Phylogenomics, Biogeography, and Morphometrics Reveal Rapid Phenotypic Evolution in Pythons After Crossing Wallace's Line

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Abstract.—Ecological opportunities can be provided to organisms that cross stringent biogeographic barriers towards environments with new ecological niches. Wallace's and Lyddeker's lines are arguably the most famous biogeographic barriers, separating the Asian and Australo-Papuan biotas. One of the most ecomorphologically diverse groups of reptiles, the pythons, is distributed across these lines, and are remarkably more diverse in phenotype and ecology east of Lyddeker's line in Australo-Papua. We used an anchored hybrid enrichment approach, with near complete taxon sampling, to extract mitochondrial genomes and 376 nuclear loci to resolve and date their phylogenetic history. Biogeographic reconstruction demonstrates that they originated in Asia around 38-45 Ma and then invaded Australo-Papua around 23 Ma. Australo-Papuan pythons display a sizeable expansion in morphological space, with shifts towards numerous new adaptive optima in head and body shape, coupled with the evolution of new micro-habitat preferences. We provide an updated taxonomy of pythons and our study also demonstrates how ecological opportunity following colonization of novel environments can promote morphological diversification in a formerly ecomorphologically conservative group. [Adaptive radiation; anchored hybrid enrichment; biogeography; morphometrics; snakes.]

Ecological opportunity is the best known mechanism to promote rapid phenotypic evolution to fill new ecological niches. It can be provided through a variety of means including the evolution of key innovations, extinction of competitors, or the arrival in a new environment rich in resources and/or depauperate of ecological competitors (Simpson 1944; Yoder et al. 2010; Stroud and Losos 2016). Colonization of new environments has ignited some of the most explosive radiations. Among the most spectacular examples are muroid rodents in South America (Schenk et al. 2013), anole lizards in the Greater Antilles (Mahler et al. 2010), colubroid snakes in the New World (Pyron and Burbrink 2012), lobeliad plants in Hawaii (Givnish et al. 2009) and cichlid fishes in African lakes (Seehausen 2006). Organisms which are able to cross biogeographic barriers may have access to new ecological opportunities not present in their current range (Yoder et al. 2010). Arguably the most famous biogeographic lines are those identified by Alfred R. Wallace that divide the Australo-Papuan and Asian biotas (Wallace 1869). These lines have been re-defined historically, but currently include Wallace's line, delimiting the Sunda shelf on the west, which contains South-East Asia as far east as Borneo and Bali, and Lyddeker's line, delimiting the Sahul shelf on the east, which contains Australia and New Guinea, i.e. Australo-Papua (Huxley 1868; Mayr 1944). The region between those lines, featuring a mixture of both Australian and Asian biota, is often called Wallacea (Michaux 2010).

The Australo-Papuan biota is a mixture of old Gondwanan lineages and groups that arrived more recently from Asia after crossing Lyddeker's line following the collision of the Eurasian and Australian plates around 25 ma (Hall 2002). Recent studies have demonstrated that most Australo-Papuan squamate reptile radiations are derived from Asian ancestors that diversified into what is today one the world's most diverse reptilian faunas (Keogh 1998; Hugall and Lee 2004; Vidal et al. 2012; Lee et al. 2016; Oliver and Hugall 2017). Although the biogeographic patterns of these extremely successful radiations are relatively well understood, the extent of phenotypic evolution undergone upon arrival to Australo-Papua remains relatively unstudied.

Pythons (Pythonidae) are an iconic group of large constrictor snakes comprising 44 species, including the largest snakes in the world, and a remarkable level of ecological and morphological diversity. For example, they range in adult body size from 50 cm dwarfs, like *Antaresia perthensis*, to 9 m giants like *Malayopython reticulatus*, and in ecological niche from the semi-aquatic *Liasis fuscus* to the fully arboreal *Morelia viridis*. They also display some of the most striking body and head shape diversity among snakes (Esquerré and Keogh 2016; Esquerré et al. 2017). Pythons have an Old World distribution: they are found in sub-Saharan Africa, in southern and south-eastern Asia, and in Australo-Papua. Most of this diversity occurs in the Australo-Papuan

region, which is home to 32 (73%) of the python species.

