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Photic niche invasions: phylogenetic history of the dim-light foraging augochlorine bees (Halictidae)

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Most bees rely on flowering plants and hence are diurnal foragers. From this ancestral state, dim-light foraging in bees requires significant adaptations to a new photic environment. We used DNA sequences to evaluate the phylogenetic history of the most diverse clade of Apoidea that is adapted to dim-light environments (Augochlorini: *Megalopta*, *Megaloptidia* and *Megommation*). The most speciose lineage, *Megalopta*, is distal to the remaining dim-light genera, and its closest diurnal relative (*Xenochlora*) is recovered as a lineage that has secondarily reverted to diurnal foraging. Tests for adaptive protein evolution indicate that long-wavelength opsin shows strong evidence of stabilizing selection, with no more than five codons (2%) under positive selection, depending on analytical procedure. In the branch leading to *Megalopta*, the amino acid of the single positively selected codon is conserved among ancestral Halictidae examined, and is homologous to codons known to influence molecular structure at the chromophore-binding pocket. Theoretically, such mutations can shift photopigment λ_{max} sensitivity and enable visual transduction in alternate photic environments. Results are discussed in light of the available evidence on photopigment structure, morphological specialization and biogeographic distributions over geological time.

Keywords: opsin; dim-light; Augochlorini; adaptive radiation; relictual taxa

1. INTRODUCTION

The invasion of a novel sensory environment represents a significant niche shift [1,2]. For photic niche shifts, photosensitivity of the eye is a target of selection, which may be associated with evolutionary diversification in many animal taxa [1,3]. Despite the independent origins of eyes, many elements of the visual system are conserved, such as the photopigment proteins (the opsins) that originate from a common metazoan ancestor [4–6]. Opsin genes are routinely used for reconstructing phylogenetic history (e.g. [7] for bees), but they also provide a potentially powerful signal for understanding the molecular basis of behavioural transitions to novel light environments, especially when viewed from a comparative phylogenetic perspective [8].

Visual pigments consist of a photon-absorbing chromophore (11-*cis*-retinal) which is surrounded by an apo-protein (opsin), embedded in the transmembrane of photoreceptor cells, and the expression of variant opsins (short/medium/long λ) permits chromatic vision [4,9]. Changes in either a small set, or single point mutations, of amino acids relative to the chromophore-binding pocket can shift spectral sensitivity [10–13]. The same result also can be achieved via gene duplication within opsin classes and differential expression of alternate copies [14,15], or the use of rhabdomeric filters to modify photopigment activation [16].

Here we explore the phylogenetic history of the obligate dim-light foraging augochlorine sweat bees (*Megalopta*, *Megaloptidia* and *Megommation*), the most diverse radiation of dim-light bees within the Apoidea (reviewed by Wcislo & Tierney [17]). These bees forage under light conditions that are orders of magnitude dimmer than related diurnal taxa [18–20] (reviewed by Wcislo & Tierney [17] and Warrant [21]), so there are reasons to expect that augochlorine opsin proteins may be under strong selection that led to adaptive radiations, as in other taxa such as cichlid fishes [8,22]. If so, opsin may be unsuitable for our phylogenetic purposes, which we test by comparison with two non-photic nuclear protein-coding genes. We estimate relative rates of non-synonymous to synonymous mutations using distance and phylogenetically informed likelihood procedures, comparing dim-light foraging taxa with their close diurnal relatives. We also use dating estimates to place the evolutionary ecology of the dim-light foraging Augochlorini within a historical context.

2. MATERIAL AND METHODS

(a) *Specimen collection and study taxa*

Bees were collected at light traps or from nests (see [23–26]) at localities given in the electronic supplementary material, table S1. The most abundant genus is *Megalopta* (approx. 30 species, including five parasites), which are distributed

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from Mexico to northern Argentina and southern Brazil, predominantly in lowlands, with one Central American montane species [24,27–31]. *Megaloptidia* (three species) occur in the Amazon basin and Guiana Shield [28,32], and *Megommation* s. str. (two species) occur in eastern Brazil, and northern Argentina and Paraguay [28,33]. Multiple specimens of the same morphospecies from different locations were used to assess potential problems associated with prior taxonomy (see electronic supplementary material, M1). Voucher specimens are located in the dry reference collection of the Smithsonian Tropical Research Institute.

(b) *DNA sequence compilation*

Bi-directional fragments of three protein-coding nuclear gene regions, long-wavelength green opsin (*LwOp*), the F2 copy of elongation factor-1 alpha (*EF-1α*) and wingless (*Wg*) were obtained (for gene maps see [34,35]). Primer oligos and polymerase chain reaction conditions are detailed in the electronic supplementary material, M2. Sequences were edited (Sequencher 4.6) and aligned (Se-Al v. 2.0a11 Carbon) to the coding region sequence of pre-existing halictid bees accessed from GenBank, and accession numbers JN106067 to JN106163 represent new sequences obtained for this study (see electronic supplementary material, table S1). Intron regions were excluded from analyses, identified in accordance with the coding regions of exemplars: *LwOp*—U26026 *Apis mellifera*; *EF-1α*—AF015267 *A. mellifera*; *Wg*—J03650 *Drosophila melanogaster*.

