Museum archives revisited: Central Asiatic hawkmoths reveal exceptionally high late Pliocene species diversification (Lepidoptera, Sphingidae)

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Submitted: 31 August 2016 Accepted: 8 January 2017 doi:10.1111/zsc.12235

Hundsdoerfer, A.K., Päckert, M., Kehlmaier, C., Strutzenberger, P. & Kitching, I.J. (2017). Museum archives revisited: central Asiatic hawkmoths reveal exceptionally high late Pliocene species diversification (Lepidoptera, Sphingidae). — Zoologica Scripta, 46, 552–570.

Three high-elevation Hyles species of Central Asia have proven difficult to sample, and thus, only a limited number of specimens are available for study. Ancient DNA techniques were applied to sequence two mitochondrial genes from 'historic' museum specimens of Hyles gallii, Hyles remneri and Hyles salangensis to elucidate the phylogenetic relationships of these species. This approach enabled us to include the holotypes and paratypes. The status of H. salangensis as a species endemic to a mountain range north of Kabul in Afghanistan is confirmed by this study. It is most closely related to Hylex nicaea and H. gallii, and quite distant from the clade comprising the species from Hyles vesperiio to Hyles tibbnyala, despite this group and H. salangensis both completely lacking an aroliun on the pretarsus. Our results show that the samples assigned to H. remneri and Hyles livornica tatsuianluica are conspecific and so we reinstate Hyles tatsuianluica stat. nov. as the valid name for this species and synonymize H. remneri syn. nov. with it. This study shows that the distribution range postulated for H. tatsuianluica extends from Nepal well into the mountains of south-western China. The distribution ranges of H. livornica and H. tatsuianluica overlap. The study confirms the previously proposed synonymies of Hyles nepalensis, Hyles gallii intermedia and Hyles gallii tibetanica with H. gallii. Extensive species sampling (over 80% of Hyles species) in this study allowed additional analyses. The dated phylogeny reveals the global Hyles hawkmoth radiation to be much more recent than previously thought: it began in the Late Miocene and culminated in a Pleistocene burst of diversification in the Northern Hemisphere. Ancestral ranges of basal nodes were reconstructed as highly equivocal, but the Neotropics has the highest probability in the two oldest nodes. Although the origins of the Madagascan and Australian species also remain ambiguous, a large crown clade of fifteen species was reconstructed to have originated in the Palaearctic. The wide distribution ranges of the two migratory species, H. livornica and H. gallii, appear to blur any traces of the biogeographic origin of the clades containing these species. Specialization in larval host plant use onto particular plant families from the ancestral condition 'polyphagous' may have led to an increased rate of speciation and phylogenetic diversification in three subgroups of Hyles (the Hawaiian clade, the Hyles centralasiae group and the Hyles euphorbiae complex).

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Introduction

The hawkmoth genus *Hyles* Hübnerr, [1819] (Lepidoptera: Sphingidae) has a global distribution with representatives on all continents (except Antarctica) and many major islands (e.g. Madagascar). The 32 species (following the ‘Sphingidae Taxonomic Inventory’; Kitching 2016) are generally identified with the help of color images using characters from the pattern and color of the wings and abdomen. However, the taxonomy based on these morphological features is highly contentious with disagreement over both the number of species and how they are related (e.g. Meerman & Smid 1988; Speidel & Hassler 1989; Danner et al. 1998; Hundsdoerfer et al. 2005, 2009; Harbach 2009). A number of studies have been devoted to elucidating the phylogeny of the genus using molecular methods (Hundsdoerfer et al. 2005, 2009). For this study, we augmented the taxon sampling to over 80% (26) of the 32 species listed in Kitching (2016) and have now included all species we consider necessary for an adequate representation of their diverse biogeographic origins. The first aim of this study was thus to elucidate the biogeographic history of the genus. Of the species not included, based on morphology alone, *Hyles wilsoni* (Rothschild, 1894) from Hawaii would simply add another branch to the Hawaiian radiation. The other five, *Hyles apocyni* (Shchetkin, 1956), *Hyles chamblya* (Denso, 1913), *Hyles chromini* Saldaías & Ivinskis, 2006, *Hyles exile* Derzhavets, 1979 (=*Hyles chuvilini* Eitschberger, Danner & Surholt, 1998) and *Hyles nervosa* (Rothschild & Jordan, 1903), would add another five Central Palaearctic species and further emphasize the importance of this region for the biogeography of this genus.

Biogeography

Currently, the most comprehensive phylogenetic hypothesis for the genus (in terms of the number of taxa and genes sampled) is based on data from one nuclear and three mitochondrial genes (Hundsdoerfer et al. 2009). From this, a biogeographic hypothesis was proposed that the genus originated in the Neotropics, given the oldest splits occur between lineages in this part of the world. Australia and Madagascar were postulated to have been colonized from there, the endemic species of these two regions, *Hyles gallic* (von Prunner, 1798) and *Hyles biguttata* (Walker, 1856), respectively, branching off next. However, the routes are uncertain due to the lack of reliable dates for these phylogenetic splits to correlate with positions of land masses and the formation of the Antarctic ice shield. Hawkmoths are large and strong flying and should have been able to cross stretches of ocean, especially if assisted by favourable winds (Rota et al. 2016 and references therein; Stewart 1975). They may also have used oceanic islands as stepping stones, or at least as resting places, even if these are not inhabited by *Hyles* today. The subsequent colonization of Hawaii could also have taken place in this manner, for example via the Fijian Islands from the south or via the Bering Strait from the north. Following this hypothesis, the Palaearctic would have been colonized from the east (Hundsdoerfer et al. 2005, 2009).

Systematics and taxonomy

Danner et al. (1998) provided the first explicit subdivision of *Hyles*, recognizing eight subgenera on the basis of perceived morphological differences. Of particular relevance to the present study are the three subgenera *Celerio* Agassiz, 1846, *Danneria* Eitschberger & Zolotuhin, 1998, and the nominotypical subgenus *Hyles*. The Neotropical species, *Hyles lineata* (Fabricius, 1775), the Palaearctic/Afrotropical *Hyles livornica* (Esper, 1892) and the Australian *H. livornioides* all have similar wing patterns that include conspicuous white highlighting of the veins, quite different from the other members of the genus, and so were placed in subgenus *Danneria*, together with a newly described species, *Hyles renneri* Eitschberger, Danner & Surholt, 1998. Similarity in wing pattern, together with larval monophagy on *Euphorbia*, also led Danner et al. (1998) to place *Hyles euphorbiarum* (Linnaeus, 1758), *H. (euphorbiarum) conspicuus* (Rothschild & Jordan, 1903) (raised to species status), *Hyles robertsi* (Butler, 1880), *Hyles nicaea* (von Prunner, 1798) and *Hyles stroebeli* Eitschberger, Danner & Surholt, 1998) together in the nominotypical subgenus *Hyles*, and also, tentatively, *Hyles salangensis* (Ebert 1969). Danner et al. (1998) also resurrected the subgenus *Celerio* for *Hyles gallic* (von Rottemburg, 1775), *Hyles nepalensis* (Daniel, 1961) (a subspecies of *H. gallic* that these authors raised to species status) and, again tentatively, *Hyles zygophylli* (Ochsenheimer, 1808). Their taxonomic actions were challenged by Kitching & Cadiou (2000), who morphologically revised the taxonomic status of several of the taxa that Danner et al. (1998) had raised to species status. Subsequently, phylogenetic reconstructions based on molecular data (Hundsdoerfer et al. 2005, 2009) showed that neither wing pattern similarity nor commonality of larval host plants necessarily reflects phylogenetic relationships within the genus *Hyles*, and consequently refuted the taxonomic validity of Danner et al.’s (1998) subgeneric concepts of *Danneria, Hyles* and *Celerio*. The currently valid taxonomy is maintained and updated in the ‘Sphingidae Taxonomic Inventory’ (Kitching 2016).

Phylogenetic brain-teasers – the challenge of this study

The second aim of this study was to resolve the phylogenetic relationships of three enigmatic high-elevation *Hyles*
species from the Central Palaearctic: *H. salangensis*, *H. renneri* and *H. nepalensis*. For a variety of reasons (e.g. inaccessibility of habitat, presence of human conflict zones), it has been difficult (and will presumably remain so) to obtain fresh tissue samples of these species for molecular analyses. To circumvent this difficulty, we analysed tissue samples obtained from ‘historic’ dry museum specimens.

