



Phylogeny and diversification of the cloud forest *Morpho sulkowskyi* group (Lepidoptera, Nymphalidae) in the evolving Andes

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The monophyletic *Morpho sulkowskyi* butterfly group, endemic of Andean cloud forests, was studied to test the respective contributions of Mio-Pliocene intense uplift period and Pleistocene glacial cycles on Andean biodiversity. We sampled nine taxa covering the whole geographical range of the group. Two mitochondrial and two nuclear genes were analysed using a Bayesian method. We established a dated phylogeny of the group using a relaxed clock method and a wide-outgroup approach. To discriminate between two hypotheses, we used a biogeographical probabilistic method. Results suggest that the ancestor of the *Morpho sulkowskyi* group originated during the Middle-Late Miocene uplift of the Eastern Cordillera in northern Peru, consistent with the divergence time of the *Chusquea* bamboos, the present hostplants of the group. Biogeographical inference suggests that the *M. sulkowskyi* and *M. lympharis* clades diverged in the northern Peruvian Andes. The subsequent divergences, from the Late Miocene to the Late Pliocene, should have resulted from a dispersal towards the Northern Andes (*M. sulkowskyi* clade), after the closure of the West Andean Portal separating the Central and Northern Andes, and a southwards dispersal along the Peruvian and Bolivian Eastern Cordilleras (*M. lympharis* clade). Only a few divergences occurred at the very end of the Pliocene or during the Pleistocene, a period when the more recent uplifts interfered with Pleistocene glacial cycles.

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INTRODUCTION

The Tropical Andes, the richest and most diverse region on Earth (Mittermeier *et al.*, 2004), are a good example of interactions between mountain building and diversification processes that decisively affected present biodiversity patterns (Hoorn *et al.*, 2013). To explain the diversification of floras and faunas throughout the vast and complex Andean mountains, different processes were considered, including dispersal and subsequent diversification driven by the rise of mountain ranges that create connections, barriers and new ecological belts, as well as more recent cycles of areas fragmentation and expansion driven by Pleistocene climatic oscillations (e.g., Descimon, 1986; Pennington & Dick, 2004; Wahlberg & Freitas, 2007; Antonelli *et al.*, 2009; Couvreur *et al.*, 2011; Condamine *et al.*, 2012; Blandin & Purser, 2013). That recent processes (Pleistocene glacial cycles) played a major role, or not, in the diversification of Andean lineages is a matter of debate (Rull, 2008, 2014, Garzón-Orduña *et al.*, 2014, 2015). Various studies, based on robust time-calibrated phylogenies, demonstrated that several plant and animal lineages diversified during the Mio-Pliocene intense uplift period (e.g., Chaves *et al.*, 2011; Fiedler & Strutzenberger, 2013; Castroviejo-Fisher *et al.*, 2014; Luebert & Muller, 2015; Sanín *et al.*, 2016). However, the role of Pleistocene geoclimatic dynamism has been suggested by other studies (e.g., García-Moreno *et al.*, 1999; Chesser, 2000; Casner & Pyrcz, 2010). As suggested by meta-analyses, the Mio-Pliocene and Pleistocene hypotheses are complementary (Turchetto-Zolet *et al.*, 2013; Rull, 2014).

Several works on diversification in the Andes focussed on butterflies or moths (e.g., Adams, 1985; Descimon, 1986; Willmott *et al.*, 2001; Hall, 2005; Elias *et al.*, 2009; Casner & Pyrcz, 2010; Strutzenberger & Fiedler, 2011; Matos-Maraví *et al.*, 2013; De-Silva *et al.*, 2016). The diversification of the Neotropics-endemic genus *Morpho* Fabricius, 1807 (Nymphalidae), that includes the emblematic, metallic blue butterflies, was studied by Penz *et al.* (2012), and its relation with the geodynamics of the Andes was discussed by Blandin & Purser (2013). This monophyletic genus (Cassildé *et al.*, 2010, 2012; Penz *et al.*, 2012) originated at ca. 32 Ma (Wahlberg *et al.*, 2009) in the east side of the proto-Andes (Penz *et al.*, 2012) and Blandin & Purser (2013). The genus diversified into species living in lowland forests, low mountain forests, and cloud forests, during a period of major and complex orogenic processes, high elevations being attained at different times in Central Andes and Northern Andes, as well as in western and eastern cordilleras (Garzzone *et al.*, 2008; Sempere *et al.*, 2008; Eude *et al.*, 2015). In order to discriminate between the Mio-Pliocene and Pleistocene diversification

hypotheses, Andean-endemic cloud forest butterflies should provide ideal models, as they diversified within a single ecological belt that could develop only when cordilleras attained suitable elevations. We have selected a group of common butterflies, the *Morpho sulkowskyi* group, that includes closely related taxa distributed from Colombia to Bolivia at 1.8-3.5 km altitudes (Krüger, 1924; Schultze, 1928; Salazar, 1998; Prieto *et al.*, 2005; personal observations in Bolivia and Peru - PB).

Here, we established a phylogeny to clarify the systematics of the group, and we used a dated phylogeny with distribution data to localize the origin of the group in time and space, and then to discriminate two biogeographical scenarios: 1) a recent vicariant diversification of a widespread species, as a result of the Pleistocene glacial cycles; 2) an older diversification, resulting from dispersal throughout the Andes driven by geodynamics processes, with two opposite predictions: i) origin in the Northern Andes (Colombia and Ecuador), with subsequent dispersal towards the Central Andes (Peru and Bolivia); or ii) origin in the Central Andes and dispersal towards the Northern Andes.

MATERIAL AND METHODS

Taxonomy

Morpho sulkowskyi Kollar, 1849 was described from Colombia. Several morphologically very similar taxa were subsequently described, which have been considered either as species or subspecies. Le Moult & Réal (1962) identified one polytypic and five monotypic species, but Lamas (2004) recognized only two polytypic species and Blandin (1993, 2007) only one (Appendix S1). In the present state of knowledge, we recognize 11 taxa of the species-group: *sulkowskyi* Kollar, 1849 (Colombian cordilleras); *lympharis* Butler, 1873 (from southern Peru (Cuzco department) to Bolivia (La Paz department)); *eros* Staudinger, 1892 (Bolivia, Cochabamba department); *sirene* Niepelt, 1911 (eastern Ecuador); *selenaris* Le Moult & Réal, 1962 (northern Peru, upper Huallaga valley, Huánuco department); *stoffeli* Le Moult & Réal, 1962 (central Peru, Pasco and Junín departments); *descimokoenigi* Blandin, 1993 (southern Peru, Cuzco department); *nieva* Lamas & Blandin, [2007] (northern Peru, Amazonas and San Martín departments); *calderoni* Blandin & Lamas, [2007] (Amazonas department); *achiras* Fisher, 2009 (northern Peru, Abiseo National Park, San Martín department); *zachi* Schäffler & Frankenbach, 2009 (northern Peru, San Martín department). Diagnostic characters are

indicated and taxa are figured, with their geographical distribution, in Appendix S1.