Earlier studies of python relationships proposed two competing hypotheses about their biogeographic origins. The first is that pythons originated in South-East Asia and from there one lineage dispersed into Africa and another into Australo-Papua (Underwood and Stimson 1990). The other phylogenetic hypothesis, based on morphology, placed the Australian semi-fossorial *Aspidites* as sister to all other pythonids and proposed that pythons originated in Australo-Papua or Gondwana (Kluge 1993). More recent molecular work on pythons has supported another hypothesis where the Afro-Asian *Python* is the sister lineage to all other pythons, implying an origin in Africa or Asia and a subsequent dispersal through South-East Asia into Australo-Papua (Rawlings et al. 2008; Pyron et al. 2013; Reynolds et al. 2014). Despite the recent development of molecular phylogenies, the systematics of pythons remains problematic, and a more robust hypothesis of relationships is necessary to test both biogeographic and evolutionary hypotheses. Here we infer the evolutionary relationships and divergence times of pythons with a phylogenomic data set of 376 nuclear exons and complete mitochondrial genomes, coupled with multiple fossil calibrations. This time-calibrated phylogeny allowed us to update the systematics and reconstruct the biogeographic history of pythons, resolving some long-standing debates concerning their origins. We also used a comprehensive morphological data set comprising head and body shape, to test the hypothesis that the invasion of Australo-Papua provided the ecological opportunity that allowed pythons to evolve in to one of the most morphologically diverse snake families.

Materials and methods

Molecular Sampling

We collected tissues from 50 specimens of pythons representing 35 of the 44 currently recognized species (Barker et al. 2015) (see Supplementary Table S1 available on Dryad at https://doi.org/10.5061/dryad.47d7wm39m). We also included *Loxocemus bicolor* and *Xenopeltis unicolor*, the closest living relatives of pythons (Reynolds et al. 2014), and ten outgroup species used exclusively for the fossil calibrations in the dating analysis described below (three for Booidea and seven for Caenophidia).

DNA Sequencing and Alignment Preparation

We extracted the DNA from approximately 1 mm^3 of tissue (stored in 100% ethanol) using a Qiagen DNeasy Blood & Tissue kit. Data were collected at the Center for Anchored Phylogenomics (www.anchoredphylogeny.com) at the Florida State University, which resulted in a final data set comprised 376 nuclear loci with an average length of 1635 bp.

Additionally, we reconstructed the mitochondrial genomes from the raw reads for each sample using a wrapper R script based on MITObim version 1.9 (Hahn et al. 2013). A link to these scripts can be found at www.github.com/IanGBrennan/mitoGenome_Assembly. We added mitochondrial sequences from the alignment reconstructed by Reynolds et al. (2014) to infer the phylogenetic placement of taxa not included in our sampling, including *P. anchietae, P. sebae, P. molurus*, and *Simalia clastolepis*. The final alignments include 35 species for the nuclear data and 39 for the mitochondrial data. Missing species all belong to recently split populations that are ecologically and morphologically very similar to species sampled in this study and therefore are unlikely to alter our macro-evolutionary inferences. See Supplementary Appendix I available on Dryad for further details on molecular data collecting.

Alignment Partition and Substitution Model Selection

To find the best partitioning scheme for both the nuclear and mitochondrial alignments and the best substitution model for each partition we used PartitionFinder 2 (Lanfear et al. 2016) using the Bayesian Information Criterion (BIC). Due to the size of the alignments we relied on a relaxed clustering algorithm that uses information from the sequences, such as base frequencies and rates of molecular evolution, to cluster similar subsets together, making the search computationally feasible (Lanfear et al. 2014). The best fits for the nuclear and mitochondrial alignments were found to be 48 and 14 partitions, respectively.

