Euarchontan Opsin Variation Brings New Focus to Primate Origins

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Associate editor: Connie Mulligan

Abstract

Debate on the adaptive origins of primates has long focused on the functional ecology of the primate visual system. For example, it is hypothesized that variable expression of short- (SWS1) and middle-to-long-wavelength sensitive (M/LWS) opsins, which confer color vision, can be used to infer ancestral activity patterns and therefore selective ecological pressures. A problem with this approach is that opsin gene variation is incompletely known in the grandorder Euarchonta, that is, the orders Scandentia (treeshrews), Dermoptera (colugos), and Primates. The ancestral state of primate color vision is therefore uncertain. Here, we report on the genes (*OPN1SW* and *OPN1LW*) that encode SWS1 and M/LWS opsins in seven species of treeshrew, including the sole nocturnal scandentian *Ptilocercus lowii*. In addition, we examined the opsin genes of the Central American woolly opossum (*Caluromys derbianus*), an enduring ecological analogue in the debate on primate origins. Our results indicate: 1) retention of ultraviolet (UV) visual sensitivity in *C. derbianus* and a shift from UV to blue spectral sensitivities at the base of Euarchonta; 2) ancient pseudogenization of *OPN1SW* in the ancestors of *P. lowii*, but a signature of purifying selection in those of *C. derbianus*; and, 3) the absence of *OPN1LW* polymorphism among diurnal treeshrews. These findings suggest functional variation in the color vision of nocturnal mammals and a distinctive visual ecology of early primates, perhaps one that demanded greater spatial resolution under light levels that could support cone-mediated color discrimination.

Key words: **color vision, sensory ecology, *Caluromys*, *Dendrogale*, Euarchonta, *Ptilocercus*, *Tupaia*.**

Introduction

The color vision of mammals is based on the expression of two opsin genes (*OPN1SW* and *OPN1LW*) that encode short- (SWS1) and middle-to-long-wavelength sensitive (M/LWS) photopigments. Some variant of this dichromatic phenotype (Peichl 2005) is the probable ancestral state of therian mammals (Wakefield et al. 2008) and every successive lineage, such as primates, that subsequently lost or gained opsin genes (Jacobs 2013; Meredith et al. 2013; Veilleux et al. 2013). Among primates, *OPN1LW* has differentiated into multiple alleles (lemurs, most New World monkeys) or paralogs (howler monkeys, Old World primates), resulting in spectrally shifted photopigments that confer allelic or routine trichromatic vision, respectively (Jacobs 2009; Kawamura et al. 2012). The M/LWS opsin variation that causes allelic trichromacy is widespread among primates (Tan et al. 2005; Melin et al. 2013) but unknown outside the order. Limited sampling, however, has precluded a formal comparative analysis of opsin genes in the grandorder Euarchonta, that is, the orders Scandentia (treeshrews), Dermoptera (colugos), and Primates. It is therefore challenging to infer the ancestral state of the primate visual system.

Opsin Sensitivity and Spatial Resolution

The peak spectral absorbance (λ_{max}) of opsins is sensitive to natural selection and varies in response to environmental

conditions and natural histories (Parry et al. 2004; Davies et al. 2012; Hunt and Peichl 2014). Crucially, functional variation of *OPN1SW* is widespread among mammals (Emerling et al. 2015). Mutations can disable function completely (Jacobs 2013) or cause large shifts in λ_{max}, conferring ultraviolet (UV) visual sensitivity (λ_{max} = 360 nm) to a mouse or blue sensitivity (λ_{max} = 444 nm) to a treeshrew, *Tupaia belangeri* (Jacobs and Neitz 1986; Jacobs et al. 2004).

The functional ecological significance of these spectral differences is uncertain, but a relationship with visual acuity has been reported. Douglas and Jeffery (2014) examined the ocular media of 38 mammalian species and found that UV transmission and sensitivity prevail in low-acuity visual systems. This result suggests that natural selection for greater visual acuity (spatial resolution) also favored UV-filtering ocular media and blue-sensitive SWS1 opsins. In theory, spectral convergence of the SWS1 and M/LWS opsins should enhance visual acuity by minimizing chromatic aberrations (Walls 1942; Thibos et al. 1990). This premise has particular relevance to primates, a lineage with exceptional levels of visual acuity (references in Moore et al. 2012; Moritz et al. 2014).

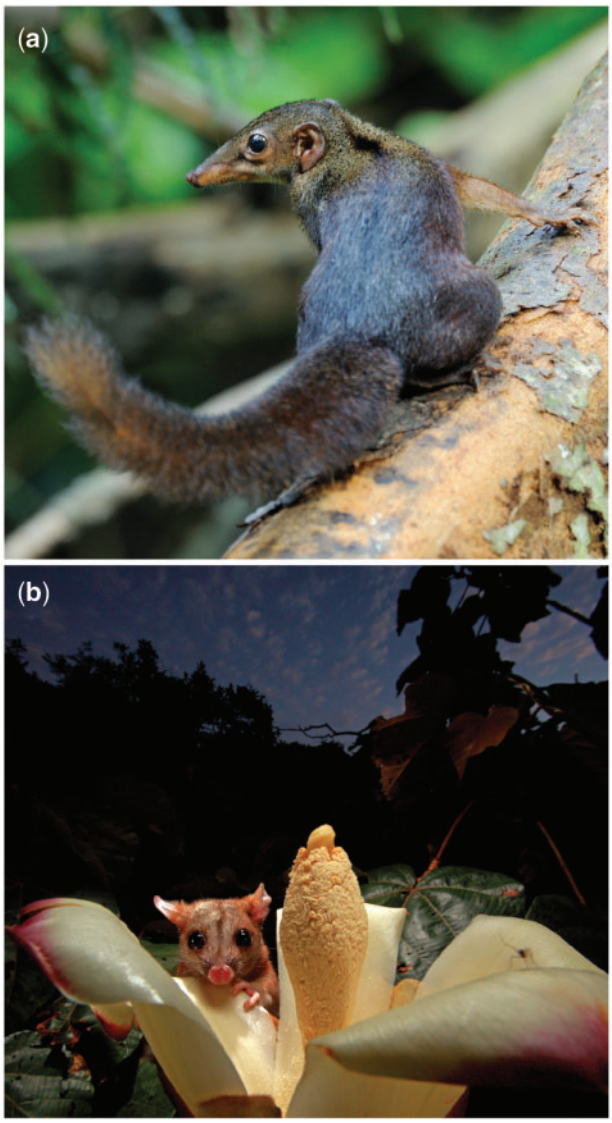

FIG. 1. Treeshrews [(*a*) *Tupaia tana*] and woolly opossums [(*b*) *Caluromys derbianus*] are phyletic and/or ecological analogues of ancestral primates, factors that invite study of their opsin genes. Panel (*b*) also depicts the pollination of balsa (*Ochroma pyramidale*) under twilight conditions [Kays et al. 2012]. Photographs by Wong Tsu Shi and Christian Ziegler, respectively, and reproduced with permission.

SWS1—a Lens on Primate Origins?