Field studies

Three recently described taxa from northern Peru (*calderoni* Blandin & Lamas, [2007], *nieva* Lamas & Blandin, [2007], *zachi* Schäffler & Frankenbach, 2009) were discovered on different slopes of the Eastern Cordillera in its northern part (Blandin & Lachaume, 2014). We performed field surveys to check for the allopatry or, alternatively, the sympatry of these taxa. After a first survey which allowed us to locate a key area close to the Carretera Transandina near the border between the Amazonas and San Martín departments, specimens were collected along two parts of the upper Río Nieva, that are separated by a mountain range that this river cut across through a narrow gorge (Fig. 1, N1 & N2). Specimens were also collected in neighbouring localities, notably in Oso Perdido (Amazonas) and El Afluente (San Martín) (Fig. 1, OP; EF). The collected specimens have been deposited either in the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (MUSM, Lima) or the Muséum National d'Histoire Naturelle (MNHN, Paris).

Molecular sampling

Field sampling along Río Nieva (Fig. 1, N1 & N2) provided specimens of *calderoni*, *nieva* and *zachi* (deposited in the MNHN). Specimens as recent as possible of *sulkowskyi* Kollar, 1849 (Colombia: Central Cordillera, Medellín area, Antioquia department), *sirene* Niepelt, 1911 (Ecuador: Baños area, Tungurahua province), *selenaris* Le Moulton & Réal, 1962 (Peru: Carpish area, Huánuco department), *lympharis* Butler, 1873 (Peru: Pillahuata area, Cuzco department), *descimokenigi* Blandin, 1993 (Peru: Cordillera Urubamba, Cuzco department), and *eros* Staudinger, 1892 (Bolivia: Incachaca area, Chapare, Cochabamba department) were selected from MNHN collections. There were no available specimens of *stoffeli* Le Moulton & Réal, 1962 (Junín department, Peru). The taxon *achiras* Fisher, 2009, from Abiseo National Park (San Martín department, Peru), could not be studied, as it is not represented in either MUSM or MNHN. Thus, we studied nine taxa and 91 specimens (Appendix S2a and Fig. 2), covering the whole geographical range of the group.

Total DNA was extracted from the legs or the abdomens of the specimens, using a Qiagen DNeasy Tissue kit. Two mitochondrial (Cytochrome oxidase 1 and Cytochrome b) and two

nuclear (EF1a - translation elongation factor 1 alpha and RPS5 - ribosomal protein S5) DNA fragments were amplified by polymerase chain reaction. For additional information on sequencing protocols, see Appendix S3.

Molecular phylogenetic analysis

The sequences of the two mitochondrial genes were concatenated for each individual, since both are from mitochondrial DNA. To conduct Bayesian analyses, the substitution model of evolution was estimated using jMODELTEST v 2.1.4 (Darriba *et al.*, 2012), and the Akaike information criterion corrected for small samples (AICc) (Akaike, 1973, 1974) was used to select the GTR+G model. Analyses were performed with MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003). Four Markov chains were run simultaneously for 50 million generations, sampling every 1000 generations to ensure independence of samples. The first 10 000 trees generated were discarded as burn-in and determined empirically from the log-likelihood values using TRACER V1.6 (Rambaut & Drummond, 2007). The remaining trees were used to construct 50% majority-rule consensus trees. Two independent runs were performed to check whether convergence was reached on the same posterior distribution and whether the final trees converged on the same topology. The statistical confidence of each node was evaluated by posterior probabilities. *Morpho anaxibia* (Esper, [1801]) (sub-genus *Iphixibia* Le Moult & Réal, 1962) was chosen as outgroup, as this species belongs to one of the two major *Morpho* clades (see Cassildé *et al.*, 2012; Penz *et al.*, 2012), while the *Morpho sulkowskyi* group belongs to the other.

Dating analyses

To minimize the effect of increased mutation rates at the intraspecific level in dating methods (Ho *et al.*, 2005; Ho, 2007), we used the Poisson Tree Processes (PTP) model to infer putative species boundaries (Zhang *et al.*, 2013). This method models speciation or branching events in terms of the number of substitutions, and can use the phylogenetic tree directly without needing the difficult and error prone procedures of time calibration required by other methods such as GMYC (Zhang *et al.*, 2013). Analyses were conducted for CO1 gene using the PTP web server (<http://species.h-its.org/ptp/>) to select one individual per cluster for dating analyses.

Because no suitable fossils for direct calibrations exist within our study group, and in order to be fully independent from regional geological knowledge, we attempted to use a balanced approach to calibrate our analyses using a wide-outgroup approach, which is considered to be a reasonable alternative (Hedges & Kumar, 2004; Strijk *et al.*, 2012). We extended a published dated genus-level dataset (Wahlberg *et al.*, 2009) with our new sequences and sequences available in GenBank for several other *Morpho* species. The sampling for the dating analysis contained 31 Nymphalidae butterflies (including 17 *Morpho* species) and three DNA fragments (CO1, EF1a and RPS5) (Appendix S2b).

To improve the estimates of node ages, we used multiple calibration points (Lee, 1999; Wang *et al.*, 1999; Sauquet *et al.*, 2012) on basal and apical nodes. We also checked whether all calibration points were compatible with the reference topology. The dating analysis was conducted using five secondary calibration points (Wahlberg *et al.*, 2009) with the entire dataset (Fig. 3A). The split between Calinaginae (*Calinaga davidis* Oberthür, 1881) and all other Nymphalidae was set at 75.7 Myr (standard deviation: 7.9 Myr, node A), the split between *Euxanthe* Hübner, [1819] and *Charaxes* Ochseneimer, 1816 at 18.1 Myr (4.6 Myr, node B); the diversification of Satyrini at 52.1 Myr (6.4 Myr, node C); the split between *Neope* Moore, 1866 and *Lethe* Hübner, [1819] at 36.6 Myr (5.0 Myr, node D), and the diversification of Morphini + Brassolini at 48.5 Myr (5.0 Myr, node E). The calibration of the node D is consistent with fossil data; two fossil *Lethe* species are known, the age of the oldest being estimated at ca. 32 Myr (Miller *et al.*, 2012).

