution was already discussed⁵⁴, as extant tarsiers, and especially those endemic to the Indonesian island of Sulawesi, have been subject to significant climatic and tectonic change^{55,56} that triggered allopatric speciation in the Malay Archipelago since the Miocene^{17,18,20,21,56–58}. Hence, the much longer history of independent evolution in environmental instability may give more credence to the theory that the salient molecular changes of the *PRDM9* zinc finger array described here have developed along the tarsiid lineage rather than initialized the anthropoid-tarsiid split.

PRDM9 variation in alleles, zinc finger motifs and especially at the key codon positions reflect the different levels of evolutionary independence of the three major tarsier clades (Western, Philippine, and Sulawesi tarsiers), provided the single non-Sulawesi specimens are representative for the respective biogeographic region. Like it has been observed in mice, chimpanzee and humans, 5'-most and 3'-most C2H2 zinc fingers are more conserved or even identical within or at least between closely related species^{29,41,59,60} even if positive selected sites are factored in. In tarsiers this appears to apply mainly to species endemic to the same biogeographic region, but is also fairly reflected in lineage-specific constraints on 5'-most C2H2 zinc fingers of Sulawesi tarsiers²¹. Considering the three DNA binding amino acids at internal zinc fingers of Sulawesi tarsiers we found several duplicated zinc finger motifs in each species/subspecies indicating their recent origin⁶¹ for most populations being estimated at less than 500,000 years ago²¹. Particularly striking are the blocks of zinc finger motifs in northern Sulawesi tarsier populations (Fig. 3). The order of their occurrence very plastically depicts how these populations very recently evolved from a single common ancestor²¹.

In conclusion, the high mutation rate of the *PRDM9* zinc finger domain^{3,7,29} and multiple events of genetic drift produced an enormous diversity of *PRDM9* alleles in tarsiers. The geographic distribution of zinc finger alleles and especially the occurrence and enrichment of specific zinc finger motifs reflect phylogeographic patterns of extant tarsiers further strengthening an involvement of *PRDM9* in population differentiation. More intriguing, however, is our discovery of a hitherto unknown and, in addition, tarsier-specific *PRDM9* allele variant that arose from two indel mutations and played a perhaps decisive role in tarsiid evolution.

Material and Methods

Sample set. The sampling of 23 individuals comprises 21 specimens of nine Sulawesi tarsier populations (1–4 individuals/population, see Fig. 7) and two non-Sulawesi tarsiers (*Tarsius bancanus* and *T. syrichta*). Samples from Sulawesi were obtained from previous studies^{18,21}, while the Western and the Philippine specimen

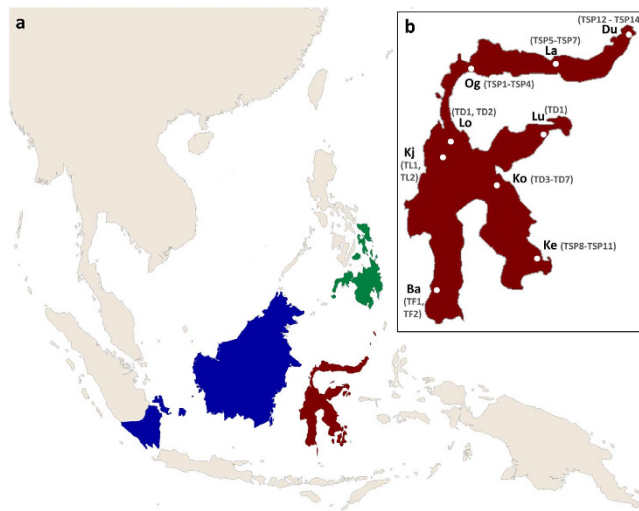


Figure 7. Maps of Southeast Asia and Sulawesi. [a] Shows the region of Southeast Asia with ranges of *Tarsius bancanus* in blue, of *Tarsius syrichta* in green and of Sulawesi tarsiers in red¹⁶. [b] Is a close-up of Sulawesi with sample sites highlighted by white dots. Alleles per population are written in parenthesis. Abbreviations: Du, Duasaudara; La, Labanu; Og, Ogametuku; Lu, Luwuk; Lo, Laone; KJ, Koja; Ko, Korosule; Ba, Bantimurung; Ke, Kendari. Base map was created in ArcGIS (ArcMap 10.0; <http://www.esri.com/software/arcgis>).