Degenerate *OPN1SW* opsin genes—resulting in monochromatic vision—are a common trait of nocturnal mammals, or those active under dark (scotopic) light conditions, such as fossorial, cave, or deep marine habitats (Jacobs et al. 1993; David-Grey et al. 2002; Zhao et al. 2009; Davies et al. 2011; Jacobs 2013; Meredith et al. 2013). At the same time, functional *OPN1SW* genes have been retained by purifying selection in some fruit bats (Wang et al. 2004) and nocturnal euarchontans (colugos: Moritz et al. 2013; primates: Kawamura and Kubotera 2004; Perry et al. 2007; Veilleux et al. 2013). Such differences are difficult to explain. Some authors view the dichromatic vision of nocturnal primates as evidence of evolutionary disequilibrium (Tan et al. 2005), whereas others propose an ecological function on the grounds that dim twilight or full moonlight is sufficient for cone-mediated color vision (Melin et al. 2012, 2013; Veilleux and Cummings 2012; Moritz 2015). This distinction between visual anachronism and adaptation is now a central topic in the debate on primate origins (Tan et al. 2005).

Thus, the functional preservation and λ_{max} of SWS1 opsins are tandem traits that can speak to the evolution and ecology of high-acuity color vision, a key derivation of primates (Ravosa and Savakova 2004; Cartmill 2012; Sussman et al. 2013). Here, we examine opsin gene variation across Euarchonta in order to explore how, when, and why enhanced visual acuity evolved. We believe that this comparative, integrated approach has the potential to inform hypotheses on the origin and evolution of primates.

Present Study

We report on the opsin genes of treeshrews (n = 7 species) and a woolly opossum (*Caluromys derbianus*) and analyze the gene sequences together with published data from other mammals. Treeshrews (fig. 1*a*) are sometimes described as "living models" of ancestral primates due to shared phyletic, morphological, and ecological affinities, albeit with a focus on locomotion (Tattersall 1984; Jenkins 1987; Martin 1990; Sargis 2004; Silcox et al. 2015; Li and Ni 2016). Similarly, *C. derbianus* (fig. 1*b*) is convergent toward primates in having a relatively large brain and eyes, small litters, and a slow life history. The arboreal agility and diet of this marsupial are therefore enduring topics in the debate on primate origins (Rasmussen 1990, 2002; Schmitt and Lemelin 2002; Gebo 2004; Sargis et al. 2007). The monophyly of treeshrews, colugos, and primates in Euarchonta is firmly established; however, the internal structure of Euarchonata is debated. Three phylogenetic hypotheses exist: 1) a sister-group relationship between

treeshrews and primates (fig. 2a), 2) a sister-group relationship between colugos and primates, that is, Primatomorpha (fig. 2b); and, 3) both treeshrews and colugos as sister to primates, that is, Sundatheria (fig. 2c).

To estimate the λ_{max} of extant and ancestral SWS1 opsins, we identified the amino acids at ten spectral tuning sites of the *OPN1SW* genes, of which two—Tyr86 and Val93, respectively—primarily determine sensitivity in the violet-blue (400–450 nm) region of the spectrum. We tested for purifying selection as a function of activity pattern; and, in the case of pseudogenes, estimated the antiquity of functional loss by comparing rates of substitution in coding versus noncoding regions of the gene. We estimated the λ_{max} of extant and ancestral M/LWS opsins on the basis of three spectral tuning sites in *OPN1LW*; and finally, we explored the potential for allelic trichromatic vision in treeshrews.

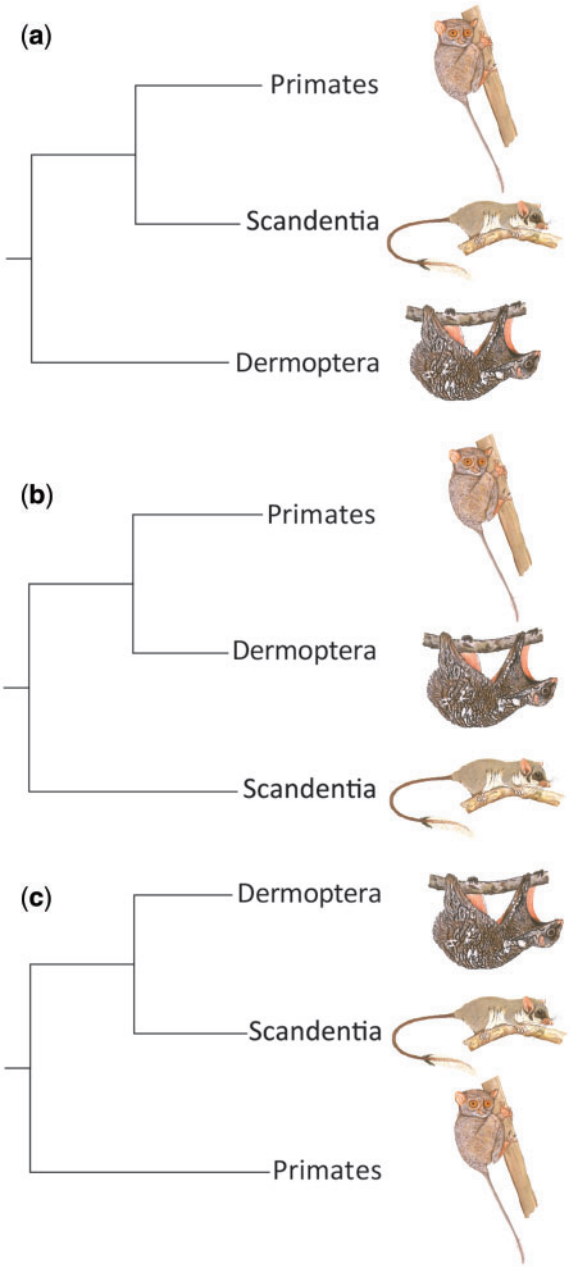

Fig. 2. The internal structure of Euarchonta is debated and revolves around three hypotheses: (*a*) a sister-group relationship between treeshrews and primates (Wible and Covert 1987; Kay et al. 1992), (*b*) a sister-group relationship between colugos and primates (Primatomorpha; Janečka et al. 2007; Meredith et al. 2011), or (*c*) both treeshrews and colugos as sister to primates (Sundatheria; Murphy et al. 2001; Sargis 2002; Bloch et al. 2007; O'Leary et al. 2013). Illustrations © The Sabah Society, reproduced with permission.

Results

We succeeded in sequencing each exon of *OPN1SW* in *Caluromys* and all tupaiids, excepting exon 2 of *Dendrogale melanura* and *Tupaia montana*. Our initial *OPN1SW* polymerase chain reactions (PCRs) failed for all exons of *P. lowii*, thus necessitating the shotgun genome sequencing approach to reconstruct this gene sequence. Partial sequencing of *OPN1LW* was successful for all species—including *P. lowii*—although two of eight individuals of *T. montana* failed repeatedly, which we attributed to low DNA quality. Whole-genome sequence reads of *P. lowii* are deposited in the NCBI Sequence Read Archive (study accession no. SRP064536) and representative sequences of each species in GenBank (supplementary table S1, Supplementary Material online).