We used a normal distribution for the tree prior to node calibration, which is particularly suitable for modelling secondary calibration points (Ho, 2007), with the same standard deviation as the Highest Posterior Density (HPD) of Wahlberg *et al.* (2009). *Calinaga davidis* was set as a sister group of all the other species and calibration nodes were constrained on the topology, while all other relationships were left free to vary, so that topological uncertainty was incorporated into posterior estimates of divergence dates. We used the Bayesian relaxed phylogenetic approach, performed in BEAST 1.7.5 (Drummond *et al.*, 2012), to estimate the relative age of divergence of the lineages studied. We partitioned the data by gene, with unlinked partitions according to the GTR+G substitution model for each and by codon for the CO1. We used the uncorrelated lognormal relaxed clock model and a Birth Death Process of speciation. We confirmed the results by using two independent analyses over 50 million generations, and sampled every 2500th generation. The two analyses converged on similar

posterior estimates. We then used Tracer 1.6 (Drummond & Rambaut, 2007) to assess convergence, measure the effective sample size of each parameter, and calculate the mean and 95% HPD interval for divergence times. We assessed whether a sample size greater than 200 was achieved for all parameters after the analyses. Results of the two runs were combined with LogCombiner 1.7.5 (Drummond *et al.*, 2012), and the Maximum Clade Credibility tree was compiled with TreeAnnotator 1.7.5 (Drummond *et al.*, 2012).

Biogeographical reconstructions

To test the dispersal and subsequent speciation hypotheses, we used a probabilistic method implemented in LAGRANGE (Ree & Smith, 2007) using biogeographical speciation scenarios and parameters for dispersal and extinction (Ree *et al.*, 2005). Terminal taxa were assigned to one or several of the following geographical areas: COL (Colombian Cordilleras), EEQ (Ecuadorian eastern cordilleras), WNP (Western Cordillera in northern Peru), ENP (Eastern Cordillera in northern Peru), ECP (Eastern Cordillera in central Peru), ESPB (eastern cordilleras in southern Peru and Bolivia). We confined possible dispersals to adjacent areas only, specifically between the following combinations: COL-EEQ, EEQ-WNP, EEQ-ENP, ECP-WNP, ECP-ENP, ECP-ESPB. The BEAST chronogram was used for LAGRANGE calculation and the root of the tree was calibrated at 8.9 Ma, as estimated age of the most recent ancestor of all lineages of the *M. sulkowskyi* group. Separate analyses were also conducted to determine the ancestral root area, using local optimizations conditional on the root state (considering either single or multiple area ranges, but with only adjacent areas, following Condamine *et al.* (2013). Three LAGRANGE models were run: an unconstrained model, where dispersals among all areas and any combination of ancestral areas are allowed (M0), and two stepping-stone models where dispersals are prohibited between non-adjacent areas and only combinations of adjacent ancestral areas are allowed: the first with dispersal allowed only from southern to northern adjacent areas (M1), while this constraint does not exist in the second (M2).

RESULTS

Field studies

Sympatry was discovered between *nieva* and *calderoni* along the upper valley of the Nieva

river (transect N1) and westwards at Oso Perdido (OP), while sympatry between *nieva* and *zachi* was observed downstream the Nieva (transect N2), and eastwards near El Afluente (EF) (Fig. 1). Specimens of *zachi* were never collected or observed with *calderoni* specimens, and reciprocally. Therefore, it is likely that *zachi* and *calderoni* populations are parapatric.

Phylogeny and divergence time estimates

The *M. sulkowskyi* group forms two clades, one including the taxa *sulkowskyi*, *sirene* and *nieva* (clade *sulkowskyi*) the other *selenaris*, *lympharis*, *eros*, *descimokoenigi*, *calderoni* and *zachi* (clade *lympharis*) (Fig. 2). In total, eight clusters were recognized under PTP, corresponding to *sulkowskyi*, *sirene*, *nieva* (two clusters), *calderoni* + *zachi*, *selenaris*, *lympharis* + *descimokoenigi*, and *eros* (Fig. 2). We selected one individual per cluster for dating analyses, except for *nieva* specimens, which were considered as belonging to a unique cluster. The two combined BEAST runs yielded high effective sample sizes (> 200) for all relevant parameters, indicating adequate sampling of the posterior distribution.

The *Morpho sulkowskyi* group diverged from the (*M. portis* (Hübner, [1821]) + *M. aega* (Hübner, [1822]) + *M. aurora* Westwood, 1851) clade at 14.2 Ma (95% HPD: 18.1 – 10.4) (Fig. 3A). The subsequent divergence into the *sulkowskyi* clade (S, Fig. 3A) and the *lympharis* clade (L, Fig. 3A) occurred at 8.9 Ma (95% HPD: 11.8–5.9 Ma). Within the *sulkowskyi* clade, two subclades originated at 3.9 Ma (95% HPD: 5.8–2.1 Ma), one corresponding to the Colombian *sulkowskyi*, the other to the Ecuadorian *sirene* and the Peruvian *nieva*. The divergence of *nieva* ancestors is estimated at 2.6 Ma (95% HPD: 4.1–1.2 Ma). Within the *lympharis* clade, two subclades originated at 3.9 Ma (95% HPD: 5.5–2.4 Ma): a northern one, corresponding to *selenaris* and *calderoni*, and a southern one, including *lympharis* and *eros*. It should be noted a topological incongruence concerning the divergence of *selenaris*, which possibly results from differences in molecular sampling (see Fig. 2 and 3); the dating analysis being based on mitochondrial and nuclear markers, its result will be used. The divergence of *selenaris* and *calderoni* ancestors is estimated at 3.1 Ma (95% HPD: 4.6–1.7 Ma). Within the Peruvian-Bolivian subclade, *eros* ancestors diverged from *lympharis* ancestors at 2.4 Ma (95% HPD: 3.8–1.3 Ma).

