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NEW INSIGHTS INTO THE SYSTEMATICS OF THE GENUS *POLYURA* BILLBERG, 1820 (NYMPHALIDAE, CHARAXINAE) WITH AN EMPHASIS ON THE *P. ATHAMAS* GROUP

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ABSTRACT. The genus *Polyura* comprises 32 species across the Oriental Region and the Indo-Australian archipelago. Its taxonomy and systematics have recently been studied using a comprehensive molecular phylogeny. Yet, certain elements of its fauna were not available for in depth study. Here, we provide a denser taxon sampling and reconstruct a new phylogenetic hypothesis for the *P. athamas* group, a morphologically cryptic complex. The known geographic range of the genus is extended to Eastern Pakistan where two species fly in sympatry. Endemics from Tawi Tawi archipelago off the northern tip of Borneo have more affinities with the fauna of Sunda Islands rather than with the Philippines. Our results also suggest three taxonomic changes; the recognition of a new species and the transfer of two subspecies to a different species.

Additional key words: Cryptic diversity; Pakistan; *Polyura attalus*; Sulu arc; Tawi Tawi archipelago.

The Nawab butterflies belong to the genus *Polyura* Billberg, 1820, a clade restricted to the Oriental Region and the Indo-Australian archipelago (IAA) (Smiles 1982, Toussaint et al. 2015). These charismatic butterflies are closely related to the genus *Charaxes* Ochsenheimer, 1816 which primarily occurs in the Afrotropics although about 30 species are found in the IAA. However, the relationships within the tribe Charaxini remain equivocal and additional work is needed to unveil the placement of *Polyura* with respect to *Charaxes* (Aduse-Poku et al. 2009, Müller et al. 2010, Toussaint et al. 2015). Recently, the first molecular phylogenetic hypothesis of *Polyura* was built to investigate species boundaries within the genus (Toussaint et al. 2015). Using a multimarker dataset and a comprehensive taxonomic coverage, Toussaint et al. (2015) recognized 32 valid species using an array of molecular species delimitation methods. Among the three species groups found in the genus, two new species were recognized, four subspecies were raised to species and one species was synonymized. Despite this stride toward a better understanding of the taxonomy and systematics of the genus, a few cases remain where the taxonomic status of genetic groupings is contentious to some degree (Toussaint et al. 2015). This is especially true in two species groups; the *P. athamas* (Drury, 1770) group and the *P. pyrrhus* (L., 1758) group. In the former, a dramatic revision was undertaken with the description of a new species *Polyura paulettae* (Toussaint 2015) and the elevation (re-elevation) of three subspecies to species rank, namely *P. alphius* (Staudinger 1886), *P. bharata* (Felder & Felder, 1867) and *P. luzonica* (Rothschild 1899). However, there is still a need to complete this taxonomic and systematic revision in the light of a denser geographic and taxonomic sampling.

The *P. athamas* group is notoriously difficult to work with because most species share a rather similar appearance and lack morphological synapomorphies (Smiles 1982, Toussaint et al. 2015). Two main monophyletic clades are found in the *P. athamas* group; (i) one including two very distinctive species with a blue wing pattern, *P. schreiber* (Godart 1824) and *P. luzonica*, and (ii) another including multiple species of elusive morphological limits, hereafter referred to as the "green species complex" (GSC) as most species present a rather similar appearance with green discal bands on the upperside of the wings (Smiles 1982, Toussaint et al. 2015). There is a clear genetic demarcation between species of the GSC, and with morphology offering little for species delimitation, their correct identification is greatly facilitated by genetic assignment (Toussaint et al. 2015). Although most of the difficult cases in the *P. athamas* group were addressed in Toussaint et al. (2015), some challenging cases still need to be tackled. It is the case with *Polyura schreiber* whose Philippine populations were recognized as a separate species, *P. luzonica*. *Polyura schreiber* has a very widespread distribution, ranging from India to Borneo, and the demarcation of Philippine populations was not extremely surprising as this archipelago has a very different geological origin compared to the Indomalayan peninsula (Figure 1). Interestingly, some subspecies occur between the Philippines and Sunda, namely *P. schreiber lindae* Schroeder & Treadaway, 2008 from Tawi Tawi archipelago and *P. schreiber praedicta* (Schroder & Treadaway 1980) from Palawan. The latter was included in Toussaint et al. (2015) where it was recovered as sister to all other *P. schreiber* populations, a relationship nicely illustrating the geological affinity of Palawan with the Sunda arc (Hall 2012, 2013). Discovering whether the populations of *P. schreiber lindae* belong to *P. luzonica* or *P. schreiber* is important for understanding the biogeography of this species complex. In the GSC, there is some haziness regarding the geographic distribution of extant described species. This is especially the case for *P. paulettae* Toussaint, 2015, a genetically distinct lineage that belongs to a morphologically homogeneous group also comprising *P. agraria* (Swinhoe 1887), *P. alphius*, *P. athamas* and *P. bharata*. *Polyura paulettae* was described from specimens collected in Myanmar and Thailand, but it is not clear if its present distribution is actually broader than these two countries. Toussaint et al. (2015) revised the geographic distributions of most species belonging to the GSC, however the ranges of a few species are still nebulously understood and there is a need to refine these in order to fully understand the evolution of these lineages.