The most likely phylogenetic trees for Euarchonta based on the synonymous and intron sites of *OPN1SW* are consistent with the concept of Primatomorpha, a sister group relationship of primates and colugos (figs. 2b and 3). Further, the most likely phyletic relationship of opsin genes within sampled treeshrews agrees well with previously reported phylogenies (Luckett 1980; Roberts et al. 2011). Some study species are omitted from the intron tree due to the absence of overlapping sequence data.

Signatures of Selection

The coding sequences of *OPN1SW* in the woolly opossum (*C. derbianus*) and diurnal treeshrews (*D. melanura*, *Tupaia* spp.), and the coding sequences of *OPN1LW* in all species, were free of indels (insertions/deletions), nonsense mutations, and premature stop codons, indicating strict conservation and functional preservation (fig. 4; supplementary fig. S1, Supplementary Material online). Greater amino acid divergence was present in *OPN1SW* (mean amino acid difference per sequence/total amino acids analyzed, SE over 1,000 bootstrap replicates: 36.53/349 = 10.47% divergence, 2.86) than in *OPN1LW* (15.46/228 = 6.78% divergence, 2.13; note: for trichromatic species, only the LWS allele/paralogue was assessed). However, this difference did not reach statistical significance (Fisher's Exact Test, $P = 0.14$).

We detected a series of frameshift deletions in the *OPN1SW* sequence of *P. lowii*. We artificially corrected the frameshifts by filling the contig gaps with the consensus bases from other taxa, and repeated the translation. While correcting the frameshifts improved the amino acid alignment, when *P. lowii* is compared with the five *Tupaia* species with exon 2 sequences, a total of ten nonsynonymous mutations out of 117 nonsynonymous sites in exon 2 (gaps sites were omitted) were altered by to amino acids unique to *Ptilocercus* in the alignment, and two stop codons occurred within the

Ptilocercus translation (fig. 5; supplementary fig. S1*a*, Supplementary Material online). There were also nine synonymous mutations out of 34 possible synonymous sites. Among *Tupaia*, which uniformly possessed intact *OPN1SW*, there were zero nonsynonymous differences and three synonymous differences. The divergence of *OPN1SW* of *P. lowii* explains why repeated attempts to amplify this gene via PCR were unsuccessful.

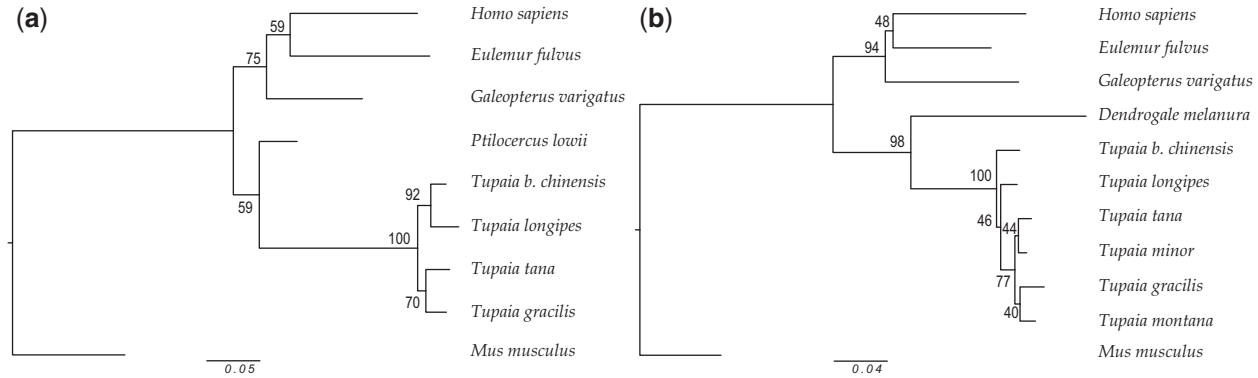

Fig. 3. Phylogenies of Euarchonta based on intron (*a*) and synonymous (*b*) sites of the *OPN1SW*. The evolutionary history was inferred by using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances are in units of the number of differences per site (Nei-Gojobori 1986). The analysis involved 9 (*a*) and 11 (*b*) nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 774 (*a*) and 354 (*b*) positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