Biogeographical inference

Adjacent areas optimizations recovered the Eastern Cordillera in northern Peru (ENP) as the most likely root state ($\log L = -22.11$). The M2 stepping stone model had a higher log-likelihood ($\ln L$) than the other models ($\ln L = -18.04$ vs. $\ln L = -19.75$ for M0 and $\ln L = -22.77$ for M1), and only results from this best-fitting model are reported (Fig. 3B). LAGRANGE inference suggests that the *M. sulkowskyi* and *M. lympharis* clades diverged in the northern Peruvian Andes. Later, ancestors of the *M. sulkowskyi* clade dispersed from northern Peru towards the Ecuadorian Andes, and subsequently towards the Colombian cordilleras (divergence between *sirene* and *sulkowskyi* subclades). The more recent divergence of *nieva* ancestors from the *sirene* lineage possibly resulted from a dispersal towards the northern end of the Peruvian Eastern Cordillera. Within the *M. lympharis* clade, the major event was the separation of a northern branch and a southern branch that occurred within the Peruvian Eastern Cordillera. Within the northern branch, *calderoni* ancestors diverged from *selenaris* ancestors in the northern end of the Eastern Cordillera, while *eros* ancestors diverged from the *lympharis* lineage in Bolivia (Fig. 3B).

DISCUSSION

Taxonomy: towards a clarification

The monophyly of the *M. sulkowskyi* group was first documented by Cassildé *et al.* (2012). Our results confirm the monophyly and clarify relationships between taxa. Their partition into a *sulkowskyi* clade and a *lympharis* clade is congruent with the intuition of Le Moult & Réal (1962), who divided the *Morpho sulkowskyi* group into a *sulkowskyi* subgroup and a *lympharis* subgroup. Moreover, our field data show that the *sulkowskyi* and *lympharis* clades are represented by sympatric populations in the north of the Peruvian eastern Cordillera (Fig. 1). Therefore, these two clades should be considered as distinct species, according to Lamas (2004) taxonomic choice. However, the analysis from PTP resulted in eight clusters, which might be considered as different species (Fig. 2). Nevertheless, it should be emphasized that the morphologically very similar taxa *sulkowskyi* and *sirene* correspond to distinct clusters, while the morphologically different taxa *zachi* and *calderoni* are included in a single cluster, as well as are the taxa *lympharis* and *descimokenigi*. On another hand, *nieva* specimens are distributed between two clusters independently of their spatial origin: in each cluster, there are specimens from N1, N2, and PN collecting areas (Fig. 1 and 2). In this context, a final

taxonomic decision needs to be supported by several analyses with different genes, combined with morphological data and geographic distribution in an integrative taxonomy approach (Dayrat, 2005; Schlick-Steiner *et al.*, 2010). At the moment, we adopt Lamas's viewpoint, and the studied taxa will be designated as follow: *M. sulkowskyi sulkowskyi* Kollar, 1849, *M. sulkowskyi sirene* Niepelt, 1911, *M. sulkowskyi nieva* Lamas & Blandin, [2007], *M. lympharis lympharis* Butler, 1873, *M. lympharis eros* Staudinger, 1892, *M. lympharis selenaris* Le Moul't & Réal, 1962, and *M. lympharis calderoni* Blandin & Lamas, [2007].

The origin of the group: when and where?

The *M. sulkowskyi* group originated between 18 and 10 Ma, according to our estimation, or even later, between 10 and 5 Ma, according to Penz *et al.* (2012). This incongruence between age estimations may be explained by different methodological choices. Recently, Garzón-Orduña *et al.* (2015) emphasized the difficulties of choosing between alternative scenarios based on divergence times estimated by different secondary calibrations that may produce incompatible ages. Moreover, Van Tuinen & Torres (2015) showed that many methodological factors influence differently the estimation of age for deep nodes and shallow nodes. Therefore, differences in species samples and calibration points may explain why we obtained an older age – ca. 48 Ma vs ca. 32 Ma – than Wahlberg *et al.* (2009). Possibly, we also overestimated other ages, including that of the *M. sulkowskyi* group and of subsequent divergences. However, we used a wide-outgroup approach and five secondary calibration points selected in Wahlberg *et al.* (2009), one of them being related to the age of a precisely dated fossil, while Penz *et al.* (2012) calibrated only the root of their tree, using the divergence time of the clade *Morpho* provided by Wahlberg *et al.* (2009). This method strongly underestimates divergence times, as demonstrated by Sauquet *et al.* (2012). Therefore, it is possible that the *M. sulkowskyi* group diverged earlier than Penz *et al.* (2012) supposed.

From a S-DIVA ancestral area reconstruction, Penz *et al.* (2012) concluded that *M. sulkowskyi* and its relatives *M. aurora*, *M. aega* and *M. portis* originated in what they called the Eastern Andean region, an immense area extending from Colombia to southern Bolivia. Blandin & Purser (2013), taking into account the other *M. sulkowskyi* and *M. lympharis* relatives (*M. absoloni* May, 1924, that lives in Peruvian southern and central lowlands and Andean piedmonts, *M. zephyritis*, Butler, 1873, that lives along the Andes in southern Peru

and Bolivia, from lowlands to low Andean slopes, and *M. rhodopteron*, Godman & Salvin, 1880, that occurs in the Sierra Nevada de Santa Marta, northern Colombia), also concluded that the ancestors of all these species originated somewhere along the eastern side of the Andes. All these species occur at elevations lower than 2.0 km, except *M. rhodopteron* that is found from 0.6 to 2.4 km, and *M. sulkowskyi* and *M. lympharis* that live from ca. 1.8 to 3.5 km (Blandin, 2007; Blandin & Purser, 2013). Therefore, we hypothesize that the divergence of the *M. sulkowskyi* group ancestors resulted from an altitudinal shift and adaptation to the cloud forest belt, in areas where altitudes suitable for the development of cloud forests were attained.