Phylogenetic Hypotheses

In order to evaluate the robustness of our results, we performed several different approaches of phylogenetic inference. First, we used the concatenated alignment of 376 loci to perform Maximum Likelihood (ML) phylogenetic inference and a Bayesian phylogenetic inference, using RAxML version 8.2 (Stamatakis 2014) and MrBayes version 3.2 (Ronquist et al. 2012), respectively. For both of these analysis we used the substitution model alignment partitions described above. Second, we generated individual gene trees for each of the loci with ML inference using RAxML, with 100 bootstrap replicates and no outgroup, since the analysis described below (Astral) requires unrooted trees. Finally, we also estimated each of the individual gene trees using StarBeast2 (Ogilvie et al. 2017). See Supplementary Appendix I available on Dryad for details.

In order to make use of the whole genomic data set in a coalescent framework we estimated a species tree using the individual gene trees generated above (from RAxML and StarBeast 2) using Astral III (Zhang et al. 2017). This is a highly computationally efficient, non-parametric and coalescent based program that finds the species tree with the highest number of shared induced quartets

within the gene trees. It can also compute branch lengths (in coalescent units) and support using local posterior probability (Sayyari and Mirarab 2016) and multi-locus bootstrapping (Seo 2008) based on 100 replicates (for the RAxML trees we used the bootstrap replicates and for the StarBEAST2 trees we used randomly selected trees from the post-burnin posterior distribution).

Fully Bayesian multispecies coalescent methods (e.g. StarBEAST 2) have been shown to reconstruct species genealogies with higher precision than concatenation and coalescent-based methods, even when using tens rather than thousands of loci (Ogilvie et al. 2016; 2017). However, programs like these still cannot handle the amount of data we have available; therefore, we used a subset of loci. First, we used the pipeline EAPhy (Blom 2015) to sort the loci according to missing taxa. Second, we used PhyDesign (López-Giráldez and Townsend 2011) to profile the informativeness of each locus through the depth of the tree and sort them by informativeness during the diversification of pythons, where phylogenetic noise would be minimal and informativeness maximized (Townsend et al. 2012). With this information, we selected the 23 most informative loci with up to only two missing taxa to run in StarBEAST 2. We set the parameters as described for the individual gene trees (Supplementary Appendix I available on Dryad), but instead we used a calibrated Yule prior on the species tree (Heled and Drummond 2012), except for runs with multiple calibrations where computational constraints forced us to use a standard Yule prior. To estimate divergence times, we incorporated fossil calibrations using the full set of snake outgroups listed in Supplementary Table S1 available on Dryad. We tried most of the calibrations proposed by Head (2015) and implemented by Esquerré et al. (2017), as well as performing a thorough review of available fossils that could be used to calibrate the pythonid tree, but could only reach convergence using three calibrations (see Supplementary Appendix II available on Dryad for details). For the runs using the calibrated Yule model we ran 5 independent MCMC chains for 2-4 billion generations, sampling every 50,000, for which a burnin of between 70-90% was required to achieve stationarity and convergence between the runs. For the runs with the standard Yule model we ran 5 chains for 600 million generations, for which a burnin of 50% was used before combining them. A maximum clade credibility (MCC) tree was obtained with TreeAnnotator 2.4.7. For all the following analyses, we use this tree and the posterior distribution of the StarBEAST2 analysis.

Biogeographic History

To infer the biogeographic history of pythons we used the R package BioGeoBEARS (Matzke 2013a). We divided the geographic range of pythons into seven major regions: Africa, Western Asia (mostly India and the Sunda plate which includes most of South-East Asia

as far east as Wallace's line) (Huxley 1868), Wallacea or Eastern Asia (the Indonesian islands between Wallace's and Lydekker's line (Mayr 1944; Michaux 2010)), New Guinea, Australia's Top End (Northern part of the Australian Northern Territory), Northern Queensland, and the rest of the Australian continent. See Figure 2 for details. We did not include the extant sister taxon of pythons, Loxocemus, in the analysis because it is from Mexico and Central America, and including it would only add noise tosister the results.