(c) *Phylogenetic inference*

Phylogenetic inference was performed using MrBayes 3.1.2. Data were partitioned by codon position within each gene. We took an objective approach ([36], see electronic supplementary material, M3), and used the most parameter rich, yet least restrictive model (GTR + I + G), for each partition and used default priors for other all parameters, which were unlinked across partitions. Default heating procedures were performed on two independent parallel runs, sampling likelihoods every 1000th generation. We ran analyses for 100 M generations so that the modelling procedure reached stationarity, and to obtain a large sample size from which to assess confidence in estimates of node divergence times. We used 10 per cent burn-in points ($n = 90$ K trees) and ran three analyses: (i) a combined three gene dataset, (ii) non-photic: *EF-1α* + *Wg*, and (iii) photic: *LwOp* only.

(d) *Tests for adaptive evolution: relative rates of dN/dS – ω*

We followed standard methods to test for signals of adaptive evolution from nucleotide sequences by assessing the relative rates (ω) of non-synonymous (dN) to synonymous (dS) substitutions. We first used distance measures (z-tests, MEGA v. 4.0) to test for positive selection (H_a: d$N >$ dS) within all gene fragments that were used to construct the phylogeny. Then we compared *LwOp* by foraging mode across our dataset, as well as independent pairwise analyses among 11 pairs of bee taxa with nocturnal versus diurnal foraging behaviour, for which comparable sequences are available (listed in the electronic supplementary material, table S1); behavioural categorizations were taken from Wcislo & Tierney [17].

Distance measures may suggest selection is operating, but do not indicate which sequenced regions are undergoing selection, and hence how selection is operating. Maximum-likelihood procedures were undertaken within a phylogenetic context (HyPhy v. 1.0) with consensus *LwOp* trees derived from Bayesian analyses. We assessed Global versus Local (specific branch) models, and *a priori* we selected clades and branches that may be expected to be under differential selection (i.e. dim-light versus diurnal foragers), and used modified data matrices (*a*, all specimens; *b*, single specimen/species; *c*, ancestral halictids added to matrix *b*) to account for the potential effect that altering outgroups may have on ingroup comparisons of ω. Finally, we used site-specific modelling procedures, employing both single likelihood ancestor counting as well as a more thorough branch-site fixed effect likelihood methodology (further details in electronic supplementary material, M4).

(e) *Divergence time estimation*

We use two relaxed clock analytical methods to estimate divergence dates for internal nodes of the Bayesian consensus tree, a simplistic path-length analysis with fine-scale optimization for smoothing substitutional rate variation (*PATHd8* v. 1.0), and a more rigorous penalized likelihood approach that optimizes smoothing rates across the tree, which then controls for extreme rate variation among branches, and importantly permits estimation of confidence measures on node age (*r8s* v. 1.71).

Justification of fossil usage, and analytical details are provided in electronic supplementary material, M5. Synthesizing the fossil (amber, pollen, compression and trace) and biogeographic evidence, the existence of ancestral halictid lineages in Maastrichtian (70.6–65.5 Myr ago) South America is plausible and conforms to molecular-derived age estimates of supra-family level for the Aculeata [37]. The most probable match of any ichnofossil to extant bee lineages is that of *Uruguay* (Maastrichtian ichnogenus) to Augochlorini (e.g. *Pseudaugochlora*), but see arguments by Michener [28, p. 101] and Genise & Bown [38]. Thus, we use the root age of 65 Ma, for the node representing the most recent common ancestor (MRCA) of Augochlorini + Caenohalictini (Halictinae), which agrees with prior phylogenetic studies of Halictidae [34,39].

We used Dominican amber inclusion fossils of halictine bees (reviewed by Engel & Peñalver [40]) as an internal minimum age constraint between 15 and 20 Ma [41]. Bees in our phylogenetic analyses that contain ancestral lineages represented in Dominican amber include: *Augochlora*, *Augochloropsis* (but see [40]), *Caenohalictus* and *Neocorynura*. To create credible boundaries for the upper and lower ages for amber calibrates, we identified two nodes: the earliest possible crown node, *Amber Early* (MRCA of *Augochloropsis* and *Augochlora*); and the most distal stem node, *Amber Late* (MRCA of *Augochlora*).