The first of these enigmatic species, *H. salangensis*, appears to be known reliably only from the original type material, 15 males collected in the 1960s from the Salang Pass, north of Kabul, Afghanistan (Ebert 1969) and a single male in NHMUK (NHMUK specimen BMNH # 812472; depository abbreviations are given in legend of Table S1) captured in 1975. *Hyles salangensis* has a wing pattern similar to that of several other species of *Hyles*, such as *Hyles annei* (Guérin-Méneville, 1839), *Hyles centralis* (Staudinger, 1887), *H. euphorbiae*, *H. robertsi* and *H. stroebelii*, but as these species have proven not to be closely related to each other, it is difficult to use wing pattern to determine the phylogenetic position of *H. salangensis*. Features of the females and larvae cannot be used as these *neri* and *554ª*

Peratures producing darker moths, and also that colours can be induced by changing the temperature experienced abdominal patterns of the two species are almost identical. Consequently, the distributional ranges and possible contact zones of the two species in the Himalaya and surrounding areas to the north and east cannot be considered to be known in any detail yet. Even the question of whether *H. livornica* occurs in Nepal remains open.

The species was considered to be endemic to Nepal, with localities being mostly in the Annapurna and Ganesh Himal at elevations between 1600 m and 4650 m, where it was said to replace *H. livornica*. Danner et al. (1998) wrote that its restriction to high elevations and insular distribution indicated that the species could be assumed to be non-migratory. In contrast, *H. livornica* is well known as a strong-flying, migratory species that is widely distributed in Africa, southern Europe and Asia. They further stated that as *H. livornica* occurs in eastern Nepal, the two species could occur sympatrically. However, it is far from clear which species actually occur(s) in eastern Nepal. The records cited by Danner et al. (1998) derive from Haruta (1994, 1995), and the seven localities given therein range in altitude from 1957 to 4200 m. Furthermore, one of the *H. livornica* localities, Muktinath (3545 m), is in the Annapurna, at the centre of the distributional range described for *H. renneri*. Thus, this record should probably be attributed to *H. renneri* rather than to *H. livornica*. Only a single specimen was illustrated by Haruta (1994) and being neither obviously very dark nor very pale, it is difficult to attribute it to either of the species.

Another taxon, *H. livornica tatsienluica* (Oberthür, 1916) from ‘Ta-tsien-lou, ‘Tibet’ (now Kangding, Sichuan) in south-western China also has a dark overall appearance. However, apparently without having studied any material first hand, Danner et al. (1998) treated it as a junior synonym of *H. livornica*. We included two specimens in this study because its wing coloration is just as dark as that of the holo- and paratype specimens of *H. renneri* collected at high elevation (2000–4000 m) in Nepal.

Kitching & Cadieux (2000) took a contrary view to Danner et al. (1998), arguing that *H. renneri* was not distinct from *H. livornica* because although moths of the former were dark, similar dark specimens could be found elsewhere within the range of *H. livornica* (including several *H. livornica tatsienluica* in NHMUK), and thus, the colour difference was not diagnostic. Furthermore, from a comparison of the genitalia they considered that the purported diagnostic features in those structures did not hold either. The geographic and altitudinal range of *H. renneri* was later greatly increased when Eitschberger (1999b) recorded a male from Gangu County, Gansu, China, at an elevation of a mere 900 m. This locality is over 2000 km from central Nepal, in a straight line across the Tibetan Plateau. Consequently, the distributional ranges and possible contact zones of the two species were both sexes (Danner et al. 1998) treated it as a junior synonym of *H. livornica*. We included two specimens in this study because its wing coloration is just as dark as that of the holo- and paratype specimens of *H. renneri* collected at high elevation (2000–4000 m) in Nepal.

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The third species whose status is open to question is the former *H. nepalensis*. Originally described as a subspecies of *H. gallii*, and based on only two specimens from Nepal, it was raised to species status by Danner *et al.* (1998). The main diagnostic characters were again a much darker overall appearance, compared to *H. gallii*, a reduction of the white patch on the upper side of the hindwing and a stronger white dorsal line on the abdomen. Danner *et al.* (1998) listed four moths of this taxon from Nepal deposited in the private collection of one of the authors (EEMEM), but illustrated a further three from the ZSM (apparently not the types). Danner *et al.* (1998) gave the distribution of *H. nepalensis* as (our translation from German): ‘Only known from Nepal and immediately adjacent areas of Tibet, where moths were caught at different localities between 2750 and 3600 m between the 21st of May and 21st of July’. However, following an assessment of the variation in adult and larval coloration, and genital structure, Kitching & Cadiou (2000) concluded there was no support for separate species, or even subspecies, status for *H. nepalensis* and synonymized it with *H. gallii*. Danner *et al.* (1998) state that *H. nepalensis* still flies at 3°C at 4400 m. Moths of *H. gallii* have been reported flying into light traps at subzero temperatures (Danner *et al.* 1998), indicating their tolerance of a cold climate at high altitude. As low temperatures during pupal development may lead to darker shades in the adult moths, the species status of the former *H. nepalensis* is questionable.

*Hyles gallii* is distributed throughout the Holarctic, including localities in the mountains of north-west India (3100–4500 m) and northern Pakistan (2300 m), the most westerly mountain range of the Himalaya–Tibetan Plateau upfolding. For comparison with our samples of the former *H. nepalensis* (which included the holotype), we included specimens from the Nearctic and the Ladakh area, specimens determined as *H. gallii* collected at low elevation in Nepal, and the holotype and two paratypes of the former *H. gallii tibetanica* Eichler, 1971 (which Danner *et al.* 1998) had synonymized with nominotypical *H. gallii*; Table S1).

**Larval host plants**

With regard to their choice of larval host plants, the genus *Hyles* includes species that range from the extremely polyphagous (e.g. *H. lineata*) to those that are restricted to a single plant family (e.g. *Hyles hippocastanum* on Elaeagnaceae, *H. zygophylli* on Zygophyllaceae and the species of the *H. euphorbiaceum* complex on Euphorbiaceae). It has been suggested that polyphagy is an adaptation to living in arid or semi-arid habitats, where the available hosts are unpredictable, in both time and space, and in terms of their taxonomic (and thus chemical) composition. Consequently, the ability to feed on whatever host plant is present would be advantageous (Physiological Efficiency Hypothesis: Singer 2008 and references therein). In contrast, specialization onto a single host plant family, particularly if that family is well protected by toxic secondary compounds, could also be evolutionarily beneficial, especially if those chemicals could be detoxified, then sequestered or otherwise co-opted for defence (Enemy-Free-Space Hypothesis: Singer 2008 and references therein). Larval host plant shifts and specialization in butterflies have also been linked with increased speciation rates (e.g. Fordyce 2010; Wahlberg *et al.* 2013; Ebel *et al.* 2015), but this has not yet been investigated in *Hyles*. The larval host plants of two of the three enigmas that are the focal species of this study are unknown, so by reconstructing the evolution of larval host plant use in the genus, our third aim of the study was to predict what are the larval host plants of *H. salangensis* and *H. remneri*.

**Methods**

**Material and laboratory techniques**

Of the 83 samples sequenced for this study (Table S1), 77 are dry museum exemplars that were collected between the years 1930 and 2007. Due to assumed degradation of ‘historic’ DNA (i.e. DNA from tissue that was not specifically preserved for later DNA analyses and is older than ~5 years), the samples were processed with ancient DNA (aDNA) techniques. Type specimens were included from all the focal taxa studied (Table S1). Sequences of the mitochondrial genes COI and COII were obtained to merge the present data set with the sequences analysed by Hundsdoerfer *et al.* (2005, 2009), following the published protocols (primers in Table S2 from Hebert *et al.* 2004; Hajibabaei *et al.* 2006; Mende & Hundsdoerfer 2013). The 2284 bp targeted were amplified in three fragments of ~860 to ~990 bp for fresh or in 13 fragments of ~110 to ~280 for the ‘historic’ specimens (details below). More recent tissue that was preserved for DNA analyses was available for the populations of *H. gallii* from Ladakh (Table S1) and Canada (both 2008).