Traditionally, the Asian (*Python molurus* and *P. bivittatus*) and African (*P. sebae* and *P. natalensis*) giant pythons of *Python* were considered to be monophyletic (Underwood and Stimson 1990; Rawlings et al. 2008). Of these species, we only have nuclear data for *P. bivittatus*, but our mitochondrial results infer all African and Asian *Python* taxa as reciprocally monophyletic, making the Afro-Asian giant *Python* group polyphyletic, since African and Asian giants are more closely related to dwarfs than to each other (Supplementary Fig. S1 available on Dryad). Since considering the lineage of *P. bivittatus* being only Asian (the African giants more related to the African dwarfs than to Asian giants) or Asian and African (the Afro-Asian giants monophyletic) has different implications, we performed the biogeographic analyses with both settings. We compared several different models each with different parameters. The models we tested were the Dispersal-Extinction-Cladogenesis or DEC (Ree and Smith 2008), and likelihood implementations of Dispersal-Vicariance-Analysis or DIVA (Ronquist 1997) and BayArea (Landis et al. 2013) models, named DIVALIKE and BAYAREALIKE respectively (Matzke 2013b). Additionally, we tested these models with two free parameters. J is a parameter that approximates long distance dispersal or founder-events, and W is a parameter that multiplies the dispersal rate matrix (Matzke 2013b). Following a similar approach to Esquerré et al. (2019), we made these models more biologically realistic by constraining adjacency (species can only occur in regions that are considered adjacent) and dispersal (species can only disperse between regions considered to be adjacent). Although frequent tectonic activity and changes in sea level have dramatically changed the geography of the Indo-Australian Archipelago between mainland Asia and Australia, the adjacency between the regions in our study have remained constant during the Cenozoic (Lohman et al. 2011). Nested models were compared with Likelihood ratio tests and all models were compared with AICc (corrected Akaike Information Criterion).

Phenotypic Evolution

We obtained geometric morphometric landmark coordinates to represent head shape and traditional morphometric measurements from 755 and 727 specimens, respectively, to study body shape of all python species included in the phylogeny (except for *Liasis*

savuensis, which is extremely similar and closely related to *L. mackloti* and *L. fuscus*) plus *Loxocemus bicolor* and *Xenopeltis unicolor* (see (see Supplementary Table S2 available on Dryad for details). Details on data collection and transformation methods (including size correction) follows Esquerré and Keogh (2016) and Esquerré et al. (2017). We reconstructed the phylomorphospace (Sidlauskas 2008) of the first two Principal Components (PC) of head and body shape to visualize the ecomorphological diversity occupied by the Australo-Papuan clade in contrast with the Afro-Asian (*Python*) and Wallacean (*Malayopython*) clades. We used data presented in Esquerré and Keogh (2016) and color coded the taxa according to microhabitat use. For head shape we used the function *plotGMPhyloMorphoSpace* of the R package *geomorph* version 3.0 (Adams et al. 2016) and for body shape we used the function *phylomorphospace* of the R package *phytools* version 0.6 (Revell 2012). To test for statistical differences in morphovolume between Australo-Papuan and Afro-Asian regions we computed the convex hull volume for the first two PCs for each group (which account for 78% and 90% percent of the variation in head and body shape, respectively), and used a Wilcoxon rank sum test to assess if the differences are statistically significant. This was done using the R package *dispRity* (Guillerme 2018).