In the southern Central Andes (Bolivia – southern Peru), at least 2.0 km elevations were attained by ca. 20-16 Ma (Sébrier *et al.*, 1988; Leier *et al.*, 2013; Saylor & Horton, 2014), and it has been suggested that cloud forests originated in this area around 20-18 Ma (Sempere *et al.*, 2005). At that time, in northern Peru, the Western Cordillera was lower than southern ranges (Picard *et al.*, 2008), but it attained locally 3 km elevations around 15 Ma (Margirier *et al.*, 2015). Thus, the divergence of *M. sulkowskyi* group was possible in either the southern Central Andes or the northern Western Cordillera. However, our biogeographical analysis indicates that the *M. sulkowskyi* group originated probably in the Eastern Cordillera, in northern Peru. Here, from ca. 24 to 17 Ma, a foredeep depozone existed in the place of the Eastern Cordillera, the uplift of which resulted from an intense horizontal shortening between 17 Ma and 8 Ma (Eude *et al.*, 2015). Our estimation for the divergence time of the *M. sulkowskyi* group (ca. 18-10 Ma) is consistent with these data. However, as suitable elevations were attained towards the end of the shortening period, Penz *et al.* (2012) estimation (ca. 10-5 Ma) is also compatible. The main point is that the group should have originated in the northern part of the Peruvian Eastern Cordillera from low or mid-altitude ancestors, when this mountain range attained cloud forest elevations, around 10 Ma.

Mio-Pliocene or Pleistocene diversification?

The first divergence within the *M. sulkowskyi* group occurred during the Late Miocene, at ca. 8.9 Ma, probably a very few millions of years after the group originated in the Peruvian Eastern Cordillera. The ancestors of the *sulkowskyi* clade possibly dispersed towards the Western Cordillera, where cloud forests today exist in large areas near the Ecuadorian border, and southwards as restricted, isolated patches surrounded by cultivated areas (Young & León,

2001). This hypothesis is supported by the discovery in one of these relictual cloud forests (Cutervo area, Naranja, 2300 m; Cajamarca department), in 1998, of four males similar to Colombian and Ecuadorian specimens and consequently identified as *sulkowskyi* by Blandin (2007), who considered *sirene* as a junior synonym of *sulkowskyi* (these specimens are housed in MUSM; see Appendix 1, Fig. A). Such a distribution, covering the north of the Peruvian Western Cordillera and the eastern Ecuadorian cordilleras, is not surprising: it is known in other cloud forest groups, for example in the hummingbird genus *Metallura* Gould, 1847, which has an Andean distribution almost similar to the distribution of the *M. sulkowskyi* group (Benham *et al.*, 2015).

The divergence of *sulkowskyi* ancestors from *sirene* ancestors occurred during the Pliocene, at ca. 3.9 Myr. Their common ancestors should have dispersed from the Peruvian Western Cordillera towards the Ecuadorian eastern cordilleras. Until the Middle Miocene, a lowland corridor situated approximately between 3°S and 5°S, the “Western Andean Portal” (WAP) separated the Ecuadorian Andes from the Peruvian Andes (Antonelli *et al.*, 2009; Hoorn & Wesselingh, 2010). The WAP landscapes included wetlands, and temporary marine incursions from the Pacific Ocean might have occurred (Hungerbühler *et al.*, 2002). Thus, the WAP constituted a long-lasting barrier for mountain organisms (Antonelli *et al.*, 2009). Starting from 13–11 Ma, the uplift of mountain ranges closed the WAP (Coltorti & Ollier, 2000; Hungerbühler *et al.*, 2002). Migration of cloud forest species towards Ecuadorian and Colombian Andes became possible when suitable elevations were attained. Luebert & Weigend (2014) quote several examples of plants that dispersed from the Central to the Northern Andes, and vice versa, during the Late Miocene and Early Pliocene, following the closure of the WAP (10–3.8 Ma). For example, the ancestors of species of the genus *Puya* (Bromeliaceae) that diversified in the high Northern Andes originated in the Central Andes and crossed the WAP around 6–4 Ma (Givnish *et al.*, 2011, 2014; Jabaily & Sytsma, 2013). We hypothesize that the ancestors of *sirene* and *sulkowskyi* lineages also crossed the WAP during this period, corresponding approximately to the Early Pliocene.

In Colombia, the Western and Central Cordilleras already had rather high elevations at the Oligocene-Miocene boundary (24–21 Ma) (Duque-Caro, 1979; Gómez *et al.*, 2005; Roddaz *et al.*, 2010). A major phase of uplift followed, and the rise of the Eastern Cordillera began at that time (Restrepo-Moreno *et al.*, 2009). This cordillera reached 2.0–2.5 km elevations by

3.7 Ma at the latitude of Bogotá (Van der Hammen & Hooghiemstra, 2001). As we estimated the divergence of *sulkowskyi* ancestors from *sirene* ancestors at ca. 3.9 Myr, a synchronous dispersal from the eastern Ecuadorian slopes towards the three cordilleras is plausible during the Late Pliocene.

The diversification of the *lympharis* clade occurred during a period encompassing the Pliocene and Pleistocene, so it is difficult to differentiate the roles, on one hand of latitudinal range expansion across existing topographical barriers and subsequent allopatric divergence, on the other of the fragmentation of already established areas provoked by glacial cycles. Our results suggest a north-to-south dispersal of the *lympharis* lineage after its divergence, in the Peruvian Eastern Cordillera, at ca. 3.9 Ma, followed around 2.4 Ma by the divergence of the southernmost *eros* lineage, perhaps as a consequence of Late Pliocene or early Pleistocene climatic changes. In our present state of knowledge, we suppose that *descimokenigi* populations, which are geographically separated from *lympharis* populations (Appendix S1), diverged during the Pleistocene.

Within the northern subclade, the divergence of *calderoni* ancestors also occurred during the Late Pliocene (ca. 3.1 Ma). It possibly resulted from a dispersal towards the north-eastern extremity of the Eastern Cordillera, but the role of climatic changes at the very end of the Pliocene or the beginning of the Pleistocene cannot be excluded. Our field studies provided evidence that *zachi* and *calderoni* populations are parapatric in the north-eastern extremity of the Eastern Cordillera. We hypothesize that their divergence resulted from Pleistocene fragmentation. However, it should be emphasized the fact that the easternmost mountain ranges raised during the very last few millions of years, and that they probably attained cloud forest altitudes during the Pleistocene; therefore, dispersal of cloud forest species from older ranges may have been controlled by climate-driven altitudinal shifts of ecosystems.

The divergence of *nieva* ancestors from the *sirene* lineage occurred approximately at the Pliocene-Pleistocene transition (ca. 2.6 Ma), logically in the north of the Peruvian Western Cordillera. As *nieva* is known only from the northernmost part of the Eastern Cordillera, which is separated from the Western Cordillera by the dry and deep Marañón valley, we hypothesize that changes in altitudinal distribution of ecological belts associated to Pleistocene climatic variations after 2.6 Ma may have favoured dispersion between the Western and the Eastern Cordilleras.

