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Inferred L/M cone opsin polymorphism of ancestral tarsiers sheds dim light on the origin of anthropoid primates

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Tarsiers are small nocturnal primates with a long history of fuelling debate on the origin and evolution of anthropoid primates. Recently, the discovery of M and L opsin genes in two sister species, *Tarsius bancanus* (Bornean tarsier) and *Tarsius syrichta* (Philippine tarsier), respectively, was interpreted as evidence of an ancestral long-to-middle (L/M) opsin polymorphism, which, in turn, suggested a diurnal or cathemeral (arrhythmic) activity pattern. This view is compatible with the hypothesis that stem tarsiers were diurnal; however, a reversion to nocturnality during the Middle Eocene, as evidenced by hyper-enlarged orbits, predates the divergence of *T. bancanus* and *T. syrichta* in the Late Miocene. Taken together, these findings suggest that some nocturnal tarsiers possessed high-acuity trichromatic vision, a concept that challenges prevailing views on the adaptive origins of the anthropoid visual system. It is, therefore, important to explore the plausibility and antiquity of trichromatic vision in the genus *Tarsius*. Here, we show that Sulawesi tarsiers (*Tarsius tarsier*), a phylogenetic out-group of Philippine and Bornean tarsiers, have an L opsin gene that is more similar to the L opsin gene of *T. syrichta* than to the M opsin gene of *T. bancanus* in non-synonymous nucleotide sequence. This result suggests that an L/M opsin polymorphism is the ancestral character state of crown tarsiers and raises the possibility that many hallmarks of the anthropoid visual system evolved under dim (mesopic) light conditions. This interpretation challenges the persistent nocturnal–diurnal dichotomy that has long informed debate on the origin of anthropoid primates.

1. Introduction

Tarsiers are an enduring source of fascination [1], both because of their many unique features and because they occupy a central position in the primate phylogenetic tree. The genus *Tarsius*—represented today by a handful of species within insular Southeast Asia—belongs to a relict lineage with an origin approximately 56 Ma [2]. Accordingly, tarsiers are not only allocated to their own family (Tarsiidae) and superfamily (Tarsioidea), but also to their own infraorder (Tarsiiformes). Debate over the phyletic position of tarsiiformes is longstanding [3], but recent phylogenies have unequivocally united tarsiers with anthropoid primates (monkeys, apes and humans) in the semiorder Haplorhini [4,5]. Thus, the functional and behavioural ecology of tarsiers has the potential to inform hypotheses focused on the origin of anthropoid primates [6].

The prevailing view of anthropoid origins emphasizes a fundamental shift from nocturnal to diurnal foraging behaviours, perhaps at a tarsier-like body size of 100 g [6]. Evidence for this view stems in part from derived attributes of the anthropoid visual system. Traits such as highly convergent orbits, high concentrations of retinal cones and ganglion cells, and extreme cortical magnification of foveal regions of the retina are all associated with enhanced visual acuity under diurnal conditions. Yet, the strength of this evidence is weakened in part by living tarsiers, which, though nocturnal, share many of these same