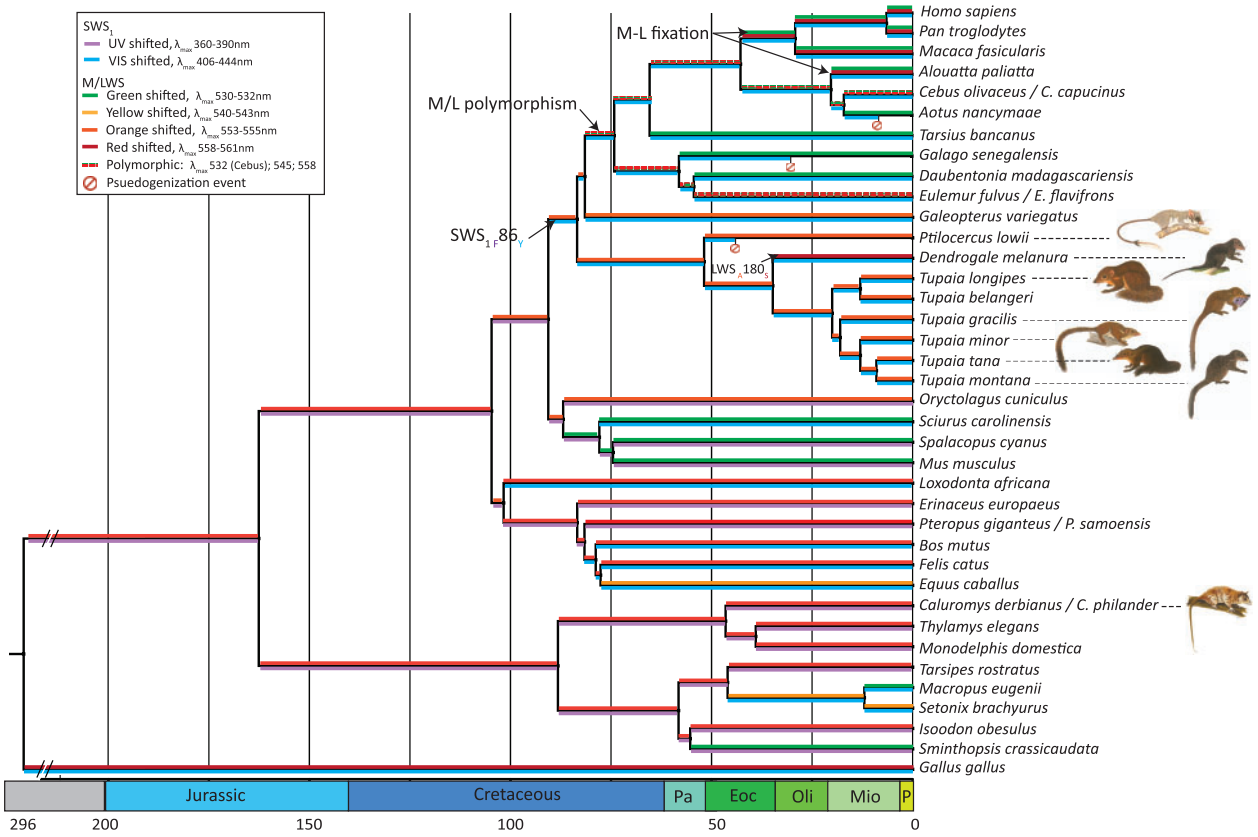

Fig. 4. Phylogenetic relationships and divergence dates for Euarchonta and outgroups were based on TimeTree (Hedges et al. 2006; accessed June 2015) and published estimates (Prideaux and Warburton 2010; Roberts et al. 2011; Fabrae et al. 2012; Song et al. 2012). Branch colors correspond with the presence and spectral tuning of opsin photopigments. Pseudogenization events are marked with a diagonally bisected circle. The inferred shift from UV to blue sensitivity in the SWS1 opsin of the ancestral euarchontan is marked with an arrow, along with the amino acids proposed to be responsible. Dashed branches indicate opsin polymorphism. The geological time scale is abbreviated as Pa, Paleocene; Eoc, Eocene; Oli, Oligocene; Mio, Miocene; P, Plio/Pleistocene. Treeshrew art © The Sabah Society, reproduced with permission.

For *OPN1SW*, purifying selection ($d_N < d_S$; $P < 0.05$) was favored over the null hypothesis of neutrality ($d_N = d_S$) for the majority of pairwise comparisons among the species in our complete data set. The notable exceptions to this pattern were the pairwise comparisons including *P. lowii*. Here, substitution rates were consistent with neutrality for the majority (25/35) of possible pairwise comparisons and nearly all (25/26) comparisons with eutherian mammals (supplementary table S2, Supplementary Material online). Taken together with the alignment results, these data indicate ancient pseudogenization of the *OPN1SW* in Ptilocercidae.

For *OPN1LW*, purifying selection ($d_N < d_S$; $P < 0.05$) was favored over the null hypothesis of neutrality ($d_N = d_S$) for the majority of paired comparisons (supplementary table S3, Supplementary Material online). Among tupaiids, neutrality was not uniformly rejected; however, the recent diversification of *Tupaia* (Roberts et al. 2011) and the short regions examined here (exons 3 and 5 only) suggest an underpowered analysis. Consistent with this interpretation is our finding that purifying selection is evident for the *OPN1LW* of *T. belangeri*, for which more sequencing data were available.

Pseudogenization of the SWS1 Opsin Gene of Ptilocercus

The ratio of the rate of nonsyonymous substitutions (d_N) to presumably neutral substitutions at synonymous and intron sites (d_{S+I}) among tupaiids, following divergence from other euarchontans, reveals moderate functional constraint (mean d_N/d_{S+I} = 0.0394/0.1060 = 0.37). *Dendrogale melanura* and *T. montana* are excluded from this analysis because we lacked sequence data for exon 2, the only region of reconstruction for *P. lowii*. The mean neutral mutation rate, *k*, of the tupaiid lineage, following the split with other euarchontans, was low (0.1060/83.43 Ma = 1.27×10^{-9} per site per year). A total of 277 nonsynonymous and 649 synonymous and intron sites were used in this analysis. The sequence data from *P. lowii* are excluded from our calculations of the neutral mutation rate because the shorter sequence recovered for this species would have unnecessarily constrained the data set used in the analysis.

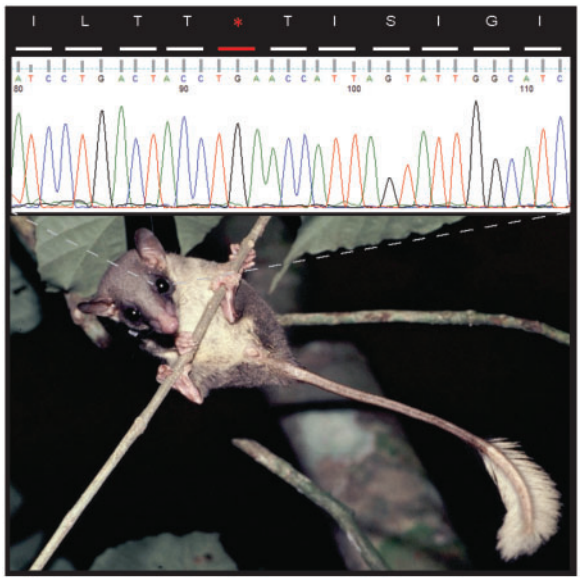

Fig. 5. *Ptilocercus lowii* and a corresponding partial amino acid sequence to demonstrate one of several stop codons in the coding region of the *OPN1SW* pseudogene. Photograph by Annette Zitzmann, reproduced with permission.

We added *P. lowii* to the data set to calculate the d_N of Ptilocercidae. The divergence of this lineage from Tupaiidae was previously estimated to be 60.19 Ma (Roberts et al. 2011). The d_N value for *P. lowii* (0.0977) is relatively high, and the f_N (fraction of neutral substitutions; 0.0977/0.0207 = 4.72) far exceeds 1, revealing an unusually high substitution rate at sites than would have been nonsynonymous in a functional gene. This result, together with the low rate of neutral evolution observed for tupaiid SWS1 opsins, prohibits an accurate estimation of the timing of pseudogenization in the lineage that gave rise to *P. lowii*. For example, using the methods of Chou et al. (2002), we calculate a date that precedes the estimated divergence of this species from other treeshrews [$t_1 = ((0.0977/1.27 \times 10^{-9}) - (0.37*60.19\ \text{Ma}))/(1 - 0.37) = 86.75$ Ma]. Analyses using alternative calculations of the timing of pseudogenization based on nucleotide position in the codon (Yokoyama et al. 2014) fare no better. Thus, the pseudogenization of *OPN1SW* cannot be dated with precision, but it is clear that the antiquity of monochromatic vision is great within Ptilocercidae.

Spectral Sensitivities of Opsins

OPN1SW Opsin Gene

Spectral tuning sites are invariant in Tupaiidae (supplementary fig. S1*a*, Supplementary Material online). The λ_{max} of the opsin is therefore expected to resemble that of *T. belangeri*, which is calculated at 444 nm (based on electroretinogram (ERG) flicker photometry, Jacobs and Neitz 1986) or 428 ± 15 nm (based on microspectrophotometry, Petry and Harosi 1990). In contrast, the spectral tuning sites of *C. derbianus* (Phe86, Thr93) predict UV sensitivity (λ_{max} = 360 nm), a result that agrees well with earlier findings from South American marsupials (Hunt et al. 2009; Palacios et al. 2010).