The most conservative use of age calibrates was a fixed *Root* of 65 Ma and a minimum age constraint of 15 Ma at *Amber Late*. We then shifted the minimum age constraint to the node *Amber Early*. Next, we removed the internal constraint, so that analyses rely solely on the Halictinae *Root* age. Finally, we modified the phylogeny into sub-trees so that the upper (*Amber Early*) and lower (*Amber Late*) internal constraint nodes are transformed to become independent fixed ages, with all ancestral taxa leading to those nodes pruned from the tree and analysis. We then explored the robustness of age estimates by adjusting age constraints at 5 Ma intervals. Thus, five broad variations on node-age calibration were performed: (i) *Root fixed* at 65 Ma, *Amber Late constrained* to 15/20/25/30/35/40/45 Ma; (ii) *Root fixed* at 65 Ma, *Amber Early constrained* to 15/20/25/30/35/40/

45 Ma; (iii) *Root fixed* at 45/50/55/60/65/70/75/80/85 Ma, no internal constraint; (iv) ancestral taxa pruned, *Amber Late fixed* at 5/10/15/20/25/30/35 Ma; and (v) ancestral taxa pruned; *Amber Early fixed* at 15/20/25/30/35/40/45 Ma.

Standard confidence interval measures are not appropriate because placing constraints on node age necessarily leads to skewed distributions, thus violating assumptions of normality. Confidence limits for node-age variability were assessed using the Bayesian analysis consensus tree as a filter constraint (PAUP* v. 4.0 b10), to yield a pool of topologically alike trees with variable branch lengths, that we then imported into *r8s* to assess variation in node age. Central distribution 95% confidence limits (CLs) were determined by the upper and lower 2.5 per cent quantile of node ages.

3. RESULTS

(a) *Phylogeny*

The combined phylogenetic data recovered 2049 aligned coding region nucleotides (*LwOp* 702 bp, *EF-1α* 754 bp, *Wg* 593 bp), with introns excluded. The consensus tree (figure 1*a*; corresponding phylogram—electronic supplementary material, figure S1*a*) gives posterior probability (PP) node support for nodes with less than 100 PP. Relationships among the diurnal augochlorine taxa are well supported. All dim-light taxa form a monophyletic clade, but with only moderate support (80 PP). The monophyletic grouping of (*Megaloptidia* + (*Megalopta* + *Xenochlora*)) is maximally supported, as is the monophyly of *Megaloptidia*. *Megalopta* is not monophyletic, as the diurnal *Xenochlora* forms a fully supported monophyly with *Megalopta atra* (the only montane *Megalopta* species), which renders *Megalopta* paraphyletic. The group (*Xenochlora* + *M. atra*) forms a sister clade to the remaining lowland *Megalopta*, which is a fully supported monophyletic group. These lowland lineages can be broadly divided by sculpturing on the basal area of the propodeum [42], into two well supported main clades: (i) one clade is comprised species with a smooth basal area of the propodeum (84 PP), which contains all specimens of *Megalopta centralis* (i.e. *Megalopta ecuadoria* in earlier publications); and (ii) one clade is comprised the parasitic *Megalopta byroni* and the remaining *Megalopta* with striate basal area of the propodeum (100 PP). Resolution among terminal branches within both of these lowland clades, however, is weak.

In order to assess the effects of including multiple specimens per morphospecies, we ran a second analysis with just one representative per taxa. This analysis generated the identical topological relationships among genera and subgenera (electronic supplementary material, figure S1*b*). Node support was also broadly equivalent, apart from the MRCA of the augochlorines that dropped from 99 to 89 PP support and the relationship between the parasite (*M. byroni*) and known host (*Megalopta genalis*) was resolved (90 PP); the remaining members of the clade with striate basal area of the propodeum collapsed into a three-way polytomy.

When the opsin fragment was removed from the matrix, very few of the above relationships hold (electronic supplementary material, figure S2). The MRCA of the augochlorines collapsed into a polytomy. The dim-light taxa no longer form a monophyletic clade; *Megommation* and *Megaloptidia* are grouped with other diurnal taxa with poor support (less than 67 PP). The only fully supported monophyletic grouping is that of *Megalopta* and *Xenochlora*, whereby (*M. atra* + *Xenochlora nigrofemorata*) form a clade (87 PP) that is sister group to a polytomous grouping of all the remaining lowland *Megalopta*.

When only opsin is used to reconstruct the phylogeny (electronic supplementary material, figure S3*a*), some resolution is lost among the diurnal outgroups but again the dim-light taxa are recovered within a common clade with strong support (94 PP). Within this clade, the grouping of *Megommation* with *Megaloptidia* is fully supported, as is *Xenochlora* with *Megalopta*. In the latter, *Xenochlora* is recovered as a distinct sister group to *M. atra*; in this analysis monophyly of the genus *Megalopta* is very poorly supported (60 PP). Within *Megalopta*, the highland *M. atra* is again isolated from the lowland *Megalopta* wherein dichotomous resolution is lost. These analyses suggest that opsin provides good resolution among the augochlorine genera included in this study, but not at the species level for *Megalopta*. However, when only a single representative per species is used, and incomplete sequences are removed, resolution somewhat improves (electronic supplementary material, figure S3*b*). If the gene is undergoing positive selection, however, then the apparent resolution it provides may be spurious.