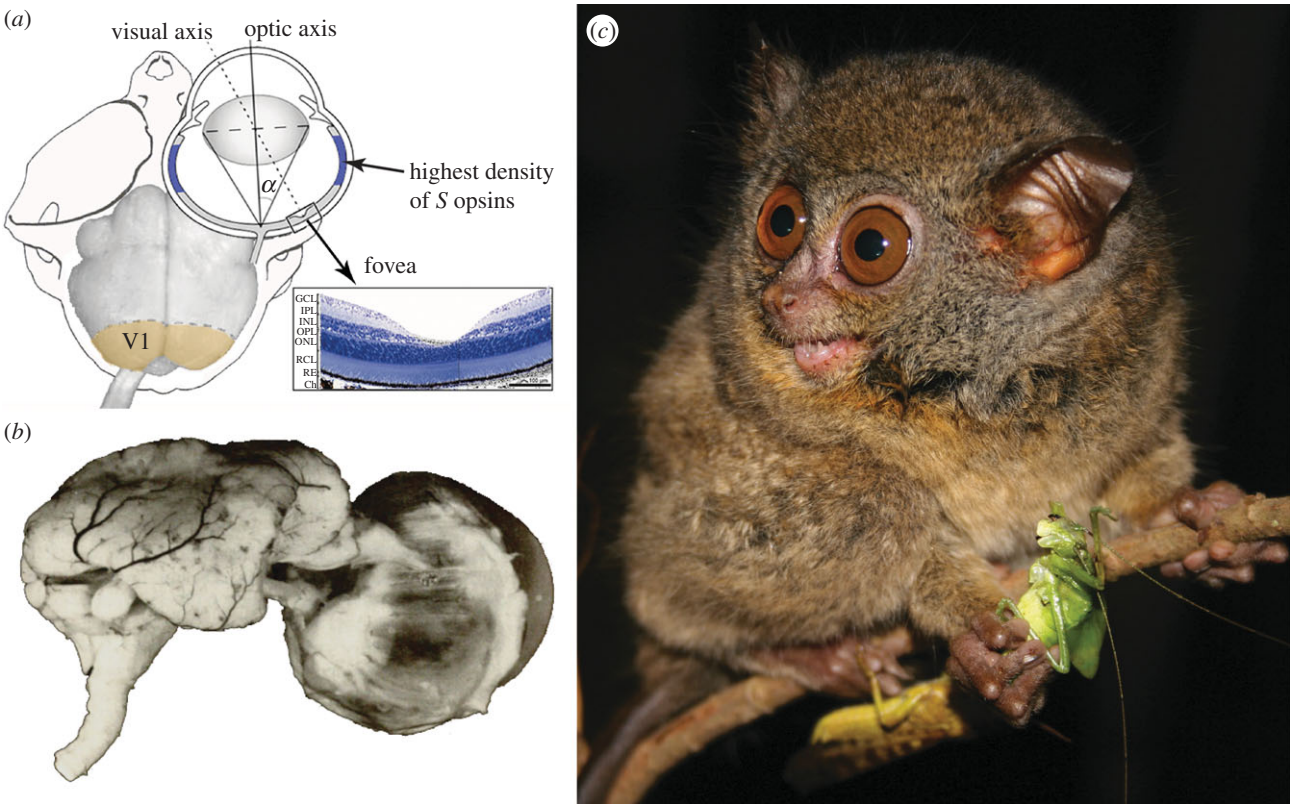


Figure 1. (*a*) The skull, eye and brain of *Tarsius bancanus*. At 21%, tarsiers have proportionally more primary visual cortex (V1) than any other primate [9]; this degree of cortical magnification is associated with foveal acuity and colour vision. The foveal pit of *Tarsius spectrum* (=*T. tarsier*) is shown in histological cross section. A curious feature of the retina is the high density of S opsins in the periphery (shown in blue). Redrawn from Hendrickson *et al*. [7], Ross [8] and Collins *et al*. [9]. (*b*) Heinrich Sprankel's preparation of the eye and brain of *T. bancanus* [11] illustrates the comparable volume of the two structures [12]; thus, the eyes of *T. bancanus* are enormous, both in absolute size and in proportion to the size of the 120–134 g animal. Polyak [13] concluded that the eye size relative to body size of tarsiers is unmatched by any mammal. (*c*) A Sulawesi tarsier (*Tarsius lariang*) on the verge of consuming an orthopteran insect (photograph by Stefan Merker, reproduced with permission).

traits. For example, tarsiers possess a fovea, a macula lutea and a central retina with relatively high densities of cones ([7–10]; figure 1*a*). These features have long provoked an important question: are these character traits convergent in tarsiers and anthropoids, and, therefore, unrelated to diurnality, or are they synapomorphic retentions from a diurnal stem haplorhine?

The consensus view—that stem haplorhines were diurnal and that this heritage is partly obscured by a reversion to nocturnality in tarsiers [3]—helps account for one of the most striking aspects of the tarsier visual system: their outsized eyes (figure 1*b*; [6]). It has long been suggested that the extreme ocular hypertrophy of tarsiers is related to the absence of a tapetum lucidum (the structure that results in the phenomenon of 'eye shine'; [14]). Most nocturnal mammals, including lorises and lemurs, possess a tapetum lucidum because it improves visual sensitivity at low light levels. Accordingly, the enlarged eyes of tarsiers are often interpreted as a compensatory trait that functions to improve visual sensitivity, and thus further evidence of a visual system that is secondarily adapted to nocturnality [14–16].