Here, we sampled and sequenced the holotype of the rare *P. schreiber lindae* from Tawi Tawi, as well as five specimens of the GSC from new localities, in order to investigate their placement with respect to the phylogenetic framework of Toussaint et al. (2015). We aim to (*i*) reconstruct the phylogenetic relationships among species of the *P. athamas* group; (*ii*) assign the newly sequenced specimens to extant species; (*iii*) provide revised maps of distribution for the GSC as well as revise the taxonomy of the group where needed.

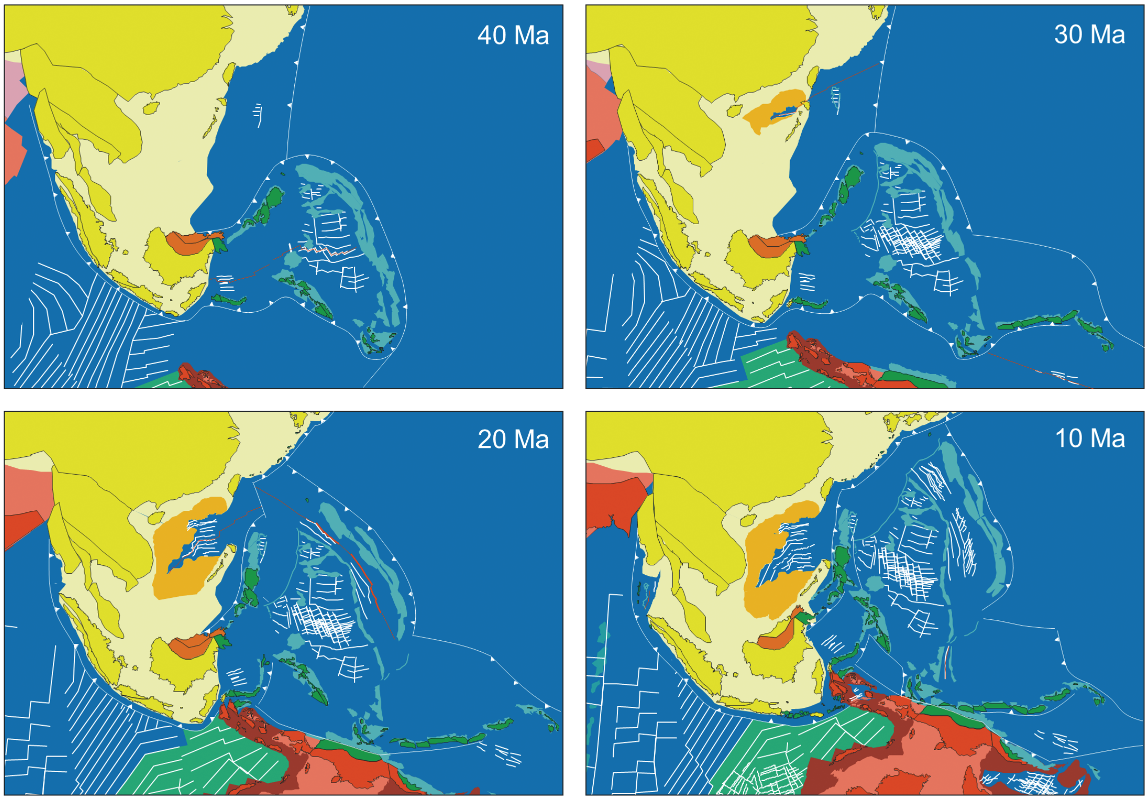

FIG. 1. Geological history of the Sunda region in the Cenozoic

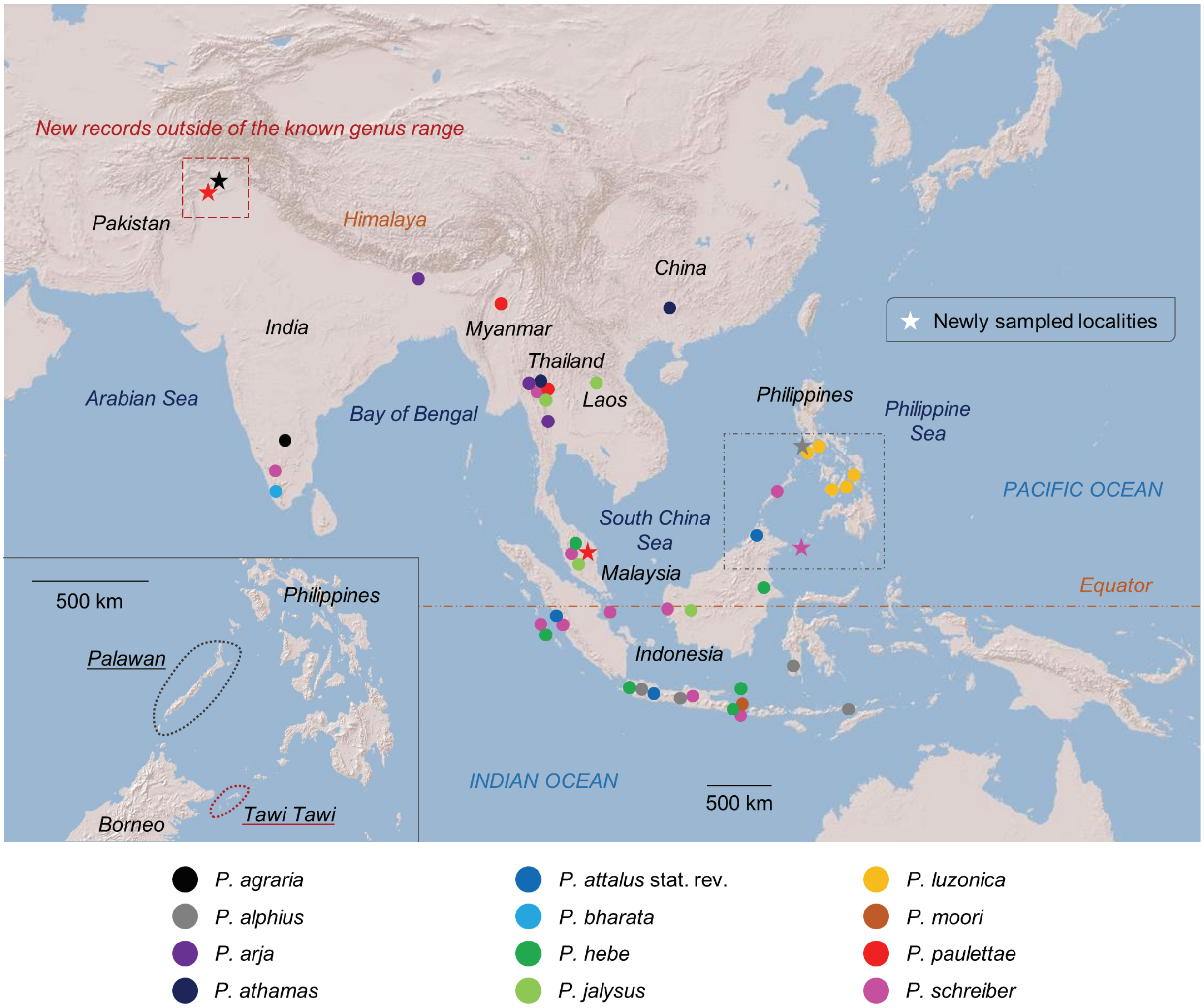

FIG. 2. Geographic sampling of the *P. athamas* group used in this study

METHODS

Taxon Sampling and Molecular Biology. We retrieved sequence data from Toussaint et al. (2015) and added several specimens of species belonging to the *P. athamas* group (Figure 2, Table 1). Total genomic DNA was extracted from leg tissues of dried collection specimens using the DNeasy kit (Qiagen, Hilden, Germany). Using PCR protocols described in Wahlberg and Wheat (2008) and Müller et al. (2010), we amplified and sequenced the following gene fragments: cytochrome oxidase subunit 1 (CO1, 471 bp), NADH dehydrogenase subunit 5 (ND5, 417 bp), ribosomal protein S5 (Rps5, 573 bp) and Wingless (396 bp). The DNA sequences were edited in Geneious R8 (Biomatters, http://www.geneious.com/), aligned using MUSCLE (Edgar 2004) and reading frames were checked under Mesquite 3.02 (http://mesquiteproject.org). The