DNA from museum specimens was extracted using an aDNA methodology in a dedicated laboratory. Such tissue consisted of either the abdomen, the contents of which were digested enzymatically following the procedures described by Hundsdoerfer & Kitching (2010), or one or two legs, which were finely chopped with small scissors prior to addition of lysis buffer from a beadex forensic kit (LGC Genomics, Berlin, Germany). Manufacturers’ instructions were followed except that the proteinase-K digestion was left overnight and the final elution was performed with 60 μL of elution buffer. The genes were amplified in up to 13 small, overlapping fragments with specifically designed *Hyles* primers (Table S2). The size of the amplified fragment depended on the degree of
degradation of the DNA and ranged from longer fragments of 300–600 bp (fragments 1a, 1b, 2a, 2b, 3a, 3b; Table S2) to shorter ones of only 110–280 bp (fragments A–M). The fragments were amplified either individually or in a two-step multiplex approach to make efficient use of the limited amount of DNA available. To conduct the multiplex PCR, primer mixes were prepared with each primer at a concentration of 2 μM (2 pmol/μL). Primer mix 1 contained fragments A, C, E, G, I, K and M, and primer mix 2 targeted fragments B, D, F, H, J and L. Each multiplex PCR was performed with 4 μL of DNA extract in a 25-μL volume containing 2.5 μL of primer mix, 12.5 μL of ready-made Master Mix 2× (Type-it Microsatellite PCR Kit, Qiagen, Hilden, Germany) and 6 μL of ultrapure H2O. Cycler settings were initially 95 °C for 5 min, then 40 cycles of 95 °C for 30 s, 57 °C for 1.5 min, 72 °C for 30 s and a final elongation at 60 °C for 30 min. Re-amplification was performed in a 20-μL volume containing 1.5 μL of a 1:10 dilution of the multiplex PCR product, 1 μL of each primer at 10 μM, 0.4 μL of dNTP mix at 10 mM of each dNTP, one unit of Taq polymerase (Bioron DFS Taq, Ludwigshafen, Germany), 2 μL PCR buffer 10× incl. 25 mM MgCl2 and 13.9 μL of ultrapure H2O. Cycler settings for re-amplification were initially 95 °C for 5 min, then 40 cycles of 95 °C for 1 min, annealing temperature (see Table S2) for 1 min, 72 °C for 1 min and a final elongation at 72 °C for 10 min. Subsequent laboratory steps, such as purification of PCR products and cycle sequencing, followed Hundsdoerfer et al. (2009). The short sequences were then assembled by hand using BioEdit (Hall 1999).

**Phylogenetic analyses**

To reconstruct phylogenetic relationships of the new specimens studied, we added the new sequences into a comprehensive mitochondrial data set comprising representative COI (1531 bp) and COII (681 bp) sequences from all Hyles species analysed by Hundsdoerfer et al. (2009), except for *Hyles cretica* Eitschberger, Danner & Surholt, 1998 and *H. robertsi*, as their samples formed an unresolved polytomy with those of *H. euphorbiae*. We omitted the intervening 71-bp tRNA-Leu from the analyses. We added a further 42 DNA barcode sequences downloaded from the public BOLD database (http://www.boldsystems.org/) or deposited in GenBank by BOLD for this study (11 Palearctic *H. gallii*, 17 Nearctic (formerly named) *Hyles gallii intermedia*, one *H. gallii tibetanica*, nine *Hyles livornica* and four *H. renneri*). The entire data set of new sequences was deposited in BOLD (see Table S1, for GenBank accession numbers). The final alignment comprised 2212 bp for 219 sequences.

The best partitioning scheme and evolutionary models were determined with PARTITIONFINDER 1.1.1 (Lanfear et al. 2012) using the AICc criterion and the greedy search algorithm (best: three-partition scheme; Table S3). Bayesian inference of phylogeny was performed using (i) BEAST v.1.8.2 (Drummond et al. 2012) with 35 000 000, trees sampled every 3000th generation, a single lognormal relaxed-clock model for both genes and a birth–death tree prior was applied (burn-in 5 000 000) and (ii) MRBAYES 3.2 (Ronquist et al. 2012) with 25 000 000 generations (25% burn-in). The BEAST log file was examined with TRACER v1.4.8 (Drummond & Rambaut 2007), and effective sample size (ESS) values of both Bayesian analyses are provided in Table S4. A maximum-likelihood (ML) tree with bootstrap support values was reconstructed using RAxML (raxmlGUI1.5b1, Silvestro & Michalak 2012; best of 10 replicate tree searches; partitioned according to the best scheme in Table S3; GTRCAT; 1000 thorough bootstrap replicates). The resulting MRBAYES tree is visualized and edited in FIGTREE (v1.4.2, Andrew Rambaut and members of his research group Molecular Evolution, Phylogenetics and Epidemiology, Edinburgh, UK) including MRBAYES and BEAST posterior probabilities (pp), as well as bootstrap support values.

Additionally, a dated tree using only one specimen for each *Hyles* species (clade), resulting in 22 taxa, was reconstructed in BEAST v.1.8.2 (Drummond et al. 2012) using a chain length of 11 million states with trees samples every 1000th state. The tree prior was set to the birth–death model (Stadler 2009). A ML tree was calculated with RAxML (Stamatakis 2014) and applied as the starting tree to provide a valid initial state. The data comprised COI, COII and additional nuclear EF1alpha (772 bp; from Hundsdoerfer et al. 2009) sequences with a total aligned length of 2985 bp (Table S5). EF1alpha sequences were missing for *H. renneri* and *H. salangensis* (filled with Ns). The best partitioning scheme and evolutionary models were determined with PARTITIONFINDER 1.1.1 (Lanfear et al. 2012) using the AICc criterion, the ‘BEAST’ model set and the greedy search algorithm. The best scheme was determined to be a seven-partition scheme; see Table S3, for details. BEAST log files were examined for convergence and sufficient ESSs with TRACER v1.6. The first 1000 trees were subsequently removed as burn-in. The remaining 10 000 trees were summarized using TREEANNOTATOR 1.8.2 (Andrew Rambaut and members of his research group Molecular Evolution, Phylogenetics and Epidemiology), and common ancestor heights were annotated on the maximum clade credibility tree. Additionally, we visualized the entire tree sample as a graphical cloud representation using DENDRITREE v2.2.1 (Bouckaert & Heled 2014) (Fig. S2). All topologies recovered in our analysis are illustrated including branch lengths. This plot shows the uncertainty with regard to topology and branch lengths (i.e. node age). Uncertainty observed at nodes with a posterior probability of one pertains
exclusively to node age and not to topology. For comparison, a ML tree was calculated with a thorough RAxML search (100 replicates best tree search; GTR+GAMMA; thorough bootstrap autoMRE, ending after 1000 replicates, Fig. S3).

For inference of divergence time estimates, we calibrated our phylogeny using three calibration points (positions in the tree and time intervals are shown in Fig. 2). Because of a lack of reliable fossils, we calibrated the root node (1) with a previous mean age estimate for *Hyles* from the fossil-biogeographic dating by Kawahara & Barber (2015). We applied a second calibration (2) to the split of the Hawaiian *Hyles* clade from its sister crown clade corresponding to the volcanic age of the oldest islands of the Hawaiian archipelago Kauai and Niihau (5.1 Ma) (see dating approaches in Fleischer et al. 1998; Lerner et al. 2011). The two endemic species from Hawaii, *Hyles calida* (Butler, 1881) and *Hyles perkinsi* (Swezey, 1920), are restricted to the younger central island group of Oahu and Molokai (*H. calida* is also present on Kauai). We therefore applied a third calibration (3) to the split among the two Hawaiian endemics according to the younger volcanic age of 3.7 Ma for the respective islands (see Fleischer et al. 1998). As recommended by Drummond & Bouckaert (2015), we validated our priors by performing BEAST runs while sampling from the prior only. We performed two test runs, one with node ages applied as hard maximum ages through uniform priors and a second run where we applied normal priors and a second run where we applied normal priors restricting the lower age to 5.1 Ma to avoid conflicts with the other priors.