(b) *Tests for adaptive evolution*

(i) *Distance measures of ω averaged across the matrix*

Results from the z-tests (electronic supplementary material, table S2), for all three gene fragments, showed evidence of stabilizing selection (all $p < 0.001$), but no evidence of positive selection (all $p = 1.0$). The same trends and significance values were found when the specimens were split into groups based on foraging mode (diurnal versus dim-light) for *LwOp*.

(ii) *Pairwise measures of ω*

Using 15 GenBank sequences of *LwOp* and five derived from the current study (electronic supplementary material, table S3), we found 11 suitable pairs of dim-light/diurnal foraging bees for pairwise measures. In two of these analyses (*Megalopta* versus *Xenochlora*, and *L.* (*Sphecodogastra*) versus *L.* (*Evylaeus*)), neutrality was not rejected. The other nine comparisons rejected neutrality and found very high support for stabilizing selection (all $p < 0.001$), and no support for positive selection.

(iii) *Maximum-likelihood measures of ω*

Likelihood measures on the *LwOp* coding sequence were performed on three dataset perturbations with incomplete sequences removed: (i) the original taxon matrix ($n = 38$) + electronic supplementary material, figure S3*a*; (ii) a single specimen/species matrix ($n = 16$) + electronic supplementary material, figure S3*b*; and (iii) all available ancestral halictids added to matrix b ($n = 33$) + electronic supplementary material, figure S3*c* (sequences sourced from GenBank; see electronic supplementary material, table S1). Akaike Information Criterion tests for *LwOp* rate procedures selected the HKY85 model. For all analyses, we used Muse–Gaut likelihood rate matrices