Primer	Sequence 5'-3' [bp]	Combinations	T (°C)	Product length (bp)
PRDM9-F1	GGGGTAGGAATCTCAAGAATTG[22]	F3-R1	54	594 (5' end)
PRDM9-F2	AAGAATTGCAAGGGTCAAGTATG[23]	F1/F2-R3	53	977–1481/963–1467
PRDM9-F3	TGGTGGGAGACAGCTTGC[18]	F3-R3	53	1501–2005
PRDM9-F4	GCCAAGAGGACGAGTAAGTCAATAG[25]	F5-R4	56	1152–1656
PRDM9-F5	CAAAGATCCACCCATGTTTC[19]	F4-R2	57	198 (3' end)
PRDM9-R1	TGACTCATCTGAGGCCTTG[21]			
PRDM9-R2	CCTCATCTCTAGTCATGAAAGTGG[24]			
PRDM9-R3	GAAGCACCTCCAAGCTG[18]			
PRDM9-R4	GGTCTCTTACACTCTGSAAGTC[23]			

Table 2. PCR primer specifications. Given are applied primer combinations, annealing temperatures and product lengths.

were provided by Y. Rumpler (Les Hôpitaux Universitaires de Strasbourg, France) and J. Brosius (University of Muenster, Germany), respectively. Whole genome amplifications (WGA) of each sample (40–80 ng/μl) were used as a template in PCR to amplify the exonic region of the *PRDM9* gene containing the zinc finger domain.

PCR and Sequencing. We PCR-amplified and sequenced the exon which encodes the zinc finger domain of the *PRDM9* gene in three parts: the 5' and 3' flanking regions and the repetitive elements in between (see Table 2).

To reduce amplification of unspecific products we conducted wax-mediated hotstart PCR. Each PCR-reaction contained 30 μl with final concentrations of 200 μM dNTPs, 2.5 units *Taq* DNA polymerase, 1x PCR buffer including 1.5 mM MgCl₂ (QIAGEN Taq PCR Core Kit), and 0.33 pM per primer. PCRs were run under the following conditions: 3 minutes of initial template denaturation at 94 °C was followed by 35 cycles of denaturation (40 sec at 94 °C), primer annealing (1 min at primer-specific temperatures) and DNA elongation (1–1.5 min at 72 °C). A final elongation step of 5 minutes at 72 °C finished the PCR. Product sizes were estimated on ethidium bromide stained 1.5% agarose gels together with O'RangeRuler 100 bp DNA Ladder and GeneRuler 100 bp Plus DNA Ladder (both Fermentas/Thermo Scientific).

In general PCR products were enzyme purified before sequencing. In some individuals PCR reactions yielded multiple different sized products, either because of size variation between the two alleles or unspecific amplicons. The separation of the two alleles or the isolation of the product from non-specific sequences was done by gel extraction with the QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were sequenced on both strands using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the corresponding PCR primer pair. After an SDS/heat treatment for elimination of unincorporated dye terminators sequences were processed on an ABI 3130xl genetic analyzer (Applied Biosystems).

For individuals with ambiguous sites present in their DNA sequences PCR products were purified by ethanol precipitation, ligated into a plasmid vector (pGem-T Vector System I, Promega) and transformed into One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen). Where gel extraction failed to separate alleles of different lengths sequences were also isolated by cloning.

At least six positive clones, three of each allele, were PCR amplified and sequenced. Colony PCR was carried out in 20 μ l reaction volumes according to standard protocols using the Taq PCR Core Kit (QIAGEN) and vector-specific primers. For long amplicons where sequencing read length was not sufficient we used the internal primer PRDM9-F2 (see Table 2) to get the middle portion of the target sequence.