**Ancient range estimation**

For reconstruction of the input tree for ancestral range estimation, ancestral distribution ranges of clades were estimated using the R package BIOGEOBEARS 0.2.1 (Matzke 2013). The range of *Hyles* was divided into 10 regions (map in Fig. 3, Table 1) and the ranges of taxa in our tree coded in a presence/absence matrix (Table S6). We used BIOGEOBEARS to test the fit of three commonly used biogeographic models on our data: dispersal–extinction–cladogenesis (DEC) (Ree & Smith 2008); ML versions of dispersal–vicariance analysis (DIVALIKE; Ronquist 1997); and Bayesian biogeographic inference (BAYAREALIKE; Landis et al. 2013). In addition, we tested whether models that allow founder event speciation (+j models; Matzke 2014) had a better fit on our data. We employed a stratified analysis, disallowing occurrence on Hawaii prior to 5.1 Ma. Model selection was carried out using the AICc criterion.

**Ancstral state reconstruction**

We used BEAST v. 1.8.2 to reconstruct ancestral states of host plant use and the condition of the arolium concurrently with estimation of phylogeny. Character matrices (Table S7, coded by AKH and IJK) were imported into BEAST as a discrete trait partition (if the host plant was unknown, it was coded as missing and the state was reconstructed). Larval host plant data were extracted from an extensive literature database maintained by IJK that also includes personal communications from amateur breeders. Output data were extracted from the maximum clade credibility tree using custom R scripts; pie charts were plotted onto trees using the R package ggtree.

**Results**

Newly generated sequences of 33 Palaearctic *H. gallii*, 14 Nearctic (formerly named) *H. gallii intermedia*, three (formerly named) *H. gallii tibetanica*, seven (formerly named) *H. costata* and 17 species of the *H. livornica* clade were imported into the R package BIOGEOBEARS to test the fit of three commonly used biogeographic models on our data: dispersal–extinction–cladogenesis (DEC) (Ree & Smith 2008); ML versions of dispersal–vicariance analysis (DIVALIKE; Ronquist 1997); and Bayesian biogeographic inference (BAYAREALIKE; Landis et al. 2013). In addition, we tested whether models that allow founder event speciation (+j models; Matzke 2014) had a better fit on our data. We employed a stratified analysis, disallowing occurrence on Hawaii prior to 5.1 Ma. Model selection was carried out using the AICc criterion.

**Table 1 Ancestral biogeography analysis**

| OTU | Occurrence codes |
|-----|------------------|
| *Hyles annel* | B |
| *Hyles biguttata* | D |
| *Hyles calida* | C |
| *Hyles centralasiae* | H, I |
| *Hyles costata* | I |
| *Hyles dahlii* | G |
| *Hyles euphorbatiae* | G, H, I |
| *Hyles euphorbiarum* | B |
| *Hyles gallii clade* | A, G, H, I, J |
| *Hyles hippophaes* | G, H, I |
| *Hyles lineata* | A, B, C |
| *Hyles livornica clade* | D, F, G, H, I, J |
| *Hyles livonicoides* | E |
| *Hyles nicaea* | G, H, J |
| *Hyles perkinsi* | C |
| *Hyles renneri clade* | J |
| *Hyles salangiensis* | H |
| *Hyles siehei* | G, H |
| *Hyles stroehlei* | H |
| *Hyles thymali* | G |
| *Hyles vespertilio* | G |
| *Hyles zygophylli* | G, H, I |

Species occurrences used for the BIOGEOBEARS analysis.

Region codes as in Fig. 3: A, Nearctic; B, Neotropics; C, Hawaii; D, Madagascar; E, Australia; F, Africa (south of Sahara); G, Western Palaearctic (incl. N Africa); H, Central Palaearctic (excluding Himalaya); I, Eastern Palaearctic; J, Himalaya.
H. nepalensis, four H. livornica, 14 H. renneri and eight H. salangensis specimens were included in the analyses. Of all included sequences, the one with the highest proportion of missing data was # 9468 (the paratype of H. gallii tibetanica) with 73% of positions missing.

**Phylogeny and systematics**

*Hyles* is reconstructed as a robust monophylum (Fig. 1) by both Bayesian implementations and ML (full support with MRBAYES & BEAST and 95% support with RAXML) aged 6.6 Ma B.P. (5.1–8.0 Ma; Fig. 2). The topologies of the three mitochondrial trees differ only in intraspecific details and interspecific groupings within the crown clade X (Fig. 1; complete loss of the arolium, see below), which is not in the focus of this paper, and an unsupported, alternative placement of *H. salangensis* in the BEAST tree (Fig. S1; details below) as sister group to all Palaearctic *Hyles*. Uncertainty in the data is further illustrated by the tree cloud based on the 22-taxon data set (mitochondrial and nuclear genes; Figs S2, S3; e.g. the placement of *H. livornica* forms the sister group of all other Palaearctic species. The species from the Neartic, Neotropics, Madagascar, Australia and Hawaii branch off first and form the basal grade (Figs 1 and 2 S1-S3). The crown group (twice full pp support; 73% ML; Fig. 1) includes all the Palaearctic species, including the migratory *H. gallii* (also occurs in the Neartic) and *H. livornica* (also occurs in Africa and on Madagascar). Our study provides molecular data of a Madagascan *H. livornica* specimen for the first time, and confirms the synonymy with this species of the former *Hyles malgassica* (Denso, 1944) (*contra* Eitschberger 1999a and Eitschberger & Surholt 1999).

*Hyles salangensis* is a fully supported lineage (2× full pp support; 100% ML) that forms the sister group of a clade comprising the Palaearctic species *H. nicaea* + the Holarctic *H. gallii/H. nepalensis* clade (Figs 1 and 2). Together, this clade has mixed support (very high in MRBAYES pp = 0.98; ML bootstrap 67%; no support in BEAST, see Fig. S1) and forms the sister group of all other Palaearctic species.

*Hyles gallii* is a clade with mixed support (Fig. 1; twice full pp support; 74% ML), with one reproducible subgroup (labelled ‘H. gallii subgroup’ in Fig. 1) that also has mixed support (BEAST pp = 0.99, MRBAYES pp = 1, ML 55%). This group comprises the majority of samples from the Neartic (23), together with the eight *H. gallii* specimens from northern India (Kashmir, Ladakh, # 4758, 4774, 4777, 4779, 4780, 4783, 5384, 6499), the three types of the former *Hyles tibetanica* (# 9452, 9468, 9469) and another *H. gallii* from Tibet (# 6521). The sequences of all three *H. gallii tibetanica* types obtained by us were identical. BOLD sequence SPUEB497-07 (individual # 1437 from the EMEM, see Table S1) is also from the holotype of *H. gallii tibetanica*. This specimen was sequenced independently by us as # 9469, and apart from the Ns, the two sequences do not differ. *Hyles gallii* as a whole forms a clade with full pp support (2× and 74% ML support; Fig. 1). Internal differentiation within the *H. gallii* clade is negligible, as the average pairwise sequence divergence is only 0.6% (p-distance; pairwise deletion). Values >2% occur only in comparisons involving # 6511 from Nepal and # 1437; there is 3.5% between # 6511 and # 1437, data not shown; the two individuals are indicated with arrows in Fig. 1.

The samples of *H. renneri* and *H. livornica* form two well-defined, but mixed clusters, each with full or near full support in BEAST (pp = 1 & 0.99, respectively; Fig. 1), but much lower support in MRBAYES (pp = 0.89 & 0.76) and under ML (74%/61%). Two moths from Nepal determined as *H. renneri* cluster with *H. livornica* (Fig. 1: # 4787, 4789). The ten other *H. renneri* moths (from Nepal and the neighbouring Tsou-la Pass in Tibet; 2020–4500 m a.s.l.; Table S1), including the holotype and three para-types, form the fully supported sister clade (Fig. 1). The two specimens of high-elevation *H. livornica tatsienluica* (# 4757 and the syntype # 6524) group in the *H. renneri* clade.

**Ancestral range analysis and dating**

Model selection with BIOGEOBEARS showed the BAYAREA-LIKE+J model to have the best fit on our data (Table 2). Ancestral ranges at nodes towards the root of our *Hyles* phylogeny were reconstructed as highly equivocal (Fig. 3), but the Neotropics has the highest probability for the two oldest nodes. Indeed, the three terminal species of these two nodes all occur in South America, two of them exclusively. According to our dating approach, the radiation of *Hyles* began in the Late Miocene (Fig. 2) with the two New World clades as the earliest offshoots.