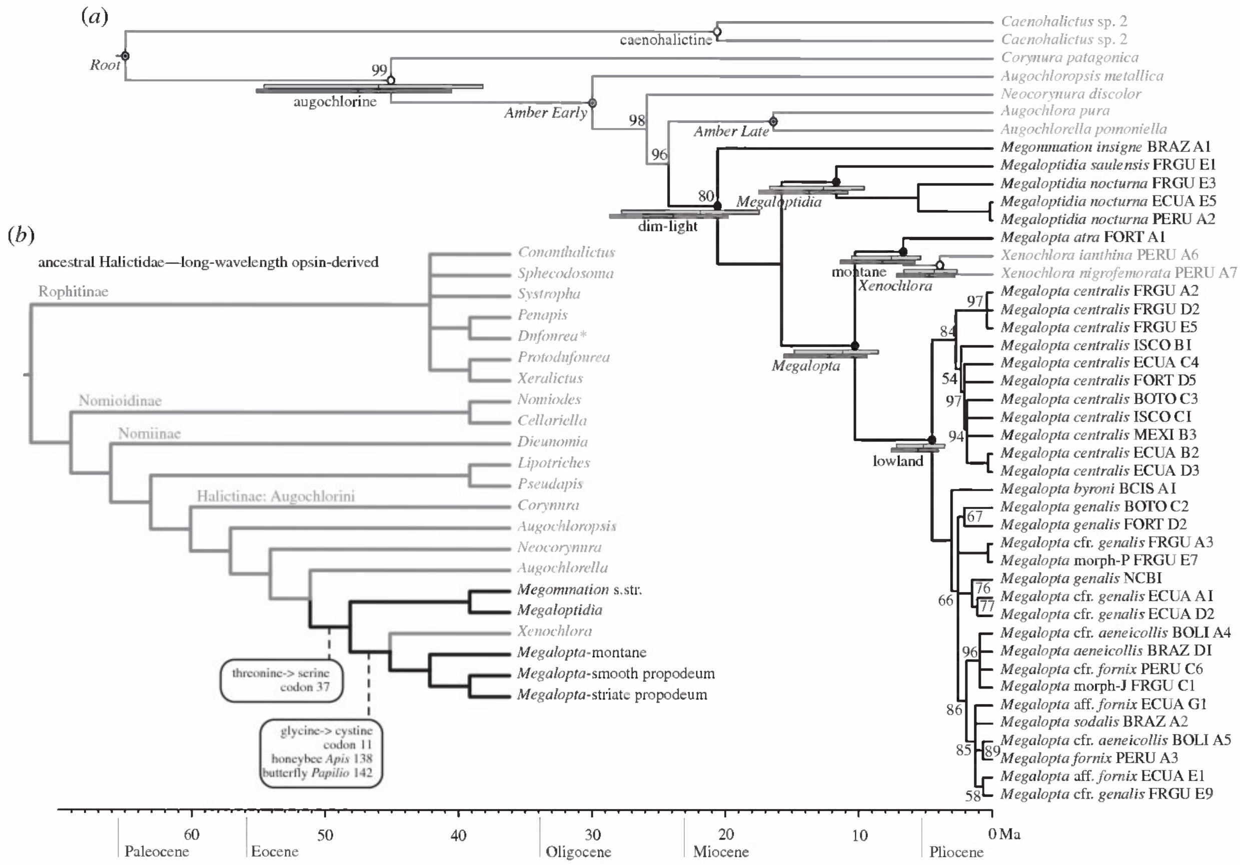

Figure 1. Total evidence and opsin-only phylogenies. (*a*) Consensus chronogram for all three genes, posterior probability node support indicated when less than 100. Foraging environment denoted by branch colour (diurnal, grey; dim-light, black), and node colour (diurnal MRCA, white circle; dim-light MRCA, black circle). Open circle with black dot denotes age-calibration node. Branch lengths derived from *r8s* analysis: *Root* fixed 65 Ma; *Amber Late* constrained 15 Ma. Horizontal bars represent 95% CL's for a 65 Ma fixed *Root* and a constraint age of 15 Ma (light grey bars) or 20 Ma (dark grey bars). Mean node age indicated by a vertical black line within these bars. (*b*) Ancestral halictid *LwOp* summary cladogram, modified from electronic supplementary material, figure S3*c*. Indicates two positions that were positively selected (all datasets) in branches leading to the MRCA of dim-light foraging clades (see electronic supplementary material, table S5). Codon Gly11Cys is homologous to the chromophore-binding pocket sites of *Papilio*-142 and bovine-123. *Megalopta* is summarized by lineage: diurnal (*Xenochlora*), montane (*Megalopta atra*) and two main lowland clades possessing either smooth (*M. centralis*) or striate basal area of propodeum (remaining morphotypes). Asterisk, *Dufourea* sequence may be questionable (see electronic supplementary material, R2).

in combination with either a HKY85 or a more parameter-rich GTR codon model, depending on whether graphical user interface or batch files were used. Consensus trees (electronic supplementary material, figure S3) were derived from the corresponding Bayesian analysis and results discussed below are presented in the electronic supplementary material, table S4. We found no evidence of recombination events in our data.

Global (shared) estimates of ω across the entire tree corroborate distance-based z-tests in rejecting neutral evolution, as confidence intervals do not overlap 1. Analyses (i) and (ii) yielded equivalent values ($\omega \sim 0.2$), while inclusion of ancestral taxa (analysis (iii)) generated a slightly weaker indication of directional selection ($\omega \sim 0.1$). Likelihood ratio tests comparing Global (H_o) versus Local (H_a) rates provided highly significant evidence for local *branch-by-branch* variation in ω for all trees. We also found evidence for *interclade* variation in ω when the tree was split by photic niche for foraging. All three matrices supported a nested model (dim-light clade + branch leading to it, in comparison with the diurnal clade), in preference to global estimates, with evidence of slightly stronger rates of stabilizing selection in the diurnal clade (ω range: 0.1–0.13), when compared with the dim-light clade (ω range: 0.34–0.36). Likelihood ratio tests comparing ω between terminal and internal branches supported H_a, indicate that terminal branches experience significantly different rates of ω compared with the rest of the tree.

The general *site and branch* Single Likelihood Ancestor Count procedure tested for both positive and stabilizing selection at each codon ($n = 234$) in the sequence. The procedure first counted across the entire tree and then counted the terminal (T) and internal (I) branches independently. Again, there is evidence of stabilizing selection, but no indications of sites under positive selection. Full counts suggested 12–41% of the sequence is undergoing stabilizing selection, depending on the matrix. Among-branch analyses of tribe Augochlorini suggest a noticeable increase in the number of codons under stabilizing selection in terminal branches versus internal branches (single specimen/species matrix *b*: $T = 7\%$, $I = 0.4\%$); these phylogenetic path-length differences are less pronounced when ancestral halictids are incorporated (matrix *c*: $T = 20\%$, $I = 15\%$).

The *site and branch* two-rate fixed effects likelihood analyses were first performed on all branches in the tree, then on sub-trees rooted at the MRCA node of the dim-light augochlorines and all derived nodes of each dim-light genus, and finally on the ancestral branches leading to all of the aforementioned nodes. Electronic supplementary material, table S4 details the sum count of directionally selected codons, followed by the position of positively selected codons. Each dataset gave rise to no more than five sites undergoing positive selection in each analysis. Each positively selected codon from data matrix *c* analyses (sub-tree and branch-site) was highlighted in an alignment relative to opsin codon positions in *Apis* (honeybee) and *Papilio* (swallowtail butterfly). Two positively selected codons were consistently recovered across matrices in branches leading to dim-light clades (see figure 1*b*; electronic supplementary materials, R2 and table S5). We identified one potentially functional amino acid mutation within the dim-light clade, relative to the homologous position of the chromophore-binding pocket.