Sequence Analyses. Raw sequences were edited and consensus sequences (KU948327 - KU948367) were generated in BioEdit 7.1.3.0⁶². To reveal the structure and integrity of the tarsier *PRDM9* gene we created alignments in BioEdit with the following approach. We compared 5'-terminal sequences including the degenerated zinc fingers in front of the C2H2 array 1) within *Tarsius*, and 2) between tarsiers, anthropoids (*Homo sapiens*, ENST00000296682; *Pan troglodytes*, GU166820.1) and rodents (*Rattus norvegicus*, ENSRNOT00000066370; *Mus musculus*, ENSMUST00000167994). We further examined the zinc finger domain by comparison of isolated zinc fingers within and between taxa of *Tarsius*, anthropoids and rodents. Structural and functional properties of tarsier *PRDM9*, in particular with regard to the type of zinc finger motifs, was also validated by peptide sequences obtained with EMBOSS Transeq v. 6.3.1^{63,64}.

Zinc finger Phylogeny. We generated two tree topologies, one including all 5'-most degenerated zinc fingers and one including both, functional C2H2-zinc fingers and degenerated first zinc fingers of the array. Haplorhine taxa represented in each data set comprised *Tarsius* (this study), *Homo sapiens* (ENST00000296682) and several other anthropoid primates, whose sequence data were adopted from Schwartz *et al.*⁷. *Microcebus murinus* served as outgroup. Corresponding sequence information was extracted from the Ensembl data base (assembly micMur1, database version 77) and by using BLAST+2.2.28⁶⁵. The zinc fingers were isolated with the first codon for cysteine as starting point. All except 5'-most degenerated and several 3'-most zinc fingers had a length of 84 bp. Lacking the first cysteine-triplet 5'-most degenerated zinc fingers start with the second codon for cysteine, and therefore being nine bp shorter. 3'-most zinc fingers can lack base pairs when interrupted by a stop codon, but they contain at least both cysteine and histidine ligands. Two 3' degenerated zinc fingers of *Tarsius bancanus* were included. Like Schwartz *et al.*⁷ we excluded the four binding triplets. To reduce the calculation time we removed identical zinc fingers within genera with the Fabox DNACollapser⁶⁶. Models of nucleotide substitution were determined with jModelTest 2.1.6^{67,68}. Phylogenetic trees were generated with the Maximum Likelihood method (PhyML 3.0⁶⁷) including an approximate likelihood ratio test (aLRT) and SH-like supports. The degenerated zinc finger of *Microcebus murinus* served as outgroup in both tree topologies. Nodes with support values below 0.5 were collapsed in TreeGraph 2.2.0–407 beta⁶⁹. The final phylogenetic trees were visualized in FigTree 1.3.1⁷⁰.

Detecting selective pressure. All functional C2H2 zinc fingers (84 bp) were tested for selective pressure on codon sites. We inferred a phylogenetic tree based on all non-identical sequences determined by Fabox DNACollapser⁶⁶ using the Maximum Likelihood method [PhyML 3.0, settings other than default: K80, bootstrap: 100]⁶⁷. The best fitting substitution model was selected using jModelTest 2.1.6^{67,68}. Tests of selection were performed with the sitewise-likelihood ratio method (SLR⁷¹) using default settings.