On the whole, the expansion and diversification of the *M. sulkowskyi* group essentially occurred before the Quaternary. However, climate-driven cycles of fragmentation and expansion possibly played a role in the ultimate diversification of the group. An almost similar pattern was observed in the butterfly genus *Lymanopoda* Westwood, 1851 (Nymphalidae, Satyrinae), a species-rich montane group distributed along the Andes. It includes a majority of cloud forest species, the larvae of which eat montane bamboos, primarily *Chusquea* species (Casner & Pyrcz, 2010). The genus first diversified between 15 and 10 Ma, the formation of major clades occurred by 10–8 Ma, and most of the species-level diversification occurred since the end of the Miocene, ca. 6 Ma, more than half of the studied species being of Pleistocene or even post-Pleistocene origin. The diversification of another high elevation (1.9–4.8 km) Andean species, the hummingbird genus *Metallura* followed an almost similar pattern, with Mio-Pliocene first divergences, but with the majority (81%) of divergence events occurring during the mid-late Pleistocene (Benham *et al.*, 2015). Thus, such examples suggest that the precise timing of diversification could differ between Andean groups, Pleistocene climatic cycles having a more or less important influence.

The origin of the group: a relationship with hostplant evolution?

Penz *et al.* (2012) supposed that caterpillars of all species of the clade including the *M. sulkowskyi* group and the (*M. portis* + *M. aega* + *M. aurora*) branch feed on monocot hostplants. The hypothesis of a “monocot clade” is supported by available data for *M. portis* and *M. aega*, that feed on Guaduiniae and Chusqueinae woody bamboos (Poaceae) (Beccaloni *et al.*, 2008), and *M. sulkowskyi* that feeds on a *Chusquea* species (Heredia & Alvarez, 2007). Four other species belong to the clade: *M. rhodopteron*, *M. aurora*, *M. absoloni*, and *M. zephyritis* (Cassildé *et al.*, 2012). The former also feeds on *Chusquea* (Montero Abril & Ortiz Perez, 2010). There is no published information on hostplants for the three other species; however, they are always found associated to dense stands of bamboo (G. Lamas pers. obs.), which makes it reasonable to assume their larvae feed on them. Available data indicate that all other *Morpho* species feed on eudicot hostplants, while other Morphinae (Antirrheina and Brassolini) feed on monocots (Beccaloni *et al.*, 2008). Consequently, Penz *et al.* (2012) suggested that the *Morpho* clade divergence was associated to a shift to eudicotyledonous larval hostplants, and that the divergence of the monocot clade was associated to a reversal to monocot hostplants. This reversal occurred during the 25–15 Ma period (Fig. 3B), when

Neotropical woody bamboos probably originated and began to diversify: the ancestors of Chusqueinae and Guaduinae originated during the Early-to-Middle Miocene (ca. 21–11 Ma), probably in the Andes (Fisher, 2011; Ruiz-Sanchez, 2011).

Heredia & Alvarez (2007) observed that the Colombian *M. sulkowskyi* feeds, at 2.4–2.5 km a.s.l., on a species closely related to *Chusquea scandens* Kunth, 1822 one of the five *Chusquea* species distributed from Bolivia to Colombia (Ohrnberger, 1999). The clade including *C. scandens* diverged between ca 14.5 and 6.5 Myr (Fisher, 2011); our estimation of the age of the *M. sulkowskyi* group is consistent with this information.

CONCLUSIONS

Simple relationships between Andean uplift and the diversification of various plant and animal groups, implying pre-Pleistocene driving processes, have been supposed by various authors. Doan (2003), for example, proposed the South-to-North Speciation Hypothesis, where the process of speciation should be related to the south-to-north progression of uplift throughout the Andes. Other authors emphasized the possible role of a rapid uplift that occurred during the Late Miocene and Early Pliocene, but often without establishing clear links between dated divergences and local geologic events (e.g., Casner & Pycz, 2010; Mulch *et al.*, 2010; Matos-Maraví *et al.*, 2013; Lagomarsino *et al.*, 2016). From a geological point of view, the concept of a progressive, general south-to-north uplift is an oversimplified view of a much more complex reality (Sempere *et al.*, 2008). In the Central Andes, palaeo-elevation histories differ not only between the south and the north, but also between the Western and the Eastern Cordilleras, notably in northern Peru (Picard *et al.*, 2008; Eude *et al.*, 2015; Margirier *et al.*, 2015). The idea that the Northern Andes, as a whole, uplifted later than the Central Andes, as suggested by Doan (2003), and often admitted by other authors, is not supported by geological studies, that also demonstrate that the timing of palaeo-elevation differed between the three Colombian Cordilleras (Restrepo-Moreno *et al.*, 2009). Consistent with many other examples, notably the clearwing Oleriina butterflies (De-Silva *et al.*, 2016), the *M. sulkowskyi* group illustrates the diversity of diversification histories throughout the Andes. It also demonstrates that Mio-Pliocene orogenic and Pleistocene climatic diversification drivers should not be opposed.