OPN1LW Opsin Gene

The three spectral tuning sites—180:A, 277:Y, 285:T—are invariant in *Ptilocercus* and *Tupaia* and we detected no intraspecific polymorphisms (supplementary figs. S1*b* and S2, Supplementary Material online). The inferred λ_{max} is therefore 555 nm (Yokoyama et al. 2008), a result that agrees well with ERG flicker photometry- and microspectrophotometry-based findings (*T. glis*: Tigges et al. 1967; *T. belangeri*: Jacobs and Neitz 1986; Petry and Harosi 1990). The three-site composition of *D. melanura* differed ($_A180_S$) from other treeshrews, predicting a λ_{max} shifted by 5–7 nm to 560–562 nm. Lastly, the three-site composition of *C. derbianus* (AYT; λ_{max} = 555 nm) agrees with earlier findings from South American marsupials (Hunt et al. 2009; Palacios et al. 2010).

Ancestral States

Our phylogenies based on *OPN1SW* intron and synonymous sites are consistent with the concept of Primatomorpha, and

we found that the ancestral state of all living and extinct (crown) euarchontans was unambiguous at eight of ten tuning sites: Phe46, Phe49, Thr52, Tyr86, Ser90, Ala114, Leu116, and Ser118. Maximum parsimony (MP) was ambiguous at two sites: Ile/Pro/Thr/Val93 and Ala/Asn97. Accordingly, we used maximum likelihood (ML) to estimate Thr as the ancestral amino acid at site 93, with shifts to Val in Scandentia, Ile in Dermoptera, and Pro in Primates. We reconstructed Ala as the ancestral amino acid at site 97, with retention in Primates and independent shifts to Asn in Dermoptera and Scandentia. Ancestral states based on a sister-group relationship between treeshrews and primates (fig. 2a) or Sundatheria (fig. 2c) are identical or nearly so, respectively. In the case of Sundatheria, the MP analysis is unambiguous concerning Ala97, but site 86 was ambiguous as either Phe or Tyr. In the ensuing ML analysis, Phe is reconstructed as the ancestral state. In all ML analyses, the posterior probabilities are greater than 0.9. Lastly, the ancestral state of the M/LWS opsin gene in crown Euarchonta is unambiguous for each spectral tuning site (Ala180, Tyr277, Thr285) in all configurations.

Discussion

Our primary conclusions are 4-fold: 1) Frameshift deletions in *OPN1SW* have an ancient origin in the lineage that gave rise to *Ptilocercus lowii*. The resulting phenotype (cone monochromacy) unites *P. lowii* with numerous mammals active under dark (scotopic) conditions (Jacobs 2013). 2) At the same time, we detected a signature of purifying selection in *OPN1SW* of *C. derbianus*, a nocturnal opossum. The preservation of a UV-sensitive SWS1 opsin in *C. derbianus* challenges the proposed incompatibility of nocturnality and dichromatic vision (Tan et al. 2005), and is consistent with other findings (Kawamura and Kubotera, 2004; Perry et al. 2007; Zhao et al. 2009). 3) The UV-sensitivity of SWS1 opsins was likely abolished at the base of Euarchonta, a result that partly diminishes the value of arboreal marsupials for modeling the visual ecology of early primates (Rasmussen 1990; Rasmussen and Sussman 2007). 4) A selective aversion to UV sensitivity in crown Euarchonta and Primates suggests a distinctive visual ecology or photic niche, that is, one that demanded greater spatial resolution under light levels that could support cone-mediated color discrimination (Emerling et al. 2015).

Such findings advance our understanding of primate origins by resolving and extending the antiquity of blue-sensitive shifts in the visual systems of Euarchonta. Previously, Carvalho et al. (2012) suggested that a substitution from phenylalanine to tyrosine at site 86 of *OPN1SW* evolved at the base of Primates. Our findings support this hypothesis and show that $_{Phe}86_{Tyr}$ is the most parsimonious ancestral state of crown Euarchonta. Site 93 is also critical to λ_{max} and our findings implicate Thr93 as the ancestral state. Combinations of Tyr86 and Thr93 exist in two Australian marsupials, the Tamar wallaby (*Macropus eugenii*) and quokka (*Setonix brachyurus*), and confer blue sensitivity (λ_{max} = 424 nm; Deeb et al. 2003; Arrese et al. 2005). Independent shifts at site 93 occurred in Scandentia ($_{Thr}93_{Val}$), Dermoptera ($_{Thr}93_{Ile}$), and Primates ($_{Thr}93_{Pro}$). Further, tandem shifts of $_{Phe}86_{Tyr}$ and $_{Thr}93_{Val}$ evolved in at least one diurnal rodent, *Sciurus carolinensis* (Carvalho et al. 2006). The arboreal proclivities of these eutherians distinguish them from the Tamar wallaby and quokka, raising the possibility that the spectral tuning afforded by Tyr86 and Thr93 are unfavorable in an arboreal milieu. Alternatively, amino acid divergence at other sites, or incompatible interactions, may have favored multiple, independent replacements of Thr93 in the latter group following the $_{Phe}86_{Tyr}$ transition. Such incompatibilities have been observed, for example, in mutant pigments created by in vitro by site-directed mutagenesis (Carvalho et al. 2012).

Future genome sequence of additional euarchontans and other mammals may resolve debate between Primatomorpha and Sundatheria. Sundatheria would implicate either Tyr86 (as above) or Phe86 as the ancestral state of crown Euarchonta. In this latter scenario, our ancestral state reconstruction indicates a transition of $_{Phe}86_{Tyr}$ in the common ancestor of Scandentia and Dermoptera and the retention of Phe86 in Primates. It follows, then, that the aye-aye (*Daubentonia madagascariensis*) is either the sole extant primate to have retained the ancestral *OPN1SW* sequence at site 86 (λ_{max} = 406 nm; Carvalho et al. 2012) or that a subsequent reversion of this amino acid occurred in the aye-aye lineage.

Implications for Primate Origins

We conclude that the color vision of ancestral primates is based on functional SWS1 and M/LWS opsins with estimated λ_{max} values of approximately 424 and 555 nm, respectively. Although precise λ_{max} values can be difficult to predict from sequence data alone (Hauser et al. 2014), blue sensitivity is strongly implicated by the inferred amino acid composition (Tyr86, Ser90, Thr93) of *OPN1SW*. Significantly, this phenotype is incompatible with the UV sensitivity of *C. derbianus* and impoverished color vision of *P. lowii*, and it follows that color vision in these species is unsuitable for evaluating the visual ecology of ancestral primates (pace Rasmussen 1990; Sargis 2004).

At the same time, differences in the nocturnality of *C. derbianus* and *P. lowii* are instructive, revealing the practical limits of the concept. The visual ecology of *C. derbianus* includes twilight (fig. 1b) and daylight activities (Reid 1997), whereas *P. lowii* is strictly nocturnal (Lyon 1913; Le Gros Clark 1926; Lim 1967; Gould 1978) and averse to moonlight (Emmons 2000). It is therefore likely that the dichromatic vision of ancestral primates is indicative of occasional or regular activity under dim (mesopic) to daylight (photopic) conditions.