References

- Mihola, O., Trachtulec, Z., Vlcek, C., Schimenti, J. C. & Forejt, J. A mouse speciation gene encodes a meiotic histone H3 methyltransferase. *Science* **323**, 373–375 (2009).
- Forejt, J. Hybrid sterility in the mouse. *Trends Genet.* **12**, 412–417 (1996).
- Oliver, P. L. *et al.* Accelerated evolution of the Prdm9 speciation gene across diverse metazoan taxa. *PLoS Genet.* **5**, e1000753 (2009).
- Hayashi, K., Yoshida, K. & Matsui, Y. A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* **438**, 374–378 (2005).
- Grey, C. *et al.* Mouse PRDM9 DNA-binding specificity determines sites of histone H3 lysine 4 trimethylation for initiation of meiotic recombination. *PLoS Biol.* **9**, e1001176 (2011).
- Brick, K., Smagulova, F., Khil, P., Camerini-Otero, R. D. & Petukhova, G. V. Genetic recombination is directed away from functional genomic elements in mice. *Nature* **485**, 642–645 (2012).
- Schwartz, J. J., Roach, D. J., Thomas, J. H. & Shendure, J. Primate evolution of the recombination regulator PRDM9. *Nat. Commun.* **5**, 4370 (2014).
- Schmitz, J., Ohme, M. & Zischler, H. SINE insertions in cladistic analyses and the phylogenetic affiliations of *Tarsius bancanus* to other primates. *Genetics* **157**, 777–784 (2001).
- Perelman, P. *et al.* A molecular phylogeny of living primates. *PLoS Genet.* **7**, e1001342 (2011).
- Beard, K. C., Qi, T., Dawson, M. R., Wang, B. & Li, C. A diverse new primate fauna from middle Eocene fissure-fillings in southeastern China. *Nature* **368**, 604–609 (1994).
- Chaimanee, Y., Lebrun, R., Yamee, C. & Jaeger, J.-J. A new Middle Miocene tarsier from Thailand and the reconstruction of its orbital morphology using a geometric-morphometric method. *Proc. R. Soc. B* **278**, 1956–1963 (2011).
- Rasmussen, D. T., Conroy, G. C. & Simons, E. L. Tarsier-like locomotor specializations in the Oligocene primate *Afrotarsius*. *Proc. Natl. Acad. Sci. USA* **95**, 14848–14850 (1998).
- Jaeger, J.-J. *et al.* Late middle Eocene epoch of Libya yields earliest known radiation of African anthropoids. *Nature* **467**, 1095–1098 (2010).
- Szalay, F. S. Systematics of the Omomyidae (Tarsiiformes, Primates): Taxonomy, phylogeny, and adaptations. *Bull. Am. Mus. Nat. Hist.* **156**, 157–450 (1976).
- Ni, X. *et al.* The oldest known primate skeleton and early haplorhine evolution. *Nature* **498**, 60–64 (2013).
- Groves, C. & Shekelle, M. The genera and species of tarsiidae. *Int. J. Primatol.* **31**, 1071–1082 (2010).
- Shekelle, M., Meier, R., Wahyu, I., Wirdateti & Ting, N. Molecular phylogenetics and chronometrics of Tarsiidae based on 12S mtDNA haplotypes. Evidence for Miocene origins of crown tarsiers and numerous species within the Sulawesi clade. *Int. J. Primatol.* **31**, 1083–1106 (2010).
- Merker, S., Driller, C., Perwitasari-Farajallah, D., Pamungkas, J. & Zischler, H. Elucidating geological and biological processes underlying the diversification of Sulawesi tarsiers. *Proc. Natl. Acad. Sci. USA* **106**, 8459–8464 (2009).
- Springer, M. S. *et al.* Macroevolutionary dynamics and historical biogeography of primate diversification inferred from a species supermatrix. *PLoS ONE* **7**, e49521 (2012).