different datasets used to infer phylogenetic relationships were generated under Mesquite. *Charaxes viola* (Butler 1866) was used as outgroup in all analyses. All sequences were deposited in GenBank (accession Nos. KU170651–KU170664).

Molecular phylogenetics. We used Bayesian Inference (BI) to reconstruct the phylogenetic relationships of the *P. athamas* group. Partitions and corresponding optimal models of substitution were searched under PartitionFinder 1.1.1 (Lanfear et al. 2012) using the greedy algorithm, and the mrbayes set of models. All genes were divided by codon positions for a total of 12 partitions to analyze. The Akaike

TABLE 1. List of specimens newly sequenced for this study

Genus	Species	Subspecies	Country	Locality	Code	CO1	ND5	Rps5	Wingless
Polyura	*agraria*	-	Pakistan	Margalla hills, Islamabad	ET0252	-	X	-	X
Polyura	*alphius*	*acuta*	Philippines	Mindoro	ET0242	X	X	X	-
Polyura	*paulettae*	-	Malaysia	Cameron Highlands, Pahang	ET0070	X	X	-	-
Polyura	*paulettae*	-	Pakistan	Margalla hills, Islamabad	ET0253	X	X	X	X
Polyura	*paulettae*	-	Pakistan	Margalla hills, Islamabad	ET0254	X	X	X	X
Polyura	*schreiber*	*lindae*	Philippines	Tawi Tawi	ET0251	-	X	-	-

Information Criterion corrected (AICc) was used to compare the fit of the different models. The BI analyses were performed using MrBayes 3.2.3 (Ronquist et al. 2012). Two simultaneous and independent runs consisting of eight Metropolis-coupled Markov chain Monte Carlo (MCMC, one cold and seven incrementally heated) running 20 million generations were used, with a tree sampling every 