In the early Pliocene [mean: 4.67 Ma, 95% highest posterior density confidence interval (HPD): 3.49–6.06 Ma], an Old World clade of the Southern Hemisphere split from a large clade of species occurring exclusively in the Northern Hemisphere. The clade formed by the Madagascan and Australian endemics, *H. biguttata* and *H. livornicoides*, has mixed support based on mitochondrial data alone (Fig. 1; MRBAYES pp = 0.90, very good BEAST support pp = 0.99; 58% ML bootstrap) and no support in the 22 taxa data set including nuclear data (BEAST pp = 0.72, Fig. 2; not supported under ML, Fig. S3). The alternative scenario, differing from that of the best tree (i.e. the maximum clade credibility tree, red in Fig. S2), would be a successive and sequential divergence of these two taxa over a short period of time. This topology occurs frequently in the total tree sample, as denoted by two separate blue lines,
Fig. 1 Phylogenetic hypothesis of *Hyles* based on mitochondrial sequences (1531 bp COI & 681 bp COID) analysed using MrBayes. OTU labels show former, partly invalid names, whereas the names to the right of clades represent the valid (and new) taxonomy. Three support values are given on branches in the form 0.98/0.99/58. Branches to OTUs of species that are not the focus of this work were collapsed for clarity and the farthest out-group species (see Table S1) were pruned. Maximal intraspecific distance of 3.5% within *Hyles gallii* is found between the two individuals marked with an arrow (# 6511 and # 1437). HT: holotype specimen, PT: paratype specimen, ST: syntype specimen. [Colour figure can be viewed at wileyonlinelibrary.com]
the first forming a branch from the stem to *H. biguttata*, and the second a branch from the stem to *H. livornicoides* (Fig. S2). The ML tree (Fig. S3) corroborates this alternative topology.

The separation of the Hawaiian lineage from its sister clade (*BEAST* pp = 1, ML bootstrap 87%; Fig. 2) occurred in the middle to late Pliocene (mean 4.01 Ma; 95% HPD: 2.95–5.12 Ma). The biogeographic origin of the most recent common ancestor is estimated to have been in the Palaearctic region. The split between the two island endemics (full Bayesian and ML support) occurred towards the end of the Pliocene or in the early Pleistocene (mean: 2.21 Ma; 95% HPD: 1.53–2.94 Ma). As expected, both of these divergences were dated as being more recent than the maximum age of the respective islands.

The sister clade of the Hawaiian lineage comprises a large group of 15 species representing a Palaearctic radiation (*BEAST* pp = 0.99, ML bootstrap 74%) that was dated to 3.53 Ma (95% HPD: 2.59–4.58 Ma; Fig. 2). With the exception of the migratory *H. livornica* and *H. gallii*, the species of this clade have purely Palaearctic distributions. Both *H. livornica* and *H. gallii* show very recent (less than ~1 Ma) dispersal events, into southern Africa/Madagascar and the Nearctic, respectively. The ancestral area of this
Fig. 3 Ancestral biogeography and larval host plant analyses of the 22-taxon tree of *Hyles* as estimated with BEAST, depicting the results of ancestral range (above nodes) and host plant estimations (below nodes). The map and bar show, respectively, the colour coding of regions (Table 1) and larval host plants (Table S7). Asterisks indicate species with unknown host plant associations (character states thus represent estimations). [Colour figure can be viewed at wileyonlinelibrary.com]
Ancestral state reconstruction: phylogeny to ecology and morphology

Reconstruction of ancestral larval host plant use (Fig. 3) returned an unambiguous result for all but three nodes, in that the largest segment is >50% (i.e. one host plant character state represents the majority). Larval host plant use at the origin of *Hyles* was reconstructed as ‘polyphagous’, as were most of the deep nodes within *Hyles* with the exception of the three basal ambiguous nodes of the stem of the crown group (*Hyles vespertilio* to *Hyles tithymali*). Three shifts to other plant families occur on internal nodes: to Rubiaceae in the Hawaiian clade; to Asphodelaceae in *Hyles siebeli* + *H. centralasias*; and to Euphorbiaceae in the crown clade. All other shifts in host plant use occur on terminal branches, including a second, independent shift to Euphorbiaceae in *H. nicaea*. The larval host plant ranges of *H. salangensis* and *H. remneri* are both unambiguously reconstructed as most likely being polyphagous (Fig. 3).

With regard to the arolium, ancestral state analysis produced unambiguous reconstructions at all nodes (Fig. S4). Complete loss of the arolium occurred at the node comprising the species from *H. vespertilio* to *H. tithymali*, with a second, independent loss along the branch leading to *H. salangensis*. A fully developed arolium is present in the Hawaiian clade. The ‘reduced and apparently non-functional’ state was reconstructed for all other nodes, including the root.

Discussion

Phylogeny and biogeography of the three enigmatic taxa

*Hyles salangensis*. The phylogenetic position of *H. salangen-
sis* is perhaps surprising in view of the similarity of its forewing pattern to those of several other *Hyles* species that occur in the same general area, particularly *H. stroeblei*, *H. robertsi* and, to a lesser extent, *H. nervosa* (Kitching 2016). Some *H. stroeblei* resemble *H. salangensis* in having paler scales near the costa and a narrower postmedial band. Other *H. stroeblei* individuals appear extremely similar to *H. nervosa* (not analysed in this study), in that the scales near the costa are rather dark olive brown and the veins traversing the broad postmedial band are highlighted with pale scales (Kitching 2016). Other *H. salangensis* are very similar to *H. robertsi*, which occurs in eastern Afghanistan as subspecies *Hyles robertsi elisabethae* Ebert, 1996. *Hyles salangensis* has also been observed to ‘resemble a dark hybrid between *H. hip-
pophaes* and *H. euphorbiace*’ (A.R. Pittaway, personal commu-
nication confirmed 27.09.2012). However, the phylogenetic positions of both *H. stroeblei* and *H. robertsi* are very far from *H. salangensis* (Hundsdoerfer et al. 2009, 2011; see also Fig. 1). We cannot comment on *H. nervosa*, as we have not yet been able to include any confirmed samples in an analy-
is. An ambiguous topological position for *H. salangensis* based on mitochondrial data alone is found by the Bayesian reconstruction with BEAST (Fig. S1). The species groups without support (pp = 0.01) on an extremely short branch as the sister taxon of all other Palaearctic species, essentially leading to a trichotomy: *H. salangensis*, *H. nicaea* + *H. gallii* and a clade of the remaining Palaearctic species, which may then be expected to result in a slightly earlier speciation of *H. salangensis*. However, MrBayes (Fig. 1) and the addition of nuclear sequence data and RAxML (Fig. 2, Fig. S3) reconstruct a more precise branching pattern with high pp support (0.97 Fig. 1, 1 in Fig. 2), albeit with a lack of high ML bootstrap support (67% Fig. 1, 65% Fig. 2, Fig. S3). Thus, given the data currently available, *H. salangensis* is a clearly delimited species that is closely related to *H. gallii* and *H. nicaea* (Figs 1 and 2).

The complete lack of an arrolium on the pretarsus in *H. salangensis* suggested that the species might be closely related to the crown clade, that is the species from *H. ves-
pertilio* to *H. titymali* (Figs 1–3, full support), members of which also lack this structure. An arrolium is present in all other *Hyles* (usually ‘reduced and apparently non-func-
tional’, but fully developed in the Hawaiian clade). Our ancestral state reconstruction indicated that the arrolium had been independently lost in the crown clade and in *H. salangensis*. The character state for the most recent common ancestor of all Palaearctic species (Fig. S4) could also be expected to change from ‘reduced and apparently non-functional’ to equivocal or absent for the alternative position of *H. salangensis* (Fig. S1). However, aside from polytomies (which essentially is the case) being unusable for reconstruction, this branching pattern is based purely on mitochondrial data and is not otherwise supported.
The ancestral biogeographic origin of *H. salangensis* was reconstructed as the Central and Eastern Palaearctic (which would not change if the above-mentioned alternative topology was to be used instead, Fig. S1). However, with so very few individuals collected, the full extent of its distributional range remains unknown. The only reliably identified specimens are the 15 males collected in the mid-1960s that constitute the type series. Although this rare species appears to be endemic to the mountain ranges of north-east Afghanistan, more extensive sampling might uncover further occurrences. The species’ host plant is unknown, but it was reconstructed as ‘polyphagous’ with around 65% probability (again, regardless of the species’ placement, Fig. S1).