The blue sensitivity of the SWS1 opsin is also telling, suggesting activities that demanded enhanced visual acuity (Douglas and Jeffrey 2014). The nature of these activities is uncertain, but the elimination of UV sensitivity could suggest that nectar was a supplemental, rather than primary, resource (cf. Sussman and Raven 1978; Gómez and Verdú 2012; Sussman et al. 2013). A diet premised on floral nectar unites most nocturnal mammals with UV-sensitive SWS1 opsins, perhaps to discriminate UV-reflecting flowers under

dim light (Fleming et al. 2009). Our supposition does not refute the importance of nectar during primate evolution, but a shift to blue sensitivity suggests a diet that commanded greater spatial resolution than necessary for discriminating flowers (Veilleux and Kirk 2014).

This premise is supported by the convergent pseudogenization of *OPN1SW* in many primate taxa (lorisids; *Allocebus*, *Cheirogaleus*, *Phaner*, and *Aotus*) and other mammals (Jacobs 2013; Veilleux et al. 2013). As with *P. lowii*, a blue-sensitive SWS1 opsin is the inferred ancestral state among euarchontans, and it is tempting to speculate that natural selection favored pseudogenization of violet–blue sensitive (400–450 nm) SWS1 opsins more readily than a reversion to UV sensitivity (360–400 nm). It follows that most *OPN1SW* pseudogenes were formerly blue-sensitive and that pseudogenization is the result of shifts to scotopic, lunarphobic conditions rather than nocturnality per se, a pattern exemplified by flying squirrels (Carvalho et al. 2006). The advantage of cone monochromacy for detecting arboreal foods with high luminance contrasts (flowers, exudates; Moritz 2015) is an argument against relaxation, suggesting that blue-sensitive SWS1 opsins are deleterious under scotopic conditions. On balance, UV- or luminance-sensitive vision appears indispensable to a nocturnal diet dependent on floral resources or exudates.

Taken together, our results suggest that the visual ecology of ancestral primates required enhanced acuity under light levels that can support color vision. This interpretation is agnostic on the debate over nocturnal or diurnal origins (Heesy and Ross 2004; Tan et al. 2005), but it does stress the role of vision under dim light. Dim (mesopic) light is perhaps a better focus of debate than crude categorical concepts such as nocturnality and diurnality (Prugh and Golden 2014). Our results also speak to the importance of enhanced visual acuity at the base of Euarchonta and Primates. Many extant euarchontans have cone-dense retinae and high visual acuity, controlling for eye size, relative to other mammals (Müller and Peichl 1989; Müller et al. 1989; Veilleux and Kirk 2014). The evolution and retention of blue-sensitive SWS1 opsins is a fresh line of evidence to support the hypothesized importance of visual acuity to early primates for resolving chromatically cryptic foods or tracking fast moving prey (Allman 1977; Cartmill 1992, 2012; Crompton 1995).

Future Directions

Retention of intact opsin genes in nocturnal mouse lemurs (*Microcebus*; Cheirogaleidae) invites study of their visual ecology. The visually-mediated foraging of *Microcebus* in an arboreal milieu, particularly under mesopic conditions, is perhaps the best living model for testing hypotheses on primate origins. Further, the absence of the M/LWS polymorphism in *Microcebus* and other strongly nocturnal primates (Tan et al. 2005) unites them with all tupaiid (diurnal) treeshrews (present study) and fruit bats (Wang et al. 2004), including day-active species (Melin et al. 2014). Together, these results reaffirm that allelic trichromatic vision is exclusive to primates with significant daytime activity. The visual acuity and corresponding visual ecology of these species are therefore germane to determining the critical combination of diet and light that favored the evolution of trichromatic vision.

To explore this premise briefly, we note the measured or estimated visual acuities of *Tupaia* (1.2–4.7 cycles/deg; Petry et al. 1984; Veilleux and Kirk 2014) and *Microcebus murinus* (4.9 c/deg; Dkhissi-Benyahya et al. 2001) and compare them with *Eulemur flavifrons* (3.8–5.1 c/deg; Veilleux and Kirk 2009), a cathemeral lemur with an M/LWS polymorphism (Veilleux and Bolnick 2009). The comparable visual acuities of *Microcebus* and *Eulemur* are instructive, suggesting that a combination of 1) blue-sensitive SWS1 opsins; 2) a visual acuity of approximately 5 or more c/deg; and, 3) increasing levels of photopic activity, are contributing factors to the diversification of M/LWS opsins. Although speculative, this conjecture invites testing. Comparative study of the visual ecologies of *Microcebus* and *Eulemur* could shed insight on the critical light thresholds that favored the evolution and diversification of primate opsins.

Materials and Methods

Study Species and Sample Collection

Treeshrews comprise a single order, Scandentia, in which two families are recognized: Ptilocercidae, containing one nocturnal species, *Ptilocercus lowii*, and Tupaiidae, containing 19 diurnal species in four genera (*Dendrogale*, *Anathana*, *Urogale*, and *Tupaia*). The present study examines the opsin gene sequences of *P. lowii*, *D. melanura*, and five species of *Tupaia* (table 1). Treeshrews were live-captured in locally-made wire-mesh cage traps (external dimensions: 25 × 25 × 40 cm) at six lowland and two montane forest sites in Borneo (years: 2002–2010, as reported in Wells et al. 2007, 2011; with supplemental captures in years 2011–2012; table 1).

We collected tissue samples (2-mm ear biopsies) under two conditions: animals were 1) lightly anesthetized (diethyl ether or isoflurane; years: 2002–2010) or 2) restrained by hand in a cloth bag with a small opening for accessing the ear (years: 2011–2012). This latter procedure has the advantage of speed (<5 min of human handling). The tissue samples were stored in 99% ethanol (years: 2002–2010) or RNAlater stabilization reagent (Ambion; years: 2011–2012). All animals were released at capture site after individual marking with subcutaneous PIT tags. Recaptured animals showed no adverse effects from our protocol, which was approved by the Institutional Animal Care and Use Committee of Dartmouth College (protocol no. 11-06-07AT).

The Central American (Derby's) woolly opossum (*C. derbianus*) is a small, arboreal (200–400 g) didelphid marsupial (Bucher and Hoffmann 1980). We biopsied muscle from a single specimen accessioned (KU 164643) in the University of Kansas Natural History Museum. This individual, a male, was found dead by RMT on December 26, 2006 at the La Selva Biological Station, Costa Rica (10°26′N, 84°0′W). Muscle tissue was preserved initially in 95% ethanol and later frozen. This protocol was approved by the Institutional Animal Care and Use Committee of the University of

Table 1. Taxonomy, Natural History, and Sampling Localities of Bornean Treeshrew Species.

Taxonomy			Natural History[b]		Sampling Localities[a]									
					Lowland							Montane		
		Species	Common Name	Activity Pattern	Foraging Height	Danum Valley	Luasong	Monggis	Poring	Tawau	Tumba-lang	Mesilou	Mount Alap	
Ptilocercidae	Ptilocercus	 *Ptilocercus lowii*	Pen-tailed treeshrew	Nocturnal	Arboreal			x						
	Dendrogale	 *Dendrogale melanura*	Smooth-tailed treeshrew	Diurnal	Terrestrial								x	
	Tupaia	 *Tupaia gracilis*	Slender treeshrew	Diurnal	Terrestrial	x	x	x	x	x	x		x	
Tupaiidae		 *Tupaia longipes*	Long-footed treeshrew	Diurnal	Terrestrial	x	x	x	x	x	x			
		 *Tupaia minor*	Lesser treeshrew	Diurnal	Arboreal	x		x	x	x	x			
		 *Tupaia montana*	Montane treeshrew	Diurnal	Terrestrial							x	x	
		 *Tupaia tana*	Large treeshrew	Diurnal	Terrestrial	x	x	x	x	x	x			

Note.—Illustrations © The Sabah Society, reproduced with permission.