1000 generations to calculate posterior probabilities (PP). In order to investigate the convergence of the runs we investigated the split frequencies and Effective Sample Size (ESS) of all the parameters, and plotted the log-likelihood of the samples against the number of generations in Tracer 1.5 (http://BEAST.bio.ed.ac.uk/Tracer). A value of ESS>200 was acknowledged as a good indicator of convergence. All posterior trees predating the time needed to reach a log-likelihood plateau were discarded as burn-in, and the remaining samples were used to generate a 50% majority rule consensus tree.

RESULTS

Phylogenetic Relationships. Under the AICc, PartitionFinder suggested a partitioning scheme comprised of seven partitions (Table 2). All MrBayes runs based on this partitioning scheme converged after only a few million generations and all parameters had an ESS>>200. The 50% majority rule consensus tree is presented in Figure 3 along with posterior probability nodal support.

Overall the phylogenetic tree is well resolved and nodal support is robust (Figure 3). We find the three species groups monophyletic with strong support. Within the *P. athamas* group, all extant described species are found monophyletic with strong support except *Polyura athamas* whose populations from mainland Asia (ssp *athamas*) are found sister to *P. bharata* + *P. arja* (Felder & Felder, 1867) + *P. hebe* (Butler, 1866) + the populations of *P. athamas* from Sunda (ssp *attalus* and *uraeus*). The latter are found sister to *P. arja* + *P. hebe* in a more derived part of the tree.