(a) Tarsiers and trichromatic colour vision

Many primates exhibit a colour vision polymorphism that results from allelic variation of the single-locus long-to-middle (L/M) wavelength opsin gene on the X chromosome. Females that are heterozygous for the gene have trichromatic vision, whereas all other individuals possess dichromatic vision. The discovery in 1999 of discrete M and L opsin genes in two sister species, *Tarsius bancanus* (Bornean tarsier) and *Tarsius syrichta* (Philippine tarsier), respectively, is provocative because it suggests that alleles of the L/M opsin gene existed in the ancestral population [17]. The heterozygous females of this population probably possessed foveate trichromatic colour vision [17], and it follows that their activity pattern resembled those of living primates with a similar combination of traits. Thus, the last common ancestor of *T. bancanus* and *T. syrichta* is hypothesized to have had a diurnal or cathemeral (arrhythmic) activity pattern [17]. This hypothesis is compatible with the view that stem tarsiers were diurnal; however, the evolution of hyper-enlarged orbits during the Middle Eocene [18] and Middle Miocene [19] predates the divergence of *T. bancanus* and *T. syrichta* in the Late Miocene [20,21]. Extraordinarily large eyes are strongly suggestive of activity under dark (scotopic) light levels. Taken together, the implication of these findings—that some nocturnal tarsiers possessed high-acuity trichromatic vision—is profound, as it challenges prevailing views on the origin of the anthropoid visual system. It is, therefore, important to explore the plausibility and antiquity of trichromatic colour vision in the genus *Tarsius*. We do so here by drawing attention to a more basal species from Sulawesi, *Tarsius tarsier* (figure 1*c*).

As demonstrated for New World (platyrrhine) monkeys [22], the simultaneous presence of L/M opsin alleles in an ancestral population, and thus the potential for trichromatic colour vision, can be inferred if the coding regions of the genes cluster together irrespective of phylogenetic distance,

while intron and synonymous-site trees reflect species relationships. Therefore, if a site tree reconstructed using non-synonymous nucleotide sequences results in *T. tarsier* clustering more closely with either *T. syrichta* (in the case of the L opsin gene) or *T. bancanus* (in the case of the M opsin gene), then this result can be interpreted as evidence of an ancestral polymorphism and fixation, versus independent derivation, of the L/M opsin gene in the various lineages. We test this prediction here by comparing the L/M opsin genes of extant tarsiers.

2. Material and methods

(a) Study subjects and data collection

Genomic DNA was extracted from the buccal cells (Gentra Puregene Buccal Cell Kit, Qiagen) of *T. tarsier* (one male, two females; $n = 5$ X chromosomes) housed at the Ueno Zoo, Tokyo. The precise alpha taxonomy of each individual is uncertain because individual provenances in Sulawesi are unknown; however, long tail tufts are an unambiguous character trait of the *T. tarsier* complex [23,24] (see the electronic supplementary material, figure S1). We also collected buccal DNA from a female Philippine tarsier at the Ueno Zoo (*T. syrichta*; $n = 2$ X chromosomes). This sampling protocol conformed to the guidelines of the Ueno Zoo and was approved by the Institutional Animal Care and Use Committee of Dartmouth College (approval no. 11-11-02AT). The genomic DNA of a male Bornean tarsier (*T. bancanus*; $n = 1$ X chromosome) was extracted from a fibroblast cell line [25].

For each tarsier, we conducted PCR amplification and sequencing of a continuous section (approx. 4 kb) of the L/M opsin gene containing exon 3, intron 3, exon 4, intron 4 and exon 5. This gene section contains three nucleotide sites that are critical for spectral tuning of the photopigment, encoding residue 180 in exon 3 and residues 277 and 285 in exon 5 (the three-sites rule [26–28]). In contrast to platyrrhine monkeys, polymorphisms are reported for strepsirrhine primates and tarsiers at site 285 only [17,29]; however, variation at any of the three sites can shift photopigment sensitivity and we, therefore, examined each. PCR primers were designed to amplify overlapping segments in this region based on conservation of nucleotide sequences among previously reported L/M opsin genes of other primates (see the electronic supplementary material, figure S2).