[a]Lowland sites: Poring, Kinabalu National Park (06°02′N, 116°42′E); Danum Valley Conservation Area (4°57′N, 117°48′E); Tawau Hills National Park (04°23′N, 117°53′E); Luasong Field Centre (4°36′N, 117°23′E); Monggis (06°13′N, 116°45′E); Tumbalang (06°08′N, 116°53′E). Montane sites: Mesilou, Mount Kinabalu (6°00′N, 116°35′E); Mount Alap, Crocker Range National Park (5°49′N, 116°20′E).

[b]Emmons 2000, Wells et al. 2004, and Wiens et al. 2008 detail the natural history and foraging ecology of these species.

Table 2. Primers and Annealing Temperatures Used in Polymerase Chain Reactions to Amplify Partial *OPN1SW* and *OPN1LW* Opsin Genes in Treeshrews (Genera: *Dendrogale*, *Ptilocercus*, *Tupaia*) and Woolly Opossum (Genus: *Caluromys*).

Gene	Genus	Region	Forward (5′ to 3′)	Reverse (5′ to 3′)	T_{anneal} °C
OPN1SW	*Ptilocercus*	Exon 2 and 3	GCC TAA AGG CTT CAA GCA GGG GG	TGC CAC AGG TCT GGT GAT AGG CT	64
	Dendrogale	Exon 1	GTA CCA CCT TGC CCC TGT CT	CCT TTC CCC TGC AGT ACC T	58
		Exons 2 and 3	GGT GAT AGG CTG GTC ATT GG	CCC AGC AGC TGA GAG TAG GA	60
		Exon 4	GCT CAG CAG CAG GAG TCA G	TTC ATG AAG CAG TAG ATG ATG G	58
		Exon 5	ATG AGG CGT CTT TTC CAC AC	TGG CTT TGT TAG CAG GAA GG	60
	Tupaia	Exon 1	AAG AAC ACA ATC GGC TTT GG	GTG GCG TAG TGT CCT TTG CT	58
		Exons 2 and 3	CAG CCC AGC CTA GAA GTT TG	CCT GAC CCT CTC AAG ACC AC	62
		Exon 4	TAA TGA ATA AGG CGG GGT GA	CTG ACA AGT CAC TGG CGA GA	58
		Exon 5	ATG AGG CGT CTT TTC CAC AC	TGG CTT TGT TAG CAG GAA GG	60
	Caluromys	Exons 1–3	TGT CAG GGG ATG AGG AGT TC	GGC CAC ACG AGC ACT GTA	62
OPN1LW	*Dendrogale, Ptilocercus*	Exon 3	CAT CAC GGG GCT CTG GTC	CTG CTC CAA CCA AAG ATG G	60
		Exon 5	AGG CTG AGA AGG AGG TGA CA	GTG GCA CTT TTG GCG AAG TA	60
	Tupaia	Exon 3	TAC CTG TCT GCT CTT CCC TGT AG	GGT CCT AAA TGA GCC ACC CTT AC	64
		Exon 5	TGC ACT GTC CCT GTC TCA CCC AG	GGC CTG CCG ATG GCC TTA CTT AC	68

Kansas (protocol no. 132-06) and follows the guidelines of the American Society of Mammalogists (Sikes et al. 2011).

DNA Extraction, Amplification, and Sequencing

We extracted genomic DNA (DNeasy Blood and Tissue Kit, Qiagen) from eight females in each of the five species of *Tupaia* (n = 16 X chromosomes per species; n = 80 X chromosomes in total across *Tupaia*). We chose animals from across trapping sites in attempt to maximize the genetic diversity in our sample. Due to low capture rates, our study was limited to two individuals of *P. lowii* (sexes unknown) and one individual of *D. melanura* (a male).

We conducted a BLAST search of the *T. belangeri* (*chinensis*) whole-genome sequence (European Nucleotide Archive; WGS Sequence Set: ALAR00000000.1) using human opsin gene sequences to identify sequences of *OPN1SW* and *OPN1LW*, which were used to design PCR primers within introns (Primer3; http://primer3.sourceforge.net, last accessed January 16, 2016) (table 2). To estimate the functionality and peak spectral absorbance (λ_{max}) of the SWS1 opsin, we amplified the spectral tuning sites in tandem with the entire coding region of the *OPN1SW*. Ten amino acid sites on exon 1—especially sites 86 and 93—are critical to λ_{max} (Shi et al. 2001; Yokoyama et al. 2006; Hunt et al. 2007; Carvalho et al. 2012). We examined the *OPN1SW* of one individual per species as this autosomal gene has low levels of functional polymorphism (Shimmin et al. 1998).

λ_{max} of the M/LWS opsin is governed by five amino acid sites spanning exons 3–5 (Yokoyama et al. 2008), of which three are variable among primates (exon 3: 180; exon 5: 277, 285) and responsible for intra- and interspecific variation in color vision (Hiramatsu et al. 2005; Kawamura et al. 2012). We therefore amplified exons 3 and 5 of all scandentians in our sample in order to estimate λ_{max} and to explore the potential for intraspecific variation.

Initial PCR amplification was poor for the two most basal treeshrews (*P. lowii* and *D. melanura*). We therefore used sequences from *Tupaia* to design primers based on highly conserved regions of each gene. This approach was successful with the exception of the *OPN1SW* of *P. lowii* (see below). The primers for *C. derbianus* (table 2) were designed from the conserved regions of the *OPN1SW* in a wide range marsupials: *Isoodon obesulus*, *Monodelphis domestica*, *Setonix brachyurus*, *Sminthopsis crassicaudata*, *Tarsipes rostratus*, and *Thylamys elegans* (supplementary table S1, Supplementary Material online). The *OPN1LW* sequence of *C. philander* was supplied by Professor David M. Hunt (University of Western Australia).

PCRs were carried out in 25 µl containing 12.5 µl of iProof HF 2x Master Mix (0.04 U/µl DNA polymerase, 2X HF buffer, 400 µM dNTPs [each]; Bio-Rad), 1.0 µM each of the forward and reverse primers, and 70–200 ng template DNA. Pure water was used as a negative control in each experiment. We carried out PCRs at 98 °C for 3 min followed by 35 cycles of 98 °C for 10 s, T_{anneal} (annealing temperature) for 30 s, 72 °C for 30 s, and concluding with 72 °C for 5 min. T_{anneal} was optimized for each gene/primer pair combination (table 2).