The endemic subspecies *P. schreiber lindae* from the Tawi Tawi archipelago is found nested in a derived clade of *P. schreiber* (Figure 3) and therefore has no affinity with the Philippine endemic *P. luzonica*. The three specimens of *Polyura* from Pakistan are found in two different parts of the tree. Two are recovered as belonging to *P. paulettae* along with the new specimen from Malaysia, whereas the remaining specimen from Pakistan is found within *P. agraria*. Finally, the Philippine specimen of *P. athamas acuta* from Mindoro is found as sister to *P. alphius*.

Taxonomic changes. Our results have three taxonomic consequences in the GSC as *P. athamas* was found polyphyletic. The Philippine endemic subspecies *P. athamas acuta* is found as sister to *P. alphius*, a species found in Sumatra, Java, the Lesser Sunda Islands and Sulawesi. Since we did not sample the other Philippine endemic subspecies, *P. athamas palawanica*, recognizing *P. athamas acuta* as a distinct valid species would be premature. Otherwise, the populations of *P. athamas* from Sunda Islands are found sister to *P. arja* and *P. hebe* as previously recovered in Toussaint et al. (2015). In this paper these populations were not elevated to species status because molecular species delimitation methods yielded incongruent results and because the nodal support of the entire *P. athamas*

TABLE 2. Best partitioning scheme for the molecular dataset as recovered under PartitionFinder

	Partition	Substitution model
P1	CO1 pos.1 + ND5 pos.1	GTR+Γ
P2	CO1 pos.2 + ND5 pos.2	HKY+I
P3	CO1 pos.3	GTR+Γ
P4	ND5 pos.3	GTR+I
P5	RPS5 pos.1 + RPS5 pos.2 + WGL pos.1 + WGL pos.2	JC+I
P6	RPS5 pos.3	HKY+Γ
P7	WGL pos.3	K80+Γ