*Hyles livornica/H. renneri*. The studied samples determined as *H. renneri* and *H. livornica* form two mixed clusters that are inconsistent with their *a priori* identifications based on a combination of adult external appearance and collection site data (in particular, altitude; Fig. 1). The two moth clusters with *H. livornica* but were initially determined as *H. renneri* (# 4787 from 1250 m a.s.l. and # 4789 from 3000 m a.s.l.; Fig. 1, Table S1) do appear somewhat paler in wing coloration when compared with the moths that form the *H. renneri* clade, including the types. This implies that these two moth samples had been misidentified as *H. renneri*. One of these paler moths (# 4787) was collected only 50 km from the type locality of *H. renneri*, but the latter is 2750 m higher. The second (# 4789) was captured at 3000 m a.s.l., the same altitude as *H. renneri* # 4788, but the two sites are almost 200 km apart.

We were also able to verify the diagnostic features of the female genitalia of *H. renneri* (much larger overall, with a longer signum compared to *H. livornica*) given by Danner et al. (1998) for the moths from Bagarchap (# 4375, 2200 m) and Tsou-la Pass (# 4373, 4500 m), thus confirming the range extension of the species into southern Xizang Zizhiqu (Tibet). As noted above, the pale Nepalese female from Kakani (2070 m; NHMUK sphingid genitalia preparation # 963) studied by Kitching & Cadiou (2000), and which in part led them to synonymize *H. renneri* with *H. livornica*, has small genitalia and a short signum. They were thus correct in determining this specimen as *H. livornica*. However, they were unable to study first-hand the genitalia of a high-elevation dark female and so missed the crucial distinction in size and signum length, leading them incorrectly to synonymize *H. renneri*. Nor, however, were Danner et al. (1998) entirely correct to separate the two species solely on geography for it is now clear that *H. renneri* and *H. livornica* both occur in central and east Nepal.

On the basis of the Nepalese and Tibetan samples studied here, *H. renneri* appears to be a higher-elevation species occurring down to about 2000 m, whereas *H. livornica* is a more low-elevation species, but ranging up to 3000 m. Thus, the two species potentially occur sympatrically between 2000 and 3000 m, although interestingly they have not yet been collected together in the same locality. At least within Nepal, wing coloration does appear to provide the means to separate the darker *H. renneri* from the paler *H. livornica*, with female genital structure providing confirmation.

The two sequenced specimens (including a female syntype, # 6524) of *H. livornica tatsienluica* also have dark wing coloration, and both group within the clade that includes the *H. renneri* holotype and several paratypes. These two specimens were, however, collected 1800 km from the type locality of *H. renneri*, and thus, they represent a major extension to the distribution range of this species from its previously known localities in Nepal and southern Xizang. We examined the genitalia of syntype # 6524 (NHMUK specimen BMNH # 812462, NHMUK sphingid genitalia preparation # 963), but they were too damaged to confirm their structure (the corpus bursae was missing). However, a third female in the NHMUK from Ta-tsen-lou (NHMUK specimen BMNH # 812888, NHMUK sphingid genitalia preparation # 962) proved to have the large genitalia and long signum diagnostic of *H. renneri* (and the locality is also at high elevation, ~3000 m a.s.l.). Unfortunately, tissue of this moth was not available for sequencing. Nevertheless, we consider that the DNA evidence from syntype # 6524, together with that of one other specimen from the type locality (Fig. 1; see above), together with the morphological evidence provided by the genitalia of BMNH # 812888, is sufficiently strong to justify reinstating *H. tatsienluica* stat. nov. as a valid species and synonymizing *H. renneri* syn. nov. with it.

However, the situation regarding both forewing colour intensity and locality elevation is less clear cut when a broader perspective is taken. A female in the NHMUK from ‘Poo Bashahr state, Schipki-la, 4000 m’ (now Shipki La, a pass on the NW India–Tibet border) has a dark forewing pattern but the small genitalia and short signum of *H. livornica*. This shows that in this area, *H. livornica* can reach the same extremely high elevations as *H. tatsienluica* does in Nepal, but also suggests that when it does so, its general coloration darkens. A second dark female in the NHMUK from ‘Amur’ also has the genitalia of *H. livornica*. Although the locality is very vague, there is very little land above 1000 m in Amurskaya and Khabarovsk Krai, and so the moth is almost certainly from a low elevation, though possibly from a relatively cool site. Although we now accept the differences in female genitalia between *H. livornica* and *H. tatsienluica*, we concur with Kitching & Cadiou (2000) that the purported diagnostic differences in the male genitalia do not hold and confirmation of identity cannot rest solely on wing colour.
In summary, *H. tattsienluica* is a valid species, separate from *H. livornica* that can be distinguished on female (but not male) genital morphology and COI–COII sequences. Where the two species occupy the same general area in Nepal (and maybe SW China), *H. tattsienluica* is darker and occurs at higher elevations, whereas *H. livornica* is paler and flies at lower elevations, but with a considerable overlap between about 2000 m and 3000 m. This may be due to seasonal movements of *H. livornica* to higher altitudes in summer or perhaps hill-topping behaviour in males (as is suspected to occur in other Sphingidae; Holloway 1987). Thus, larval habitats may be located well away from localities in which adults are observed. The species is a well-known migrant in Africa and Western Europe, and there is no reason to suspect such behaviour is absent in the Nepalese populations, though its ecology there remains to be investigated. The type locality of *H. renneri* would seem to be near the far western end of the distribution of *H. tattsienluica* and is simply the place where the species is currently most accessible. The Tsou-la Pass locality in southern Xizang may be near the centre of the species’ range, connecting the western end in Nepal with Ta-tsien-lou, which may represent the eastern end of its distribution. *Hyles tattsienluica* would thus appear to be a Tibetan Plateau species, but the exact limits of its range have yet to be fully determined.

With respect to the distribution range of *H. livornica*, contra Eitschberger (1999a) and Eitschberger & Surholt (1999) and in agreement with the revision by Kitching & Cadiou (2000), our data confirm the synonymy of *H. malgassica* with *H. livornica* (Fig. 1).

*Hyles gallii*. In contrast to the situation with *H. tattsienluica* and *H. livornica*, the seven individuals of the former *H. nepalensis* studied (all of which come from very high elevations of 3000–4000 m) show no distinct cluster formation. They group among *H. gallii* from the UK, Germany, Czech Republic, Finland and China (Shaanxi) (Fig. 1) with very little genetic differentiation. The type locality of *H. gallii* is Germany and as the present clade includes two samples from that country, as well as the holotype of *H. nepalensis*, we consider it to represent the ‘true’ *H. gallii* and hence agree with Kitching & Cadiou (2000) that the name *H. nepalensis* is a synonym of *H. gallii*.

Nearctic *H. gallii* can be separated morphologically from most Palaearctic *H. gallii* by the colour of the hindwing medial band and anal patch. Generally, in Palaearctic moths, the pink medial band distal to vein CuA2 is largely white and the anal patch is large, circular and white. The hindwings of moths from Nepal and from Lanak La in NW Xizang are likewise pale and so conform to the Palaearctic phenotype. In contrast, the entire medial area in Nearctic moths is more uniformly suffused with pink and the anal spot reduced in size (see illustrations in d’Abrera [1987]: 184) and this was the main feature that has been used to justify subspecies status for the Nearctic populations as *H. gallii intermedia* (Kirby, 1837). However, the hindwings of *H. gallii* specimens from the Ladakh region of NW India near the border to Pakistan, about 850 km from the type locality of *H. nepalensis*, also show the same pink-suffused hindwing medial band as Nearctic moths, and it was just such specimens that Kitching & Cadiou (2000) used as evidence to justify the synonymy of *H. gallii intermedia*. Thus, we were most surprised to find that while some Canadian samples grouped with the phenotypically similar Indian moths in the *H. gallii* subclade (Fig. 1), others were scattered among samples from lowland Palaearctic sites in the basal grade (Fig. 1). If coordinates of the localities are plotted on a map, the Nearctic samples show no geographic pattern (data not shown). DNA sequence data provide no support for *H. gallii intermedia* as a separate Nearctic subspecies, suggesting an apparently very recent colonization of the Nearctic, or even for a broader geographic group based on a pinker hindwing medial band. Indeed, the low level of differentiation within the *H. gallii* complex as a whole reinforces the rejection, and confirms the synonyms, of all (former) subordinate taxa: *H. gallii intermedia*, *H. nepalensis* and *H. gallii tibetana*.