We purified amplicons with the QIAquick PCR Purification Kit or, when nontarget sequences were also amplified, the QIAquick Gel Extraction Kit (Qiagen). Purified DNA was sequenced directly using an Applied Biosystems Model 3100 automatic sequencer with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits v3.0 with AmpliTaq DNA polymerase. Primers designed for PCR were used during sequencing reactions. DNA sequences were assembled and edited manually using Sequencher v 5.1 (Gene Codes). Heterozygous sites were scored manually using a single-letter nucleic acid code (IUPAC nomenclature) when chromatograms displayed peaks of nearly equal height.

Whole-Genome Sequencing

Amplification of the *OPN1SW* of *P. lowii* failed repeatedly. We therefore pursued massive parallel sequencing using whole-genome shotgun sequencing and a reference-assisted assembly strategy. We prepared an Illumina sequencing library (Meyer and Kircher 2010), and sequenced it to low (~3–5×) coverage on a single lane of an Illumina HiSeq 2000 at the Penn State Genomics Core Facility. After quality control filtering, we formatted the short read data set as a BLAST database and queried the *OPN1SW* sequences of human and *Tupaia*, revealing reads that likely originated in the *OPN1SW* of *P. lowii*. We pairwise-aligned these candidate reads to the

coding regions of the human *OPN1SW* using Geneious version 5.6.7, and manually extended high-confidence local contigs of *P. lowii* by querying the ends for overlapping regions in the short read data set until they reached coverage gaps on either side. Using this strategy, we recovered a 569-bp region of the *OPN1SW* sequence including the complete sequence of exon 2. Our contig aligned to the corresponding human *OPN1SW* region with 77.1% pairwise identity, whereas it failed to align meaningfully to other human opsin genes, strongly implicating an origin in the *OPN1SW* of *P. lowii* rather than similar regions.

To verify the contig sequence, we PCR-amplified a region containing the complete exon 2 using the KAPA HiFi Hotstart PCR Kit according to the manufacturer's protocol. The resulting PCR product was purified with homemade Solid Phase Reversible Immobilization (SPRI) beads (Rohland and Reich 2012) and Sanger-sequenced from both ends using PCR primers on an Applied Biosystems 3730XL capillary system. We aligned the product (336 bp) with the short read contig containing exon 2; the two sequences were identical, confirming the fidelity of the SWS1 opsin gene sequence. The presence of premature stop codons and indels indicates pseudogenization and release from functional constraint. Degradation of this gene explains the failure of the initial experiments using conserved primers.

Opsin Sequence Analyses

For the *OPN1SW* (1,062 nt positions) and partial *OPN1LW* (exons 3–5; 692 nt positions) opsin genes, we constructed a multiple alignment with each species in our study, together with sequences from selected mammals (supplementary table S1, Supplementary Material online). We used congeners when species-level data for both opsins were unavailable. In the case of *Spalacopus cyanus*, sequence data for the *OPN1LW* were unavailable but the λ_{max} of the opsin is known (Peichl et al. 2005). Multiple individuals per species were included in the analysis of treeshrew *OPN1LW* opsin genes to assess the potential for polymorphism. We compiled and aligned nucleotide sequences using the Clustal W procedure in MEGA6 (Tamura et al. 2013) and refined the alignment manually. The coding regions were then translated into amino acid sequences.

To study exon-wide patterns of genetic drift and natural selection, we calculated the rates of nonsynonymous substitutions per nonsynonymous site (d_N) and synonymous (d_S) substitutions per synonymous site (Nei and Gojobori 1986) using MEGA6 (Tamura et al. 2013). All ambiguous positions were removed for each sequence pair. We used the Z-test of purifying selection to assess whether differences between d_N and d_S differed significantly from 0.

Spectral Tuning Sites and Ancestral Character States

Ancestral tuning sites of *OPN1SW* and *OPN1LW* were inferred using the MP algorithm in MEGA6 with a 70% site coverage cutoff (Tamura et al. 2013). When results were ambiguous, we used ML analysis, using the Jones–Taylor–Thornton matrix-based model and assumed uniform rates among sites to select the most likely candidate amino acid. We then used TimeTree (Hedges et al. 2006; accessed June 2015) and published divergence dates (Prideaux and Warburton 2010; Roberts et al. 2011; Fabrae et al. 2012; Song et al. 2012) to determine the most likely phylogenetic structure of Euarchonta and calculate divergence times. We tested all possible phylogenetic configurations of Euarchonta (i.e., phylogenetic structure following the Primatomorpha, Sundatheria, and treeshrew–primate sister group relationship hypotheses, respectively), as outlined in figure 2.

SWS1 Opsin Pseudogenization

The timing of pseudogenization of genes has been estimated in other studies by examining substitutions of nucleotides that would have been nonsynonymous in a functional gene, relative to the neutral mutation rate and calibrated against species divergence estimates (Chou et al. 2002; Stedman et al. 2004). We follow similar methods to estimate the loss of function of *OPN1SW* in *P. lowii*. Briefly, the nonsynonymous substitution rate in a functional gene is defined as the neutral mutation rate, k, multiplied by the fraction of neutral substitutions f_N. f_N is inversely related to the degree of functional constraint on a gene, where 1 = neutral evolution (no constraint) and small values indicate strong purifying selection. Following pseudogenization, $f_N = 1$ and the substitution rate increases to k, a property we used to estimate the timing of pseudogenization. We defined t_1 as the time of inactivation and t as the time of divergence between the taxon with the pseudogene and a related lineage with a functional orthologous gene. We estimated t_1 using the formula: $t_1 = (d_{N\ (pseudogene)}/k - f_N t)/(1-f_N)$. In this study, k = mean per site substitutions at synonymous and intron sites (d_{S+I}) among taxa/estimated divergence time. We combined intron data with synonymous data to increase the sample size. Although selective constraints on introns and synonymous sites might vary slightly, both are predominantly subject to neutral evolution. Analyses were conducted in PAML with codeml; d_N was calculated for coding sequences only, whereas d_{S+I} was calculated by concatenating coding sequences with the intron data set in which the nucleotides T and C were inserted before each intronic site using a custom Perl script. In this way, PAML treated the intron sites (x) as synonymous positions of Serine (TCx).

Supplementary Material

Supplementary figures S1 and S2 and tables S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

The authors dedicate this article to D.T. Rasmussen (1958–2014), whose work on *C. derbianus* motivated crucial aspects of this research. The authors acknowledge with gratitude the practical assistance of A. Fatah Bin Amir, A. Biun, R.M. Brown, M.S. Callahan, C.F. Danosi, L.H. Emmons, D.M. Hunt, R. Kays, M. Montague, C. Sendall, and F. Tuh Yit Yu. Collection and export of treeshrew tissues was approved by Sabah Parks, the Sabah Biodiversity Council, and the Economic Planning Unit of Malaysia (permit nos. JKM/

MBS.1000-2/2(26) and JKM/MBS.1000-2/3(30)). This work was supported by the Natural Sciences and Engineering Research Council of Canada (PDF-420810-2012 to A.D.M), the German Academic Exchange Service (DAAD; scholarship to K.W.), the Cramer Fund of the Department of Biological Sciences, Dartmouth College (to G.L.M.), and the David and Lucile Packard Foundation (Fellowship in Science and Engineering no. 2007-31754 to N.J.D.).

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