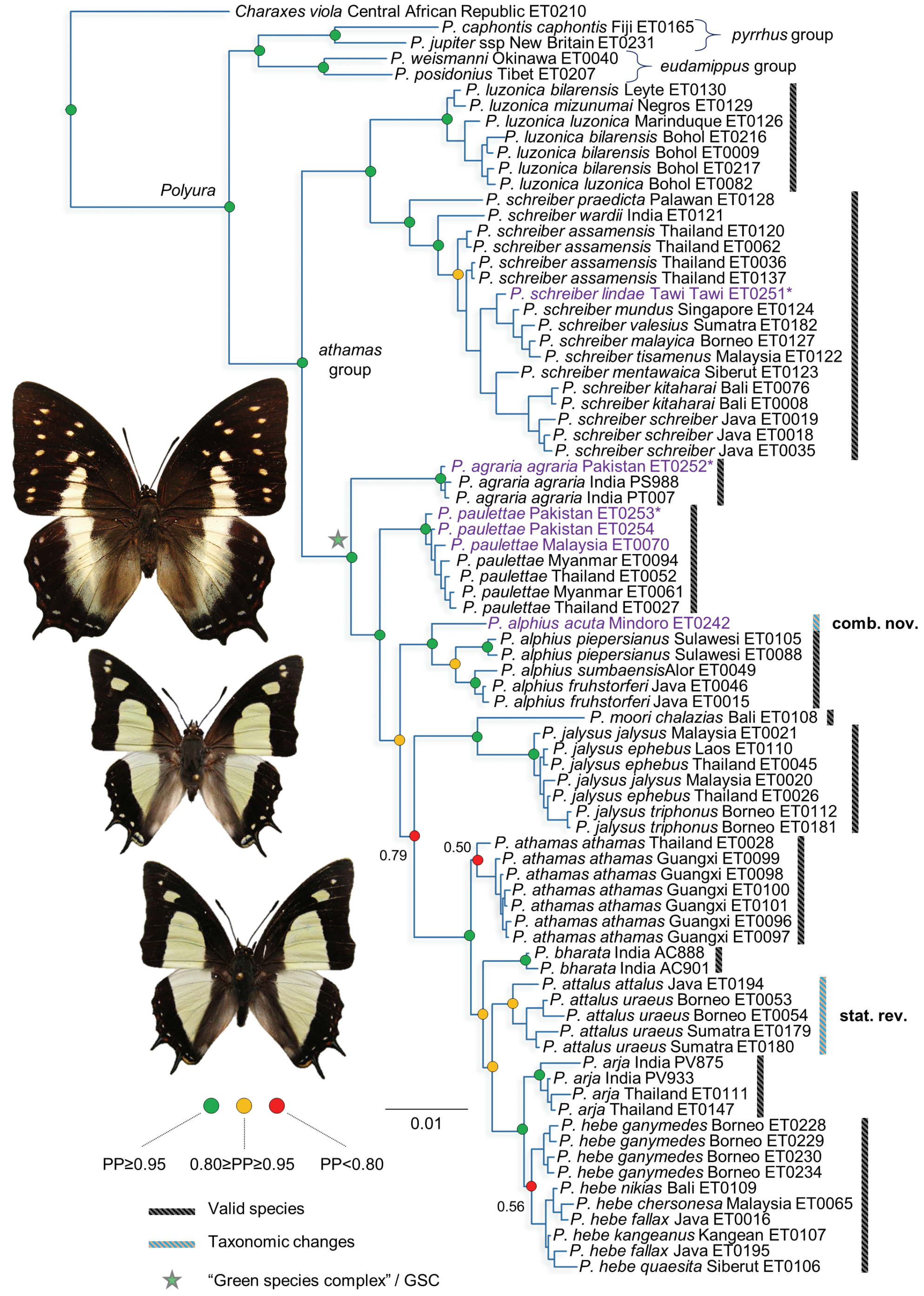

FIG. 3. Bayesian molecular phylogeny of the *Polyura athamas* group. The phylogenetic tree presented is a 50% majority rule consensus of post burnin posterior trees from the MrBayes analysis based on the best partitioning scheme selected under PartitionFinder (Table 2). Nodal support is highlighted for the major nodes of the phylogeny following the color code shown at the bottom left of the figure. Habitus of three specimens sampled in this study are presented as indicated with asterisks after the name of the specimens in the phylogeny. From top to bottom: *Polyura schreiber lindae* from Tawi Tawi archipelago, *Polyura agraria* from Pakistan and *Polyura paulettae* from Pakistan.

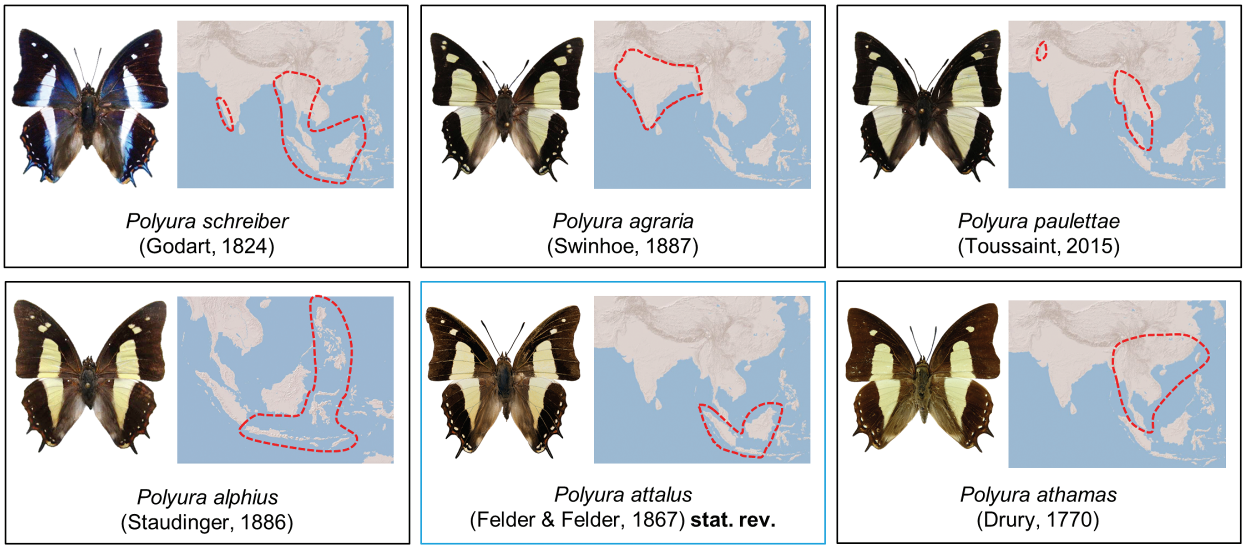
Polyura schreiber (Godart, 1824)
Polyura agraria (Swinhoe, 1887)
Polyura paulettae (Toussaint, 2015)
Polyura alphius (Staudinger, 1886)
Polyura attalus (Felder & Felder, 1867) **stat. rev.**
Polyura athamas (Drury, 1770)