(c) *Divergence estimates*

We used the total data consensus tree for all *PATHd8* analyses, and as the constraint topology to filter trees ($n = 5144$) to gain 95% CLs for *r8s* analyses. Figure 1*a* presents a chronogram with branch lengths derived from a conservative *r8s* analysis. Results from all analyses are presented in electronic supplementary material, table S6 and thoroughly compared in electronic supplementary material, R3. However, both methods provided similar age estimates and no unexpected results arose when age constraints were liberally extended. Table 1 shows a subset of *r8s* analyses that independently yield equivalent results. Removal of internal constraints or alternate fixed-age placement strengthen the intuitive *a priori* choice of rooting the tree at 65 Ma with placement of an internal minimum constraint of 15 Ma at the *Amber Late* node. The *Amber Late* node is located quite high in the tree, but the alternate independent use of calibration point nodes (and even the removal of *Megommation*—analysis 4*X*, table 1), suggest our estimates are robust. These calibrating procedures and their confidence intervals suggest that the MRCA of the augochlorine taxa used in this phylogenetic reconstruction is at least 46 Ma (95% CL 38–55 Myr ago). The obligate dim-light foraging augochlorine bees share a common ancestor that is at least 22 Ma (95% CL 18–28 Myr ago). The most speciose dim-light foraging genus *Megalopta* has an MRCA that is at least 11 Ma (95% CL 7–16 Myr ago), and the two most geographically widespread lowland *Megalopta* clades are estimated to have diverged and radiated within the last 5 Ma (95% CL 4–7 Myr ago). A credible upper boundary (*Root* fixed at 65 Ma; *Amber Late* constrained to 20 Ma) yields very similar 95% CL's for all nodes, never exceeding more than 3.3 Ma difference at either tail.

4. DISCUSSION

(a) *Opsin evolution*

The functionality of our positively selected amino acid sites, relative to distantly related opsin proteins, remains an open question. Recent empirical work shows that mutations of long-wavelength opsins at positions homologous to bovine tuning sites alter sensitivity of *Drosophila* chromophores [13]. Our codon 11 (*Apis* 138, *Papilio* 142, bovine 123 [43]) is the only positively selected codon on the ancestral branch leading to MRCA of the most diverse dim-light foraging augochlorine clade (*Megalopta*); the amino acid is conserved in ancestral lineages and then switches Gly11Cys (figure 1*b*, electronic supplementary material, table S5). Homology modelling of the crystal structure and ultimately mutagenic experiments are required to assess the functionality of this mutation. Comparisons to previous studies on Lepidoptera and bees [43], however, suggest that this codon may be associated with structural changes that influence the chromophore-binding pocket, and potentially shift the absorption λ_{max} of the visual pigment.

The functional consequences of differential opsin expression are beginning to be resolved for bees. A fully nocturnal carpenter bee (*Xylocopa*) is capable of colour discrimination under very dim light [44], whereas honeybees (*Apis*) switch and use achromatic vision at low light intensities [45]. Bumble-bees express *LwOp* at much faster rates than ultraviolet (UV) or blue opsins [46], suggesting *LwOp* may play a role in photoreceptors measuring optic flow. As with diurnal bees, manipulation of horizontal flow in the visual field alters flight speed in *Megalopta*, even at low light intensities [47]. In addition, *Megalopta* possess a number of other neuro-physiological and anatomical adaptations for vision in dim light (reviewed by Wcislo & Tierney [17] and Warrant [21]). Future research aims to link these adaptations with studies of opsin expression.

Are data from long-wavelength opsin valid for recovering phylogenetic history of bees that are likely to experience strong selection on traits related to their visual ecology? An examination of rates of d*N*/d*S* shows that this fragment is under stabilizing selection. This finding is consistent with other studies examining predominantly diurnal bees (apids, megachilids, colletids and halictids), whereby the majority of mutations were at synonymous third codons (e.g. [7,34,35,48]). In augochlorines, only a handful of codon sites are under positive selection, as might be expected if point mutations result in spectral tuning of photopigment wavelength sensitivity. In general, *LwOp* provided good resolution at the generic level, except for the relationship between *Megalopta* and *Xenochlora* (see below).

Gene duplication is one mechanism to shift photopigment sensitivity; duplicate copies *LwOp* occur in some bees and butterflies [49]. To assess whether we had sequenced an alternate copy of *LwOp*, we re-analysed our data incorporating all known copies of bee *LwOp*-Rh2 (Apidae—*Apis*, *Bombus* and *Diadasia*; Megachilidae—*Osmia*) [50,51]. The resulting tree (electronic supplementary material, figure S3*d*) suggests that the *LwOp* copy used in the majority of bee phylogenetic studies [34,52] has an affinity to *LwOp*-Rh1. This implies that we have sequenced the *LwOp* copy expressed in the compound eyes, as *LwOp*-Rh2 is only known from bee ocelli [51]. The ocelli of *Megalopta* appear to functionally resemble cockroaches, more so than other bees, in that they are UV insensitive [53].