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Figures

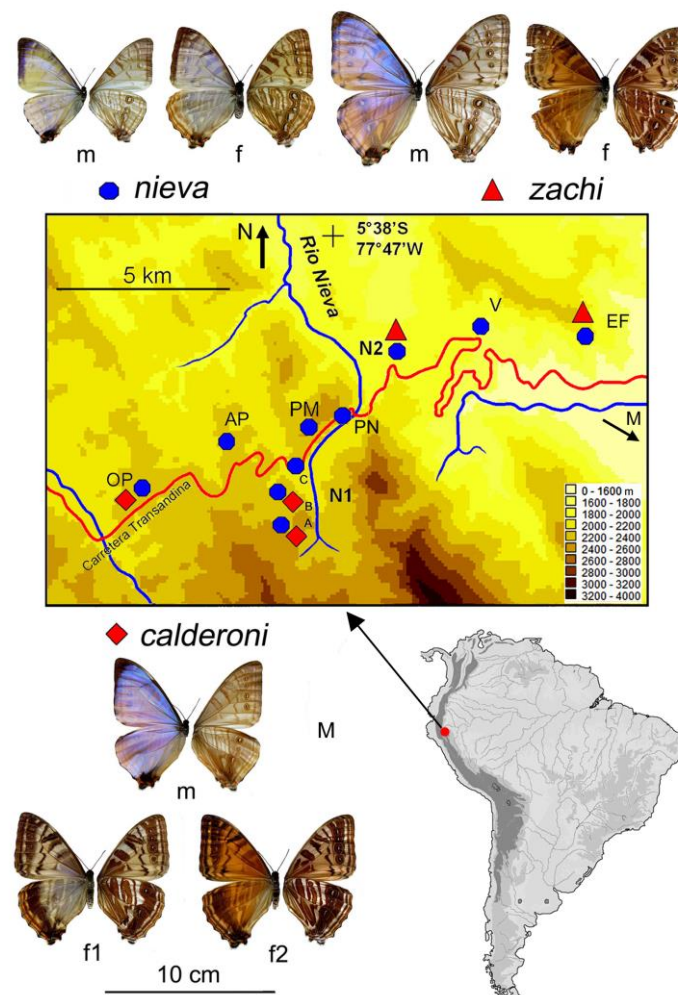


Figure 1. Map of the region where field studies were carried out, with habitus of the taxa *calderoni*, *zachi* and *nieva* (m: male; f: female; f1 and f2: female morphs within the *calderoni* population). N1 and N2: sampling areas along the upper Río Nieva. Other localities where specimens were collected: AP: Abra Patricia; EF: El Afluente; OP: Oso Perdido; PM: Abra Pardo Miguel; V: Venceremos. Two specimens of *nieva* were also collected at Santa Cruz del Mirador (M), at ~20 km ESE from El Afluente.

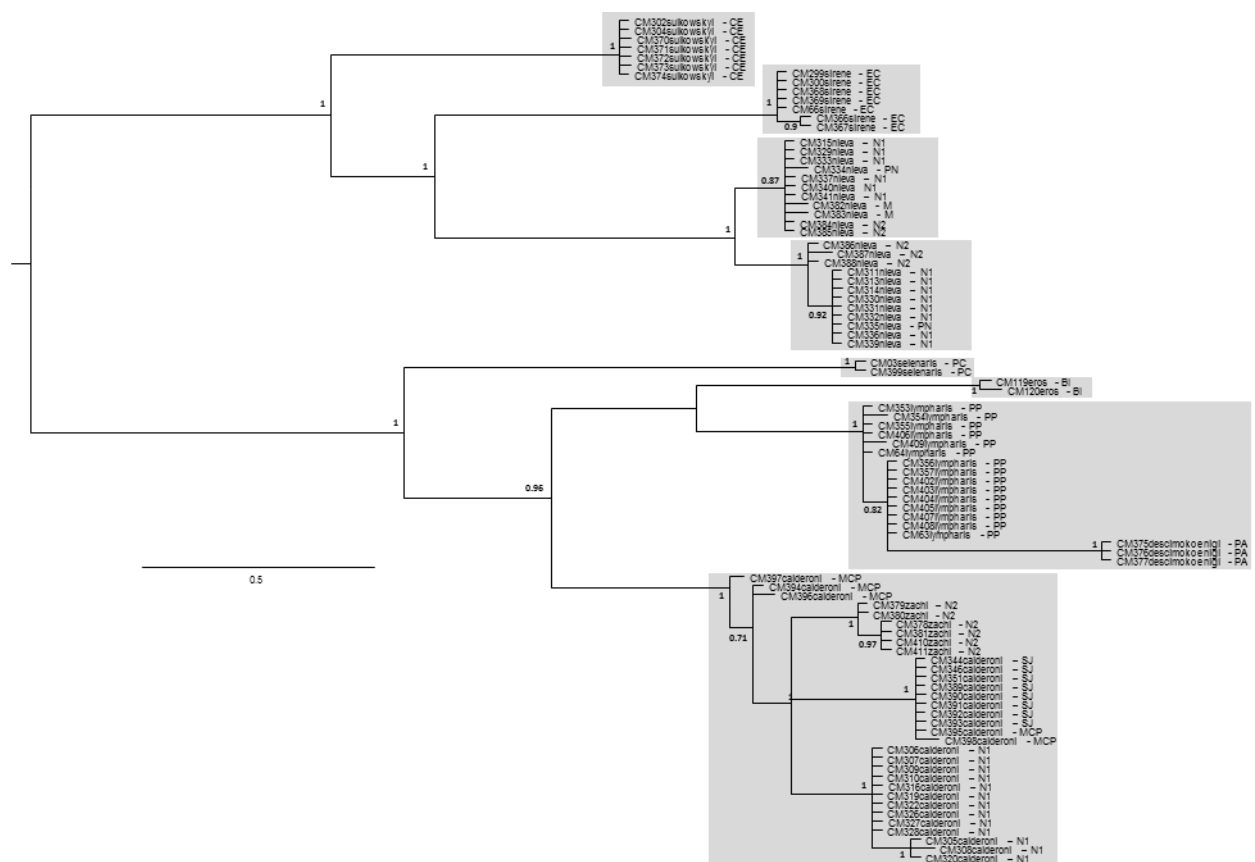


Figure 2. Topology obtained by Bayesian inference and clusters recognized after analyses conducted for Cytb on the PTP web server (<http://species.h-its.org/ptp>).