20. Stelbrink, B., Albrecht, C., Hall, R. & Rintelen, T. von. The biogeography of Sulawesi revisited. Is there evidence for a vicariant origin of taxa on wallace's "anomalous island"? *Evolution* **66**, 2252–2271 (2012).
21. Driller, C. *et al.* Stop and Go – Waves of tarsier dispersal mirror the genesis of Sulawesi island. *PLoS ONE* **10**, e0141212 (2015).
22. Looman, C., Abrink, M., Mark, C. & Hellman, L. KRAB zinc finger proteins: an analysis of the molecular mechanisms governing their increase in numbers and complexity during evolution. *Mol. Biol. Evol.* **19**, 2118–2130 (2002).
23. Ng, P. C. *et al.* Genetic variation in an individual human exome. *PLoS Genet.* **4**, e1000160 (2008).
24. Toth-Petroczy, A. & Tawfik, D. S. Protein Insertions and Deletions Enabled by Neutral Roaming in Sequence Space. *Mol. Biol. Evol.* **30**, 761–771 (2013).
25. Williams, L. E. & Wernegreen, J. J. Sequence context of indel mutations and their effect on protein evolution in a bacterial endosymbiont. *Genome Biol. Evol.* **5**, 599–605 (2013).
26. Zhang, Z. D., Frankish, A., Hunt, T., Harrow, J. & Gerstein, M. Identification and analysis of unitary pseudogenes. Historic and contemporary gene losses in humans and other primates. *Genome Biol.* **11**, R26 (2010).
27. Levin, B. R., Perrot, V. & Walker, N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* **154**, 985–997 (2000).
28. Poon, A. The rate of compensatory mutation in the DNA bacteriophage X174. *Genetics* **170**, 989–999 (2005).
29. Buard, J. *et al.* Diversity of Prdm9 zinc finger array in wild mice unravels new facets of the evolutionary turnover of this coding minisatellite. *PLoS ONE* **9**, e85021 (2014).
30. Stubbs, L., Sun, Y. & Caetano-Anolles, D. Function and evolution of C2H2 zinc finger arrays. *Subcell. Biochem.* **52**, 75–94 (2011).
31. Shannon, M., Hamilton, A. T., Gordon, L., Branscomb, E. & Stubbs, L. Differential expansion of zinc-finger transcription factor loci in homologous human and mouse gene clusters. *Genome Res.* **13**, 1097–1110 (2003).
32. Hamilton, A. T. *et al.* Evolutionary expansion and divergence in the ZNF91 subfamily of primate-specific zinc finger genes. *Genome Res.* **16**, 584–594 (2006).
33. Emerson, R. O., Thomas, J. H. & Myers, S. Adaptive evolution in zinc finger transcription factors. *PLoS Genet.* **5**, e1000325 (2009).
34. Thomas, J. H., Emerson, R. O. & Shendure, J. Extraordinary molecular evolution in the PRDM9 fertility gene. *PLoS ONE* **4**, e8505 (2009).
35. Nowick, K. *et al.* Gain, loss and divergence in primate zinc-finger genes. A rich resource for evolution of gene regulatory differences between species. *PLoS ONE* **6**, e21553 (2011).
36. Baker, C. L. *et al.* PRDM9 drives evolutionary erosion of hotspots in *Mus musculus* through haplotype-specific initiation of meiotic recombination. *PLoS Genet.* **11**, e1004916 (2015).
37. Boulton, A., Myers, R. S. & Redfield, R. J. The hotspot conversion paradox and the evolution of meiotic recombination. *Proc. Natl. Acad. Sci. USA* **94**, 8058–8063 (1997).
38. Baker, C. L. *et al.* Multimer formation explains allelic suppression of PRDM9 recombination hotspots. *PLoS Genet.* **11**, e1005512 (2015).
39. Kondrashov, A. S., Sunyaev, S. & Kondrashov, F. A. Dobzhansky-Muller incompatibilities in protein evolution. *Proc. Natl. Acad. Sci. USA* **99**, 14878–14883 (2002).
40. DePristo, M. A., Weinreich, D. M. & Hartl, D. L. Missense meanderings in sequence space. A biophysical view of protein evolution. *Nat. Rev. Genet.* **6**, 678–687 (2005).
41. Berg, I. L. *et al.* PRDM9 variation strongly influences recombination hot-spot activity and meiotic instability in humans. *Nat. Genet.* **42**, 859–863 (2010).
42. Patwa, Z. & Wahl, L. M. The fixation probability of beneficial mutations. *J. R. Soc. Interface* **5**, 1279–1289 (2008).
43. Uecker, H. & Hermisson, J. On the fixation process of a beneficial mutation in a variable environment. *Genetics* **188**, 915–930 (2011).
44. Meredith, R. W. *et al.* Impacts of the Cretaceous terrestrial revolution and KPg extinction on mammal diversification. *Science* **334**, 521–524 (2011).
45. Tavaré, S., Marshall, C. R., Will, O., Soligo, C. & Martin, R. D. Using the fossil record to estimate the age of the last common ancestor of extant primates. *Nature* **416**, 726–729 (2002).