PCRs were carried out in volumes of 50 µl containing 1.5 units of Ex Taq polymerase hot start version (Takara, Tokyo, Japan) with 1× Ex Taq Buffer, 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 1 µM of the forward and reverse primers, and *ca* 1 ng of the tarsier genomic DNA. Cycles were set at 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 30–120 s. Pure water was used as the template for the negative control in every reaction. The amplified DNA fragments were purified using UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Solana Beach, CA, USA). Both strands of the purified DNA samples were directly sequenced using Applied Biosystems model 3130 automatic sequencer with Big Dye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems Japan, Tokyo) and the PCR primers and other primers designed inside the amplified region.

The PCR products of the L/M opsin gene from female samples were subjected to DNA cloning to distinguish the X-chromosomal allelic sequences. The amplified DNA fragments were cloned into pGEM-T vectors (Promega, Madison, WI, USA) and were sequenced as described above. We confirmed their nucleotide sequences in duplicate experiments.

Nucleotide sequences were aligned using CLUSTAL W and alignment was refined visually. We used MEGA5 [30] to conduct phylogenetic analyses of aligned nucleotide sequences. The number of nucleotide differences was corrected for multiple substitutions by the Juke–Cantor formula [31]. The number of nucleotide substitutions per synonymous site (d_S) and per non-synonymous site (d_N) for each pair of sequences was estimated using the Nei–Gojobori model [32]. In our calculations, gap sites were removed in a pairwise fashion. Phylogenetic trees were constructed following the neighbour-joining method [33] and their reliabilities were evaluated via bootstrap analyses with 1000 replications.

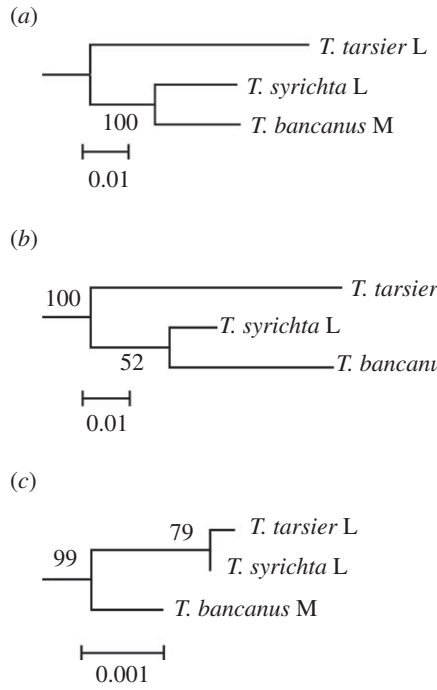
3. Results

Distinct sequence types were found and a sequence from each species—*T. tarsier* L no. 1; *T. syrichta* L no. 1 and *T. bancanus* M (see the electronic supplementary material, figure S3)—was deposited in the DDBJ/EMBL/GenBank Database under accession nos AB675925, AB675926 and AB675927, respectively. The four sequence types of *T. tarsier* and both sequence types of *T. syrichta* were identical in amino acid composition at the three critical tuning sites, having Ala, Tyr and Thr at residues 180, 277 and 285 (designated Ala/Tyr/Thr), respectively. This amino acid composition is in agreement with a previous report on *T. syrichta* [17], which inferred an L opsin with a peak spectral absorbance (λ_{max}) of 553 nm based on the three-sites rule [27,28]. The amino acid composition of *T. bancanus* (Ala/Tyr/Ala) also agrees with a previous report [17], which inferred an M opsin with a λ_{max} of 538 nm [27,28].

We reconstructed phylogenic trees of the tarsier L/M opsin genes based on introns 3 and 4 (figure 2*a*) and the synonymous (figure 2*b*) and non-synonymous sites (figure 2*c*) of the exons 3, 4 and 5. The intron and synonymous trees agreed well with the established phyletic relationships of tarsiers. The L opsin gene of *T. tarsier* was cast as the most basal lineage, whereas the M opsin gene of *T. bancanus* and the L opsin gene of *T syrichta* were closely related. We used the first sequence listed for each species (see the electronic supplementary material, figure S3) when constructing the tree, but the choice of sequences did not affect the tree topology and reliability (data not presented). Intraspecific differences in the four sequence types of *T. tarsier* ranged from 0.1 to 1.0 per cent of the intron sites and 0 to 1.5 per cent of the synonymous exon sites. The two sequences of *T. syrichta* differed in 0.4 per cent of the intron sites and were identical in the synonymous sites. In contrast to the phylogenies based on the intron and synonymous sites, the non-synonymous-site phylogeny grouped the L opsin genes of *T. tarsier* and *T. syrichta* with each other, to the exclusion of the M opsin gene of *T. bancanus* (figure 2*c*).