Parallel and convergent evolution has been identified in the rhodopsin gene of bats [54]. In augochlorine bees, the non-photic gene matrix (*EF1-α* and *Wg*) did not group the dim-light lineages within a single clade, but the alternate paraphyletic arrangement was not statistically supported (electronic supplementary material,

Table 1. Divergence age estimates. (Subset of penalized likelihood (*r8s*) results for alternate age-calibration procedures, indicating the time calibrate for each analysis, the mean node-age estimate in millions of years and 95% CL.)

node	*Root*	augochlorine	*Amber Early*	caenohalictine	*Amber Late*	dim light	*Megaloptidia*	*Megalopta*	montane	lowland	*Xenochlora*
1a calibrates:	**65[a]**				**15[b]**						
mean ($n = 5142$)	—	45.99	31.22	21.00	17.18	21.93	12.75	11.18	7.57	5.06	4.36
95% CL	—	38.15—54.64	25.63—38.29	15.08—28.44	15.00—22.33	17.51—27.86	9.70—16.76	8.46—14.92	5.27—10.58	3.63—7.14	2.65—6.66
1b calibrates:	**65[a]**				**20[b]**						
mean ($n = 5144$)	—	47.49	33.45	21.02	20.17	23.91	13.86	12.16	8.23	5.49	4.73
95% CL	—	40.49—55.07	28.84—39.09	15.09—28.46	20.00—22.33	19.86—28.70	10.87—17.47	9.35—15.70	5.85—11.28	3.98—7.56	2.90—7.10
2d calibrates:	**65[a]**		**30[b]**								
mean ($n = 5144$)	—	46.47	31.90	21.01	17.36	22.33	12.97	11.37	7.70	5.14	4.43
95% CL	—	39.86—54.58	30.00—38.20	15.08—28.45	13.39—22.32	18.44—27.77	10.12—16.75	8.81—14.89	5.42—10.56	3.73—7.14	2.71—6.67
3e calibrate:	**65[a]**										
mean ($n = 5144$)	—	45.85	31.03	21.00	16.92	21.76	12.66	11.09	7.52	5.02	4.33
95% CL	—	37.82—54.56	25.13—38.20	15.08—28.42	12.64—22.32	17.09—27.76	9.53—16.68	8.30—14.86	5.18—10.50	3.7—7.2	2.61—6.63
5d calibrate:			**30[a]**								
mean ($n = 5142$)	—	—	—	—	16.26	20.90	12.10	10.63	7.20	4.80	4.13
95% CL	—	—	—	—	12.78—19.98	17.52—24.32	9.54—15.07	8.20—13.56	5.10—9.79	3.46—6.65	2.54—6.14
4c calibrate:					**15[a]**						
mean ($n = 5143$)	—	—	—	—	—	22.84	13.07	11.34	7.65	5.12	4.40
95% CL	—	—	—	—	—	16.24—29.53	8.89—17.35	7.71—15.10	4.78—10.54	3.32—6.87	2.44—6.52
4Xiii calibrate:					**15[a]**						
mean ($n = 5141$)	—	—	—	—	—	—	14.14	12.30	8.29	5.53	5.20
95% CL	—	—	—	—	—	—	8.91—18.16	7.67—15.64	4.76—11.27	3.27—7.18	2.41—6.81

[a]Fixed age.
[b]Minimum constraint age.

figure S2). A phylogenetic study of *Megalopta* using morphological characters [55] yielded a topology similar to our *LwOp*-only topology (figure 1*b*), but the study did not include the other dim-light augochlorines, *Megommation* or *Megaloptidia*.

(b) *Reversion to diurnal foraging*

Xenochlora, the closest diurnal relative of *Megalopta*, was elevated to generic status [56] based on a suite of morphological characters (e.g. coloration and ocellar size), but otherwise appears to resemble *Megalopta* in form, social behaviour and nesting biology [25]. Owing to a lack of ethological data, we cannot rule out facultative crepuscular activity, but we do know they are diurnal foragers (D. W. Roubik 1991, unpublished observation, cited in Engel *et al.* [56]). Our data support the incorporation of *Xenochlora* within *Megalopta*, forming a fully supported sister clade with *M. atra*. Males of *Xenochlora* are unknown; Michener [28, p. 412] considered that the phylogenetic position of *Xenochlora* was uncertain, but based on available evidence he would have treated it as a basal subgenus within *Megalopta*. Our data show that *Megalopta* is paraphyletic, and imply that the common ancestor for this genus foraged in dim light. If substantiated, *Megalopta* (*Xenochlora*) represents a reversion to diurnal foraging. There are various examples in vertebrate evolution where both dim-light vision and colour vision have reversed (reviewed by Yokoyama [8]). A morphological study retains *X. nigrofemorata* as the sister taxon to *Megalopta* [55], as per our *LwOp* results, but we recovered poor support for *M. atra* as sister clade to the remaining lowland *Megalopta* (electronic supplementary material, figure S3*a*). Our total evidence tree recovers the arrangement of ((*M.* (*Xenochlora*) + *M. atra*), (lowland *Megalopta*)) with maximal support (figure 1*a*).