46. Keller, G. *et al.* Chicxulub impact predates the K-T boundary mass extinction. *Proc. Natl. Acad. Sci. USA* **101**, 3753–3758 (2004).
47. Gingerich, P. D. Environment and evolution through the Paleocene-Eocene thermal maximum. *Trends Ecol. Evol.* **21**, 246–253 (2006).
48. Prothero, D. R. The late Eocene-Oligocene extinctions. *Annu. Rev. Earth Planet Sci.* **22**, 145–165 (1994).
49. Beard, K. C., Krishtalka, L. & Stucky, R. K. First skulls of the early Eocene primate *Shoshonius cooperi* and the anthropoid-tarsier dichotomy. *Nature* **349**, 64–67 (1991).
50. Kay, R. F., Ross, C. & Williams, B. A. Anthropoid origins. *Science* **275**, 797–804 (1997).
51. Williams, B. A., Kay, R. F. & Kirk, E. C. New perspectives on anthropoid origins. *Proc. Natl. Acad. Sci. USA* **107**, 4797–4804 (2010).
52. Jablonski, N. G. The evolution of the tarsiid niche in *Tarsiers* (eds Wright, P. C., Simons, E. L. & Gursky, S.) 35–49 (Rutgers University Press, 2003).
53. Jablonski, N. G. Primate homeland: Forests and the evolution of primates during the Tertiary and Quaternary in Asia. *Anthropol. Sci.* **113**, 117–122 (2005).
54. Merker, S. *et al.* Control region length dynamics potentially drives amino acid evolution in tarsier mitochondrial genomes. *J. Mol. Evol.* **79**, 40–51 (2014).
55. Haq, B. U., Hardenbol, J. & Vail, P. R. Chronology of fluctuating sea levels since the Triassic. *Science* **235**, 1156–1167 (1987).
56. Hall, R. Southeast Asia's changing palaeogeography. *Blumea* **54**, 148–161 (2009).
57. Evans, B. J. *et al.* Monkeys and toads define areas of endemism on Sulawesi. *Evolution* **57**, 1436–1443 (2003).
58. Merker, S. *et al.* *Tarsius wallacei*, a new tarsier species from central Sulawesi occupies a discontinuous range. *Int. J. Primatol.* **31**, 1107–1122 (2010).
59. Baudat, F. *et al.* PRDM9 is a major determinant of meiotic recombination hotspots in humans and mice. *Science* **327**, 836–840 (2010).
60. Groeneveld, L. F., Atencia, R., Garriga, R. M., Vigilant, L. & Mailund, T. High diversity at PRDM9 in chimpanzees and bonobos. *PLoS ONE* **7**, e39064 (2012).
61. Ponting, C. P. What are the genomic drivers of the rapid evolution of PRDM9? *Trends Genet.* **27**, 165–171 (2011).
62. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98 (1999).
63. Rice, P., Longden, I. & Bleasby, A. EMBOSS: the European molecular biology open software suite. *Trends Genet.* **16**, 276–277 (2000).
64. Goujon, M. *et al.* A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res.* **38**, W695–W699 (2010).
65. Camacho, C. *et al.* BLAST+. Architecture and applications. *BMC bioinformatics* **10**, 421 (2009).
66. Villesen, P. FaBox: an online toolbox for fasta sequences. *Mol. Ecol. Notes* **7**, 965–968 (2007).
67. Guindon, S. & Gascuel, O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biol.* **52**, 696–704 (2003).
68. Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. jModelTest 2. More models, new heuristics and parallel computing. *Nat. Methods* **9**, 772 (2012).
69. Stöver, B. C. & Müller, K. F. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC bioinformatics* **11**, 7 (2010).

70. Rambaut, A. *FigTree Version 1.3.1*. Available at <http://tree.bio.ed.ac.uk/software/figtree>. (Accessed: 1st December 2014) (2009).
71. Massingham, T. & Goldman, N. Detecting amino acid sites under positive selection and purifying selection. *Genetics* **169**, 1753–1762 (2005).

Acknowledgements

This work was supported by grants (ZI568/6-1 and ZI568/6-2) from the Deutsche Forschungsgemeinschaft (DFG, <http://www.dfg.de>) allocated to H.Z.

Author Contributions

H.Z. and C.D. conceived the study; S.H. performed experiments; S.H. analysed data; H.Z. and D.P.-F. contributed reagents, materials and analysis tools; C.D. and S.M. provided samples; S.H. and C.D. wrote the manuscript; H.Z., S.M. and D.P.-F. revised the manuscript.

Additional Information

Accession codes: The DNA sequences have been deposited in GenBank under the accession codes: KU948327-KU948367.

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Heerschop, S. *et al.* The pioneering role of *PRDM9* indel mutations in tarsier evolution. *Sci. Rep.* **6**, 34618; doi: 10.1038/srep34618 (2016).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016