4. Discussion

We have replicated an earlier report of M opsin genes in Bornean tarsiers (*T. bancanus*) and L opsin genes in Philippine tarsiers (*T. syrichta*) [17]. In addition, we have shown that Sulawesi tarsiers (*T. tarsier*) possess an L opsin gene. In accordance with the species phylogeny, the L opsin gene of *T. tarsier* fell outside the *T. bancanus*–*T. syrichta* cluster in the intron and the synonymous-site trees. However, in the non-synonymous-site tree, the L opsin gene of *T. tarsier* clustered with the L opsin gene of *T. syrichta*. This result suggests that alleles of the L/M opsin gene were present in the last



	Tta L	Tsy L	Tba M
Tta L		3418	3414
Tsy L	262		3484
Tba M	259	124	

	Tta L	Tsy L	Tba M
Tta L		138.4	138.4
Tsy L	13		138.5
Tba M	11	6	

	Tta L	Tsy L	Tba M
Tta L		434.6	434.6
Tsy L	0		434.5
Tba M	1	1	

Figure 2. Phylogenetic trees of the tarsier L/M opsin gene using (*a*) introns 3 and 4, (*b*) the synonymous sites of exons 3, 4 and 5, and (*c*) the non-synonymous sites of exons 3, 4 and 5. For each species by species comparison, the length of the nucleotide sequence (bp) used and the number of nucleotides differing between each pair are presented, above and below the diagonal, respectively, in the tables adjacent to each phylogeny. The bootstrap probability is given at each node. We determined the phylogenetic root for tree (*a*) using the common marmoset L/M opsin gene (Ensembl Marmoset release 70, Genome assembly C_jacchus3.2.1 (GCA 000004665.1) (http://www.ensembl.org/Callithrix_jacchus/Info/Index), X:140703132–140704540 for intron 3 and X:140704707–140706490 for intron 4) and for trees (*b*) and (*c*) using three spectral alleles of the common marmoset L/M opsin gene (GenBank AB046546–AB046548) as the out-group for each tree. Scale bars indicate the number of nucleotide substitutions per site.

common ancestor of crown tarsiers and that polymorphic trichromatic vision persisted until the divergence of *T. syrichta* and *T. bancanus* in the Late Miocene [20,21]. This pattern is consistent with homogenization of the alleles in the introns and synonymous sites by recombination, and natural selection (balancing selection) against homogenization of the non-synonymous sites to maintain the spectral polymorphism of the alleles.

An alternate interpretation of our results is that the last common ancestor of crown tarsiers was uniformly dichromatic and similar to all living tarsiers studied to date. If so, based on the intron and synonymous-site trees, the M opsin gene of *T. bancanus* is most plausibly a derivation of the L opsin gene of *T. syrichta* by a nucleotide substitution at codon 285. Yet, the L/M opsin genes of each species are otherwise identical at non-synonymous sites throughout exons 3–5 (i.e. in amino acid sequence). It is highly improbable that the only substitution present among the approximately 435 possible non-synonymous sites, and among the three possible directions of nucleotide change, occurred at a spectral tuning site by chance. Given the strict conservation of the amino acid sequence, the chance of a non-synonymous mutation being selectively neutral is doubtful because the fixation probability of a neutral mutation is generally low. Thus, if the M opsin gene is derived from the L opsin gene and not fixed via random drift alone, strong positive selection should have favoured the M opsin gene in Bornean tarsiers. This raises the question of why a shift from deutan to protan dichromacy, with an intermittent stage of polymorphic vision including trichromacy, was adaptive for tarsiers inhabiting the rainforests of Borneo versus those of Sulawesi or the Philippines.

(a) Implications for anthropoid origins

Our results indicate that the last common ancestor of crown tarsiers possessed polymorphic trichromatic vision. Such a finding would normally suggest a diurnal activity pattern because high-acuity trichromatic vision is strongly associated with bright (photopic) conditions; however, this Middle Miocene population of tarsiers [21,34] postdates or is coincident with the existence of hyper-enlarged orbits in the genus ([18,19]; figure 3). The evolution of extraordinary eye size is functionally incompatible with diurnality and inconsistent with cathemerality, an activity pattern that favours intermediate ocular morphologies [35–38]. This paradoxical tandem of polymorphic trichromatic vision and enlarged eyes is, therefore, puzzling, and it raises the possibility that dim (mesopic) light rather than a particular diel pattern was an important factor during tarsier evolution. Indeed, recent findings suggest that full moonlight and twilight are sufficient for cone-mediated colour vision yet dim enough to favour enlarged eyes for greater sensitivity [39].