FIG. 4. Updated distribution maps of the species belonging to the *P. athamas* group

group was fairly low. Here, we recover a better resolution and better nodal supports compared to Toussaint et al. (2015). Moreover both clades from continental Asia and from Sunda Islands have been shown to be clearly differentiated genetically even when considering the nuclear genome only (Toussaint et al. 2015). Based on our results and need of a taxonomic act to solve the paraphyly of *P. athamas* we suggest the following changes:

POLYURA BILLBERG, 1820

Polyura attalus (Felder & Felder), 1867, **new status**

Charaxes attalus Felder & Felder, 1867: 438.
Eulepsis attalus (Felder & Felder): Moore 1896: 263.
Eulepsis athamas attalus (Felder & Felder): Rothschild & Jordan 1899: 257.
Eriboea athamas attalus (Felder & Felder): Fruhstorfer, 1914: 719; Roepke 1932: 95.
Charaxes athamas attalus (Felder & Felder): Roepke 1938: 348.
Polyura athamas attalus (Felder & Felder): Stichel 1939: 560; Smiles 1982: 172.

Polyura attalus uraeus (Rothschild), 1899, **new combination**

Eulepsis athamas uraeus Rothschild, 1899: 254.
Eriboea athamas uraeus (Rothschild): Fruhstorfer, 1914: 719.
Polyura athamas uraeus (Rothschild): Stichel 1939: 558; Smiles 1982: 169.

Polyura alphius acuta (Rothschild, 1899), **new combination**

Eulepis athamas acutus Rothschild, 1899: 256.
Eriboea athamas acutus (Rothschild): Fruhstorfer, 1914: 720.
Polyura athamas acutus (Rothschild): Stichel 1939: 563.
Polyura athamas acuta (Rothschild): Smiles 1982: 171.

DISCUSSION

Our phylogenetic reconstruction provides a clear resolution for the *P. athamas* group with interesting results regarding both species geographic ranges and taxonomy. First, we find that the endemic population from Tawi Tawi archipelago belongs to *P. schreiber* and is closely related with other populations from Borneo, Malaysia and Singapore. This pattern is similar to the one found in *P. schreiber praedicta* endemic to Palawan. The latter is found sister to all other populations of *P. schreiber* and therefore is more closely related to the fauna of Sunda than to the Philippine one. The affinity between this island endemic and the fauna of Sunda is congruent with the geological history of the region (Figure 1). Indeed, Palawan is mainly composed of continental margin material and is closely related to the Sunda shelf (Hall 2012, 2013). As a result, Palawan was likely connected with the rest of the Sunda shelf by land bridges during periods of decreased sea level (Hall 2012, 2013). Because *P. schreiber praedicta* is found as sister to all other *P. schreiber* populations, this subspecies might constitute a trace of a colonization