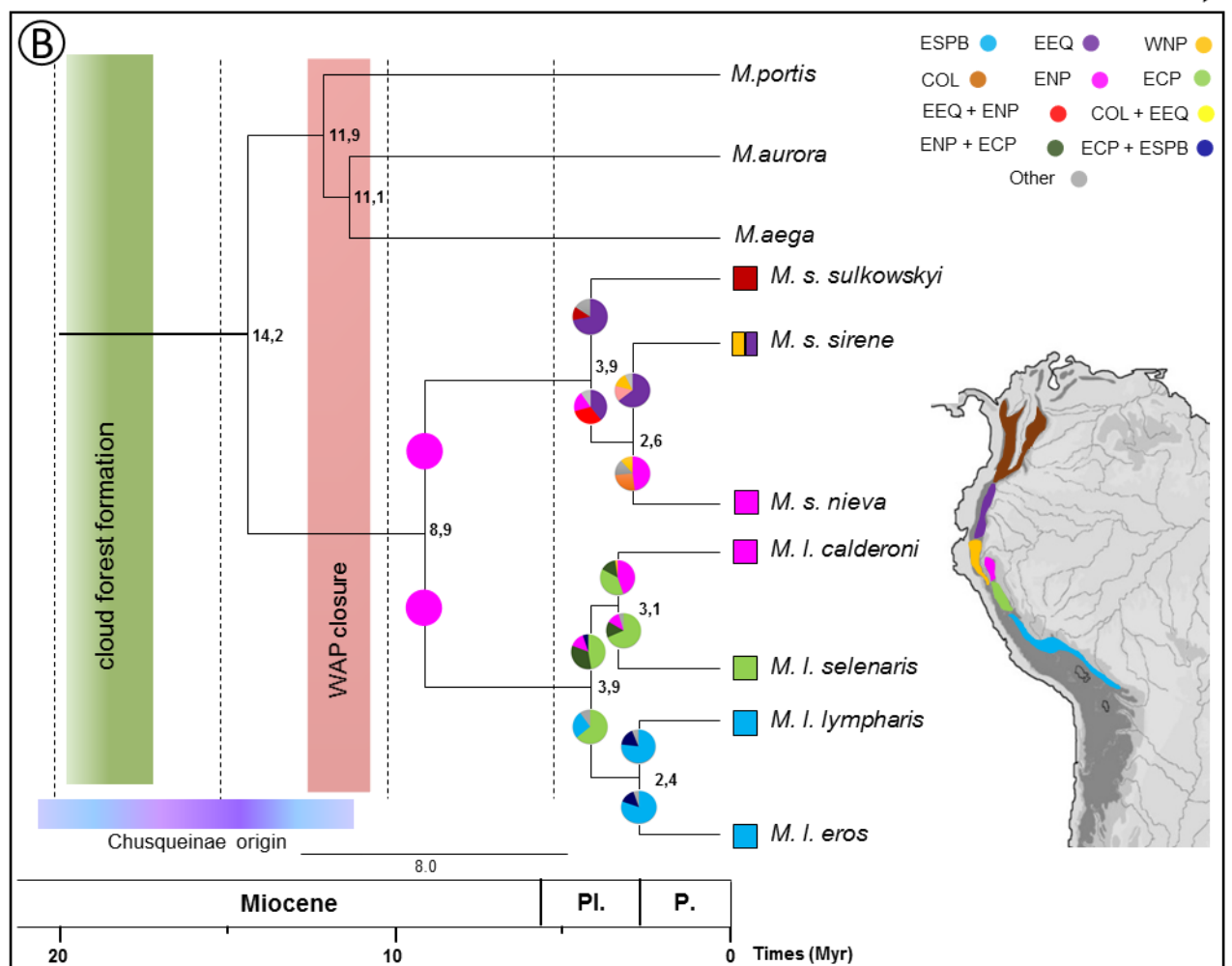
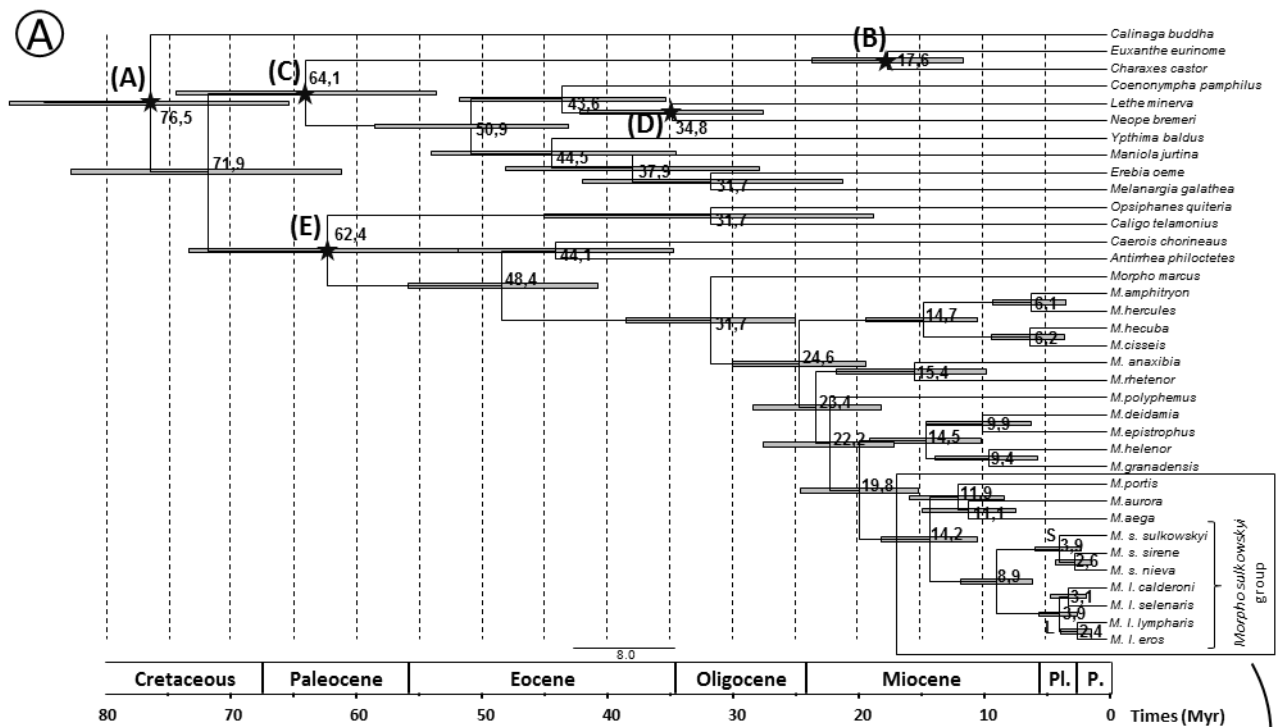


Figure 3. A) Chronogram representing the divergence times of the principal lineages of nymphalid butterflies including the *Morpho sulkowskyi* group. B) Chronogram representing the divergence times of the *Morpho sulkowskyi* group. Node positions indicate mean estimated divergence times, and pie charts represent relative probabilities of ancestral ranges.

Supporting Information

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

Appendix S1. The *Morpho sulkowskyi* group: geographical distribution and taxonomy.

Appendix S2. List of all taxa included in the analyses and their Genbank accession numbers: a - phylogenetic analysis and species delimitation; b – dating analysis. In GenBank, geographical origins of many specimens are not available (n/a).

Appendix S3. Additional information on sequencing protocols.