Our findings call attention to a light environment that is under-appreciated in the literature on tarsier visual adaptations and, by extension, the origin of anthropoid primates [40]. Accordingly, some speculation is warranted on why early tarsiers were plausibly active under mesopic light levels. Evidence suggests that Sundaland was exceedingly rainy during the Eocene and Miocene [41,42]; and rain is expected to dampen the foraging efficiency of a sit-and-wait ambush predator by immobilizing invertebrate prey [43] or masking prey movement amid broadband noise [44]. Accordingly, we suggest that rain-induced sensory deprivation led tarsiers to expand their foraging activities under mesopic light levels, conditions that favoured high-acuity stereoscopic vision for detecting

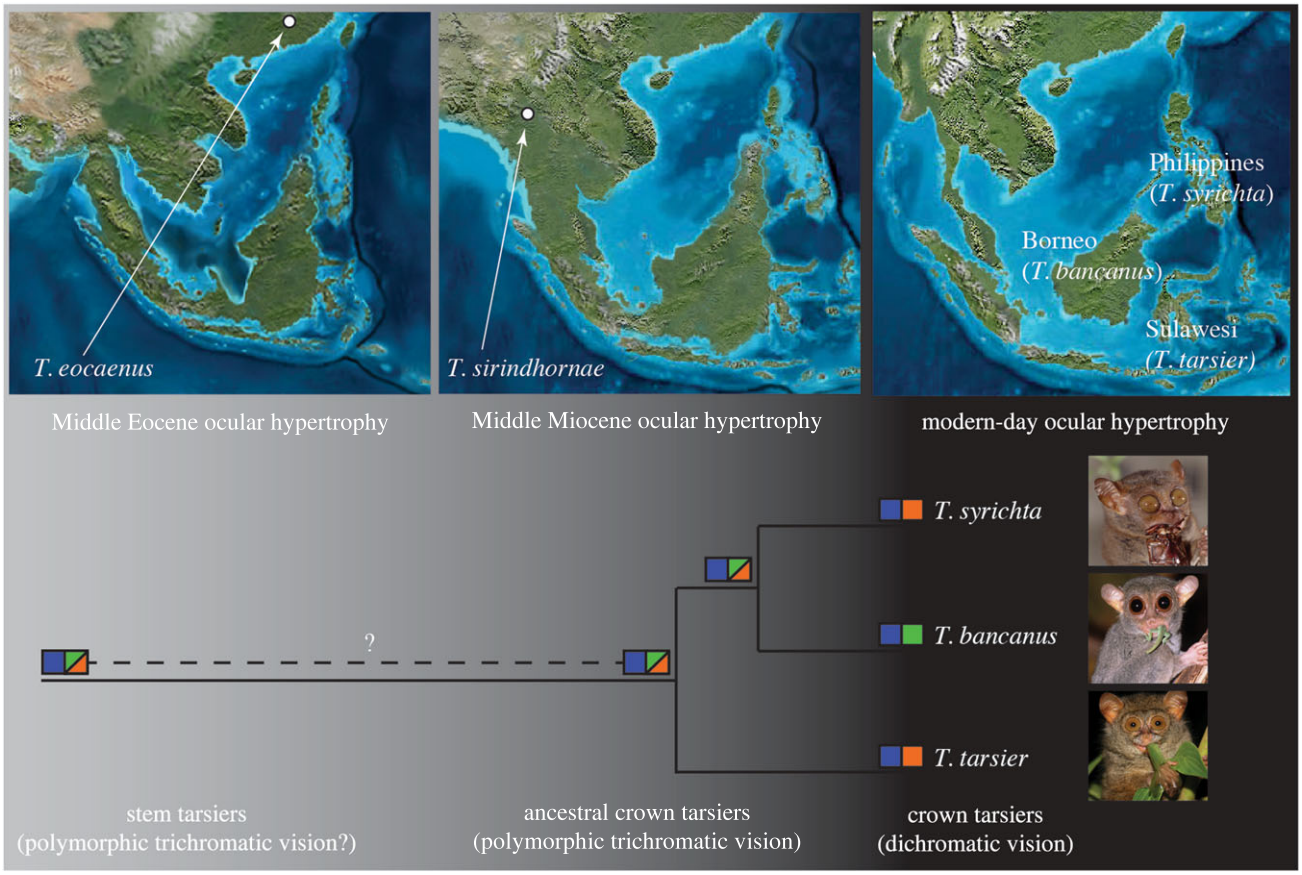


Figure 3. The ocular hypertrophy of *Tarsius eocaenus* [18] and *Tarsius sirindhornae* [19] predates or is coeval with the inferred L/M cone opsin polymorphism in the last common ancestor of crown tarsiers. Squares depict discrete opsins and their spectral sensitivities. The combination of hyper-enlarged eyes and trichromatic vision during the Middle Miocene suggests an activity pattern that includes dim (mesopic) light levels (indicated in grey). By contrast, contemporary tarsiers are active mainly under dark (scotopic) light levels (indicated in black). Palaeogeographic maps depict Sundaland during the Late Eocene (35 Ma) and Early Miocene (20 Ma) (Ron Blakeley and Colorado Plateau Geosystems, Inc.). The photographs of *T. syrichta* (Daniel Heuclin), *T. bancanus* (David Haring) and a member of the *T. tarsier* complex (=*T. lariang*; Stefan Merker) are reproduced with permission.

motionless prey [45]. Perhaps compellingly, polymorphic trichromatic vision is associated with the consumption of green foods at low light levels [46]; and, it could be advantageous for discriminating green generalist orthopteran insects [47], an important food item for many tarsiers ([48,49]; figure 1*c*). In addition, tarsiers are themselves vulnerable to predation, especially from twilight-active felids and viverrids [50–53]; trichromatic vision is likely to be advantageous for detecting these russet-coloured predators [54]. Mesopic light, then, could have exerted a strong selective tension on the visual system of tarsiers, favouring an evolutionary compromise between sensitivity and high-acuity polymorphic trichromatic vision. Testing the plausibility of this hypothesis will hinge on several factors, which we detail in the supporting information.