The origin of *Hyles* and possible early colonization routes

The genus *Hyles* was first postulated to be of New World origin during the Oligocene/Eocene and from there two major radiations across the Southern and the Northern Hemisphere were suggested (Hundsdoerfer et al. 2005, 2009). However, the estimated ancestral ranges for the three basalmost nodes of our *Hyles* phylogeny were highly equivocal (Fig. 3), so our analyses could not corroborate or refute such a scenario, so calling into question yet again the geographic origin of *Hyles* hawkmoths. If the alternative topology (Fig. S3) were to be used for the reconstruction (which, as a ML tree, it cannot), the nodes along the main stem would also be inferred as equivocal and so not change the result. In addition, the temporal scenario must also be reconsidered, because recent molecular dating approaches have suggested a much more recent origin and radiation of *Hyles* hawkmoths. Although examples of Oligocene to Early Miocene globally distributed butterfly radiations exist (*Junonia* Hübner, 1819, Nymphalidae, Kodandaramaiah & Wahlberg 2007; *Mycalesina* Reuter, 1896, Nymphalidae, Aduse-Poku et al. 2015), the origin of *Hyles* was recently dated by Kawahara & Barber (2015) to the Middle to Late Miocene, when proto-*Hyles* separated from the Neotropical *Xylophanes* and African *Chaerocina*.
about 10 Ma. During this period, several events of transcontinental faunal interchange between the Old World, particularly East Asia, and the Nearctic via Beringia have been suggested for some butterfly genera (Papilio Linnaeus, 1758, Papilionidae, Wu et al. 2015; Oeneis Hübner, [1819], Nymphalidae, Kleckova et al. 2015; Polyommatus (von Rottemburg, 1775), Lycaenidae, Vila et al. 2011).

Diversification within Hyles itself, however, has to be considered much more recent and was presumably unrelated to Miocene climatic oscillations and successive submergence or emergence of land bridges. According to our time-calibrated phylogeny, the global Hyles hawkmoth radiation began in the Late Miocene.

Australia and Madagascar were colonized by the grouping without statistical support comprising H. livornicoides and H. biguttata, which split from all other Hyles 4.7 Ma B.P. (3.5–6.1; Fig. 2). Colonization routes have been previously postulated via Antarctica or Fiji (Hundsdoerfer et al. 2005) under the assumption of the alternative tree topology in which the Australian and Madagascan species branch off successively (Figs S2 and S3). The route via Antarctica still remains a plausible scenario for both Australia and Madagascar with the topology recovered in this study, in which H. livornicoides and H. biguttata are sister species. Although ice sheets have been present on Antarctica over the past 40 million years, they have been extremely dynamic (Zachos et al. 2001). A major ice sheet had re-established on Antarctica by 10 Ma, but the early Pliocene is marked by a subtle warming trend between 6 and ~3.2 Ma (see Zachos et al. 2001 and references therein). Thus, Antarctica may have been able to serve as at least a stopover for the ancestors of this clade. The alternative hypothesis of a westward dispersal via (proto-) Polynesian, Melanesian, Micronesian islands now seems less likely, considering the newly recovered sister group relationship between H. biguttata and H. livornicoides. However, this topology received no bootstrap support in the ML analyses (58% Fig. 1, ≤50% Fig. 2 and 0 Fig. S3) and the ancestral range was estimated to be highly equivocal, thus leaving this aspect of Hyles biogeography open and requiring further study. In contrast to previous hypotheses, our results indicate that the ancestors of the Hawaiian clade colonized Hawaii from the Palaearctic and not the Nearctic, corroborating the scenario postulated by Hundsdoerfer et al. (2005) (via the Bering Strait). This would not change if the alternative topology (Fig. S3) were to be used for the reconstruction.

**Pliocene origin and Pleistocene diversification in the Palaearctic**

The Hyles hawkmoth radiation culminated in a burst of diversification towards the end of the Pliocene (starting at ~4 Ma) resulting in no fewer than fifteen extant Palaearctic species, and from where two migratory species, H. gallii and H. livornica, more recently colonized the Nearctic and Africa/Madagascar, respectively. Despite some topological ambiguity within the crown group comprising the species from H. vespertilio to H. titymbali (Fig. 2 vs. Fig. S3), and in the position of H. salangensis (Fig. S1), the ancestral ranges of these stem nodes are all reconstructed as Palaearctic (except the Himalaya) without ambiguity. This result is not expected to change with alternative branching patterns.

At a first glance, our age estimate of ~6.5 Ma for Hyles appears quite young considering that during this short time interval, Hyles hawkmoths colonized all continents. However, similar temporal scenarios have been reconstructed for a number of butterfly taxa. For example, from the early Pliocene onwards, three subgroups of cold-adapted butterflies of the genus Oeneis diversified and progressively dispersed from Central Asian ancestral ranges into the Northern and Western Palaearctic and the Nearctic (Kleckova et al. 2015). Similarly, at about 5 Ma several ‘Out-of-Asia’ dispersals into Africa, Australia and the New World were hypothesized for butterflies of the genus Junonia (Kodandaramaiah & Wahlberg 2007). For these global butterfly radiations, dispersal was considered a more important factor than vicariance (Kodandaramaiah & Wahlberg 2007) and climatic niche diversification was shown to promote speciation processes in cold-adapted taxa (Kleckova et al. 2015). These findings are paralleled by a number of Pliocene bursts of diversification in birds. At about 6 Ma, Turdus Linnaeus, 1758 thrushes began diversifying in a global radiation that included repeated transatlantic dispersals (Voelker et al. 2009), and an early Pliocene onset of an ‘Out-of-Africa’ radiation including several transcontinental dispersals gave rise to sixteen species of Apus Scopoli, 1777 swifts (Päckert et al. 2012; Tietze et al. 2015).

Furthermore, the effect of global climate cooling towards the end of the Pliocene is most striking in mountain ecosystems, where cold-adapted species started occupying the newly emerging temperate and boreal niches, for example, in the Himalayas (global review in Fjelds et al. 2012; birds: Päckert et al. 2012). Particularly in birds, there is evidence that the elevational aspect of the niche occupied by species evolves late during evolutionary history, with a steep increase in elevational disparity occurring from about 5 Ma to a peak at the beginning of the Pleistocene (Price et al. 2014). Elevational parapatry of Hyles hawkmoths in the Himalayas is complex and also dates back to a mid-Pliocene lineage separation. The elevational ranges of the sister species H. livornica (low elevations) and H. tattsienluica (moderate to high elevations up to alpine environments at 4000 m and above) partly overlap. H. gallii and H. nicaealatyrus (Walker, 1856)
occur in local sympathy depending on the abundance of the larval host plant, *Euphorbia stracheyi* (Kitching & Cadiou 2000; Smetacek & Kitching 2012).