(c) *Single tribal origin of dim-light foraging*

Our results place *Megommation*, *Megaloptidia* and *Megalopta* within a monophyletic clade, suggesting a single origin of obligate dim-light foraging, with a reversion to diurnal foraging from a dim-light common ancestor. Our conclusions should be considered tentative given the limited generic sampling in the tribe (9 of 25 (36%) augochlorine genera recognized by Michener [28]), and inconsistent support for the node leading to *Megommation* (moderate or high support in the total evidence tree and *LwOp* tree, respectively). A précis of previous augochlorine systematics is provided in the electronic supplementary material, D1. Our tree differs from these analyses [42,57,58], in that *Megalopta* is distal to *Megommation* and the sister clade of *Megaloptidia*. This finding is probably not a sampling artefact, as the arrangement is robust to data matrix modifications.

Our recovery of *Megommation* as basal sister group to the remaining dim-light augochlorines is consistent with nesting behaviour. The primitive state for Augochlorini is ground nesting [59], and *Megommation* is a ground nester [60]. Nesting behaviour in *Megaloptidia* is unknown. Anatomical features of mandibles, and scale-like setae surrounding the median pseudopygidial slit, are convergent for wood nesting augochlorines [61]; *Megaloptidia* possess a broad mandible with a subapical tooth [32], but differ from *Megalopta* in lacking: (i) teeth on the inner surface of the mandible [28, p. 408]; and (ii) tergal scale-setae. Both *Megalopta* and *Xenochlora* are stem nesters [23–26], which may be ecologically advantageous in the humid tropics.

(d) *Antiquity of dim-light augochlorines: ecological association with night-flowering plants and biogeography*

Our temporal estimates were robust to perturbations of fossil-derived calibrates and our root age of 65 Ma for the origin of Halictinae is consistent with independent studies of bee phylogenetics [34,39]. Palaeopalynological evidence suggests that the structure of low latitude South American forest communities have remained relatively stable since the Early Eocene [62], which roughly equates with our estimates for the origin of the Augochlorini. Our results also suggest that dim-light augochlorines predate the origin of phyllostomid bats [63]. *Megalopta* bees use more than 60 angiosperm species at one site in central Panama (I. Lopez, A. R. Smith & W. Wcislo 2007, unpublished data), but little is known of their role as potential pollinators. Hopkins *et al.* [64] noted that *Megalopta* was the most abundant visitor to *Parkia velutina* in Brazil, and hypothesized that nocturnal bees may have played a role in opening a new niche (night-blooming flowers), which was subsequently exploited by bats (for a more detailed discussion, see [17]).

Our arrangement places (*M. atra* + *M.* (*Xenochlora*)) as the basal sister clade to all other *Megalopta*. *Megalopta atra* is unique in its montane distribution, found only at mid-elevations (approx. 1000–1500 m) in Costa Rica and Panama [24,27]. Mountain peaks as species isolation mechanisms have been empirically demonstrated [65], and should be more extreme in the tropics [66], owing to a decreased range in temperature tolerance relative to temperate species. Our results suggest a Late Miocene origin for *Megalopta* (approx. 11.2 Ma) and the MRCA of (*M. atra* + *M.* (*Xenochlora*)) (approx. 7.6 Ma). Current estimates indicate that the final closure of the Panamanian isthmus occurred in the Pliocene [67], and it is feasible [68] that ancestral *Megalopta* lineages traversed the Panamanic Seaway before final closure. Colonization of cloud forests during cooler climes (more broadly distributed at lower elevations) and subsequent isolation from younger lineages may be accounted for by more recent (Quaternary) climatic events, or by competitive exclusion [69,70]. The contemporary lowland lineages radiated less than 5 Ma. It seems unlikely that the common ancestor of *Megalopta* was a Central American cloud forest bee, and we hypothesize that *M. atra* represents a relictual highland species (for other examples, see [48,71,72]).

5. SUMMARY

This study provides a phylogenetic platform from which evolutionary inferences on vision and behaviour in dim-light augochlorine bees can proceed. Results suggest a tribal origin of dim-light foraging in the Late Miocene, with a secondary reversion to diurnal foraging (*Xenochlora*) within the distal and most diverse lineage *Megalopta*. Adaptive selection tests suggest that *LwOp* is broadly under stabilizing selection, with a handful of sites under positive selection. Further investigation is required to fully determine the modes of visual transduction among these bees, and to relate the molecular evolution of all opsin proteins with rates of expression.

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