A potential problem with our hypothesis is that extant tarsiers remain active under twilight and bright moonlight [55,56], conditions that appear to be mesopic [39]. Thus, it is unclear why tarsiers were independently released from the selective advantages of trichromatic vision during the past 5 Myr. Although the possibility of allelic fixation owing to stochastic processes such as genetic bottlenecks cannot be ruled out, the parallel loss of polymorphic trichromatic vision in at least three species could be associated with diminished rainfall across insular southeast Asia during the Pleistocene [41]. Reduced rainfall is expected to favour a greater commitment to nocturnality among tarsiers and a reversion to audition as the primary sensory modality. Our suggestion that the selective advantages of visually mediated foraging are relaxed under drier conditions is consistent with field observations in Sulawesi. During the dry season, tarsiers preferentially attend to the rustling of invertebrate prey in leaf litter [48], a broadband acoustic cue [57] to which at least one species is exceedingly sensitive [1].

The tarsiid niche is an exemplary model of long-term stability [58], and we suggest that tarsiers achieved dietary stasis by vacillating between scotopic and mesopic light levels, conditions that would alternately stress their unique auditory and visual systems. Furthermore, we suggest that the canon of haplorhine visual adaptations—convergent orbits, high concentrations of retinal cones and ganglion cells, and extreme cortical magnification of foveal regions of the retina—are perhaps better interpreted as mesopic traits that were preadaptive to a diurnal activity pattern. Although speculative, a shared mesopic origin for the last common ancestor of crown tarsiers and stem anthropoids has the advantage of parsimony over the prevailing hypothesis: that the attributes of the anthropoid visual system evolved when stem haplorhines invaded a diurnal niche, a view that entails a full diurnal–nocturnal reversal during tarsier evolution. The present findings challenge this persistent nocturnal–diurnal dichotomy and shed new (dim) light on the evolution of haplorhine and anthropoid primates.

The sampling protocol conformed to the guidelines of the Ueno Zoo and was approved by the Institutional Animal Care and Use Committee of Dartmouth College (approval no. 11-11.02AT).

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