Furthermore, *Hyles* diversification during the Pleistocene epoch was limited exclusively to the Northern Hemisphere. Examples of Pleistocene diversification in other Lepidoptera mostly refer to single species or species pairs that (like *Hyles* hawkmoths) split into several genetic lineages across wide distribution ranges. One of the most widely distributed butterfly species, *Lampides boeticus* (Linnaeus, 1767) (Lycaenidae), started diversifying in the early Pleistocene (2.3–1.5 Ma) and its demographic history and extant distribution across the entire Old World were apparently shaped by the species’ ability to undertake regional migrations (Lohman et al. 2008). In *Melitaea cinxia* (Linnaeus, 1758) (Nymphalidae), seven phylogroups correspond to a characteristic circum-Mediterranean diversification that includes outliers in the East Palaearctic and western China (Wahlberg & Saccheri 2007). Other examples of more recent late Pleistocene lineage separations were found in three *Parasinus* Linnaeus, 1758 (Papilionidae) species (Gratton et al. 2008; Todisco et al. 2012), the genus *Maniola* Linnaeus, 1758 (Nymphalidae; Kreuzinger et al. 2015) and *Lopinga achine* (Scopoli, 1763) (Nymphalidae; Kodandaramaiah et al. 2012). Even the enigmatic intraspecific structure found in *H. gallii* with two very young haplotype lineages distributed across the entire Holarctic is partly reflected in other butterfly groups, for example *Coenonympha tullia* (O. F. Müller, 1764) and allies (Kodandaramaiah & Wahlberg 2009) and four species (-pairs) within the Holarctic crown clade of Polyommatini (Lycaenidae, Vila et al. 2011). Alpine butterflies of the Holarctic *Parasinus phebus*, (Fabricius, 1793) complex also diverged into a Eurasian–Beringian and a Neartic lineage within the last 100 000 years (Todisco et al. 2012). However, *H. gallii* differs from these latter two examples in the lack of any clear phyleogeographic structure within or among Eurasian and Neartic haplogroups, which might be explained by a higher level of gene flow among *Hyles* hawkmoth refugia due to greater dispersal ability. The absence of support for a pattern of vicariance in the *H. gallii* clade contrasts markedly with the support found for intraspecific east–west splits in other clades, for example the Western and Central Palaearctic lineages of *H. hippophaes* and *H. nicaea* (not shown in Fig. 1 due to collapse of the clades concerned). A Pleistocene origin of Palaearctic east–west disjunctions is frequently suggested in passerine birds (Haring et al. 2007, 2012; Päckert et al. 2012) but has also been corroborated by divergence time estimates in butterfly species pairs/species triplets from the Western, Central and/or Eastern Palaearctic (*Oeneis glaucalis* Moll, 1763, *Oeneis norna* (Thunberg, 1791) and *Oeneis filla* (Eversmann, 1851), Kleckova et al. 2015; *Coenonympha* Hübner, 1819, several terminal clades, Kodandaramaiah & Wahlberg 2009).

**Hyles ecology in the light of phylogeny**

Compared with the butterfly taxa discussed above, the burst of Pleistocene diversification in *Hyles* hawkmoths is exceptional. Phylogenetic comparison across butterfly genera suggested that increased speciation rates were frequently associated with host plant shifts in the respective clades (Fordyce 2010; Ebel et al. 2015). In *Hyles*, the Palaearctic stem species was likely polyphagous (Fig. 3). Within the clade reconstructed to have originated in the Palaearctic (including the Hawaiian species pair), specializations onto single plant families were detected at three internal nodes (in addition to four more that are restricted to single species: *H. nicaea*, *H. vespertilio*, *H. zygophylli* and *Hyles costata*). Utilizing Rubiaceae may have enabled the radiation into three species on Hawaii (*H. wilsoni* was not sampled) and specialization on Elaeagnaceae (and specifically the flowers and young fruits rather than the leaves) may have facilitated the radiation of three species in Central Asia (see Kitching 2016). Utilizing *Euphorbia* as host plant may have led to an increased speciation rate (and at least five species) within the crown group, the *Hyles euphorbiae* complex (HEC). However, the phylogenetic relationships within the HEC are not supported, and much uncertainty is seen in the tree cloud (Fig. S2), corroborating the ambiguity surrounding species boundaries in this group found by Mende et al. (2016). This observation may simply be due to the extremely young age of this group (1.1 Ma, 95% HPD 1.53–0.69 Ma; Fig. 2), one in which speciation processes appear to be still underway. Even so, the data do indicate that accelerated speciation rates could be associated with host plant shifts in the respective clades (as in Ebel et al. 2015; Fordyce 2010), although this association is much under debate for Lepidoptera (e.g. Hardy & Otto 2014; Janz et al. 2016).

The function of the arolium as a means of clinging to smooth surfaces is well documented, although the fine structure, and thus the mechanism, varies among different groups of insects. While not apparently studied in detail, the lepidopteran arolium is a flat flexible cuticle that probably functions as an adhesive suction cup (as in Hymenoptera and Orthoptera). The closest relatives of *Hyles*, most of which live in relatively humid biotopes, all have large and well-developed arolia that may assist in clinging onto smooth surfaces such as waxy leaves, but in the arid/semiarid habitats that most *Hyles* inhabit, such an adhesive device may be functionally impaired (e.g. by dust or low humidity), accounting for its widespread reduction or loss in the genus. The fully developed arolium of the Hawaiian...
species, whether retained from the most recent common ancestor of the genus or re-acquired by character reversal, could then be hypothesized as having an as yet unknown adaptive advantage on these islands. Close ultrastructural study of the arolia of *Hyles* species may thus yield general insights into both the function and evolution of this organ.

**Conclusions**

The enigmas of the three high-elevation Himalayan *Hyles* brain-teasers were elucidated in the present study. *Hyles salangensis* and *H. tatsienluica* were both found to be valid species, with *H. renneri* being a junior synonym of the latter. The known range of *H. tatsienluica* was confirmed as extending from the mountains of south-western China to southern Xizang/Tibet and Nepal. Identification of specimens from the Himalaya and surrounding areas as *H. tatsienluica* on the basis of an overall darker appearance of the moths is not infallible. At present, only mtDNA sequencing of COI-COII and/or study of the female genitalia appear to allow unequivocal separation of the species from *H. licornica*, some uncertainty yet remaining due to the lack of nuclear data. In contrast, the status of the former *H. nepalensis*, *H. gallii tibetiana* and *H. gallii intermedia* as junior synonyms of the more widespread *H. gallii* is strongly supported by this study. The low genetic divergence within *H. gallii* indicates very recent trans-Holarctic dispersal and/or ongoing gene exchange across the immense Holarctic distribution range of this species. The vast distribution ranges and migratory behaviour of both *H. gallii* (Western Europe to Eastern North America) and *H. licornica* (Western Europe to Eastern Russia and Africa, Madagascar) appear to be significant hindrances to ancestral biogeographic analyses in *Hyles*. Our analyses were neither able to confirm nor refute a New World origin of the genus, nor postulate a plausible scenario for the colonization of both Australia and Madagascar by their respective endemic *Hyles* species. According to our time-calibrated phylogeny, the global *Hyles* hawkmoth radiation must be considered much more recent than previously thought: it began in the Late Miocene and culminated in a Pleistocene burst of diversification in the Northern Hemisphere. Our data indicate accelerated speciation rates may be associated with host plant shifts in three clades, for an example utilizing *Euaboria* as host plant in the crown group, the *H. euphorbiacae* complex.

**Acknowledgements**

We thank the curators who allowed us to study the specimens in their collections, especially M. Nuss (MDT Dresden), G. Martin (NHMUK London), Dr. S. Gaal-Hazsler (NHMV Vienna), T. J. Witt, Dr. W. Speidel & T. J. Greifenstein (MWM Munich), Dr. A. Hausmann & U. Buchsbaum (ZSM Munich) and Felix Sperling (UASM Edmonton). Thanks to Giovanni Faguen Gonzalez and Danny Shpeley (Edmonton) and Anja Rauh (Dresden) for insect leg sampling. This work was supported by the DFG (grant numbers HU 1561/1-1, 1–2 and PA 1818/3-1); and the EU’s Improving Human Potential programme SYNTHESYS (grant numbers GB-TAF-3410, GB-TAF-5177). Special thanks go to Elisabeth Hintelmann and the prize committee for the R.J.H. Hintelmann Prize (2007) for enabling analyses of the specimens from the museums in Munich (ZSM and MWM). We thank Anja Rauh (Dresden) for additional laboratory work to include more samples from the Nearctic and Eastern Palaearctic and Anke Mueller for laboratory support. Thanks go to Ingo Michalak (Leipzig) for valuable comments and suggestions on an earlier version of the manuscript. We thank Rodolphe Rougerie, Jeremy de Waard and the BOLD consortium, as well as the respective providers, for the DNA barcodes included for comparison. Finally, we thank Lutz Bachmann, two anonymous reviewers, as well as Matthias Nuss (also reviewer), for valuable suggestions that certainly much improved the manuscript.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

**Figure S1.** BEAST reconstruction of *Hyles* phylogeny.

**Figure S2.** “Tree cloud” representation of the phylogeny of *Hyles.*

**Figure S3.** Maximum likelihood reconstruction of the phylogeny of *Hyles.*

**Figure S4.** Reconstruction of the ancestral state of the arolium.

**Table S1.** Specimens studied
**Table S2.** Primers
**Table S3.** Best partitioning schemes and evolutionary models
**Table S4.** ESS values
**Table S5.** Sequences used for the 22-taxa dataset
**Table S6.** Input matrix for ancestral range estimation with BIOGEOBEARS
**Table S7.** Character matrix used for reconstruction of ancestral host plant use and the ancestral state of the arolium.