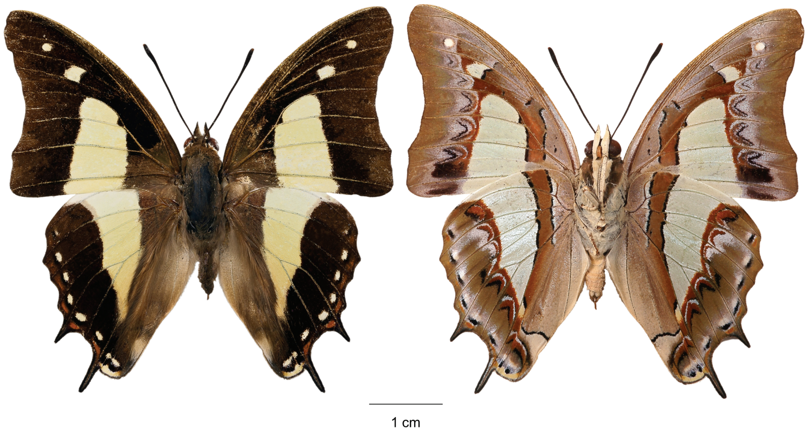

FIG. 5. Habitus of *Polyura attalus* (Felder & Felder, 1867) new status from Java. Dorsal and ventral pictures of a dry specimen from West Java stored at the Museum of Natural History of Toulouse (France). Picture credit: Didier Descouens.

event from the Philippines to continental Asia and the Sunda shelf. In the case of *P. schreiber lindae*, the geological history of the Tawi Tawi archipelago differs greatly from that of Palawan (Figure 1). The Tawi Tawi archipelago belongs to the Sulu arc, a young volcanic chain, which was a subduction-related arc from the Middle to Late Miocene (Hall 2012, 2013). The recent volcanic activity in this region has a somewhat unusual character: it is not obviously related to subduction and there is no subducted slab or seismicity beneath the islands today (Hall pers. comm.). The Tawi Tawi archipelago therefore has no clear geological affinity with either the Sunda arc or the Philippines. With respect to the directionality of biogeographic events, it is interesting to find that part of the fauna dwelling in this archipelago is more closely related to the fauna of Sunda than to the Philippines one. The Tawi Tawi archipelago was likely colonized from the Sunda shelf during periods of low sea level. Such sea level fluctuations have been recorded during the Quaternary climate change (QCC) that began 2.6 million years ago (Ma) (Voris 2000). The rather low genetic divergence between Tawi Tawi endemics and other *P. schreiber* populations, as well as the close relationships of this taxon with Sunda populations, might indicate that the origin of this subspecies could stem from the QCC and be associated with isolation caused by sea level fluctuations. Yet, the hypotheses regarding the origin and evolution of Palawan and Tawi Tawi endemics remain to be tested using a dated phylogeny and proper models of historical biogeography.

Second, we successfully assigned the Pakistani specimens to *P. agraria* and *P. paulettae*, two lineages that were not known to occur in sympatry. The presence of *P. agraria* in Eastern Pakistan is not surprising as this species flies throughout India. However, *P. paulettae* was only known from Thailand and Myanmar, from which this species was recently described (Toussaint et al. 2015). This finding, along with the assignment of a new specimen from Malaysia to *P. paulettae*, suggests that this species might be much more widespread than previously thought. This is relatively surprising considering that this is a recently described species and clearly highlights the complexity of this morphologically homogenous group. These results extend the range of *Polyura* westward to Pakistan (Figure 4). Additional fieldwork in India is needed to fully comprehend the distribution of *Polyura paulettae* as it presently has a disjunct distribution between Eastern Pakistan and Myanmar. Knowing if this gap is an artefact due to a sampling bias, or if it is real, would be crucial to understand the evolution of this species in Indomalaya. From our results we can hypothesize that the present-day disjunction is likely to be due to a sampling bias and *P. paulettae* should be present in India because we find a very shallow genetic difference between the populations across its geographic range. This hypothesis however remains to be tested in a proper phylogeographic framework with a denser geographic sampling.

In this study we revised the geographic distribution, taxonomy and systematics of *Polyura* species found in the *P. athamas* group. The genus range is extended to Pakistan and six species have an updated distribution. Most notably, the recently described *P. paulettae* has a putative disjunct distribution that requires additional sampling effort in India. *Polyura alphius* has also a wider geographic range encompassing the Philippines where the subspecies *P. alphius acuta* new combination is found. Finally, the "umbrella" species *P. athamas* has a reduced distribution in mainland Asia, only because its populations from the Sunda Islands are reinstated as a valid species *P. attalus* new status. With these new insights, the *P. athamas* group now has a clearer taxonomy that will allow the study of biogeography and diversification dynamics of the entire genus.

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