We used the R package *l1ou* (Khabbazian et al. 2016) to detect the phylogenetic position of phenotypic shifts towards different adaptive optima. This method, which can handle large trees and trait matrices and is based on the lasso (Tibshirani and Taylor 2011), models changes in trait evolution along a changing adaptive landscape over time and models lineages under an Ornstein–Uhlenbeck (OU) process (Hansen 1997). The model can then detect which regimes are convergent towards the same adaptive optima. We used the package's implemented phylogenetic Bayesian Information Criterion (pBIC), which offers a phylogenetically informed and more conservative model comparison without over-fitting (Khabbazian et al. 2016). To account for phylogenetic uncertainty, we performed this test on 100 trees randomly sampled from the post-burnin posterior distribution and the MCC tree. We performed this separately on the Procrustes coordinates of head shape and the size corrected body measurements. To visualize the relative amount of times each shift was detected on a clade we added a short branch elongation on that clade to the MCC tree.

To test the power to detect shifts in phenotypic evolution of pythons we performed a series of exercises which leverage simulated data and our empirical phylogenetic hypotheses. The ability to fit more complex models, such as those with multiple "adaptive optima" as proposed in *l1ou* may be limited by the number of tips present in the tree, total tree depth, and dimensionality of the data (Beaulieu et al. 2012). Given the limited size of our phylogeny (31 species) we used a sample of 100 phylogenetic trees from the posterior of our dating analysis to test that we can accurately recover models

of varied complexity, and that false recovery rates are low enough to limit concern that there is bias towards specific model types (Cooper et al. 2016).

To determine the true positive rates of our models, we started by fitting four evolutionary models to our set of 100 trees and phenotypic data sets. These included commonly employed evolutionary models: Brownian Motion (BM), single-peak Ornstein Uhlenbeck (OU), and Early Burst (EB), and a multi-optima OU model which does not require *a priori* designation of adaptive regimes (*l1ou*). Because of the high dimensionality of our data, we fit these models using the R package *mvMORPH* (Clavel et al. 2015), which allows a multivariate implementation of those commonly used evolutionary models. From these model fits we extracted the appropriate parameters (BM— σ; OU— σ, α; EB— σ, β; *l1ou*— σ, α per regime, number and placement of regimes), and simulated data under each model using empirical parameter estimates. For *l1ou* we created trees with branches 'painted' according to the recovered regimes using *make.SIMMAP* in *phytools*. Finally, we fitted the 100 data sets simulated under each model back to the four models to determine if we could accurately recover the generating process.

RESULTS

Phylogenetic Hypotheses

The coalescent-based tree inferred by Astral (using RAxML gene trees), and the concatenated trees inferred by RAxML and MrBayes, have the same topologies, with just one exception in the inference of the relationship between the *Morelia spilota* subspecies (Supplementary Fig. S2 available on Dryad). The Astral tree using the StarBEAST2 trees, and the multi-species coalescent tree using the 23 most informative loci, recover the same topology but differ from the previous analyses in the placement of *Simalia oenpelliensis*. This species is either sister to *Antaresia* or *Morelia*, but with low support for either scenario and a very short inter-nodal distance (Fig. 1 and Supplementary Fig. S2 available on Dryad). This either suggests rapid divergence between the three lineages that is extremely hard to resolve or past introgression. *Simalia oenpelliensis* is closely related closely to the other species currently placed in *Simalia*, and it is also morphologically very distinct, therefore we describe the genus *Nawaran* gen. nov. for the species *Nawaran oenpelliensis* (see Supplementary Appendix III available on Dryad and Taxonomy section for details) to address the taxonomic issue raised by our results.

The relationships among all the other taxa are concordant across all the phylogenetic analyses. The Afro-Asian clade comprising *Python* is sister to the Indo-Australian clade *sensu* Rawlings et al. (2008), comprising the South-East Asian *Malayopython* and the Australo-Papuan clade. Within *Python*, the central African *P. regius* is the sister to the rest of the species in the genus, which comprise two clades, the Asian *P. molurus* complex (represented here by *P. bivittatus*) and the South-East Asian
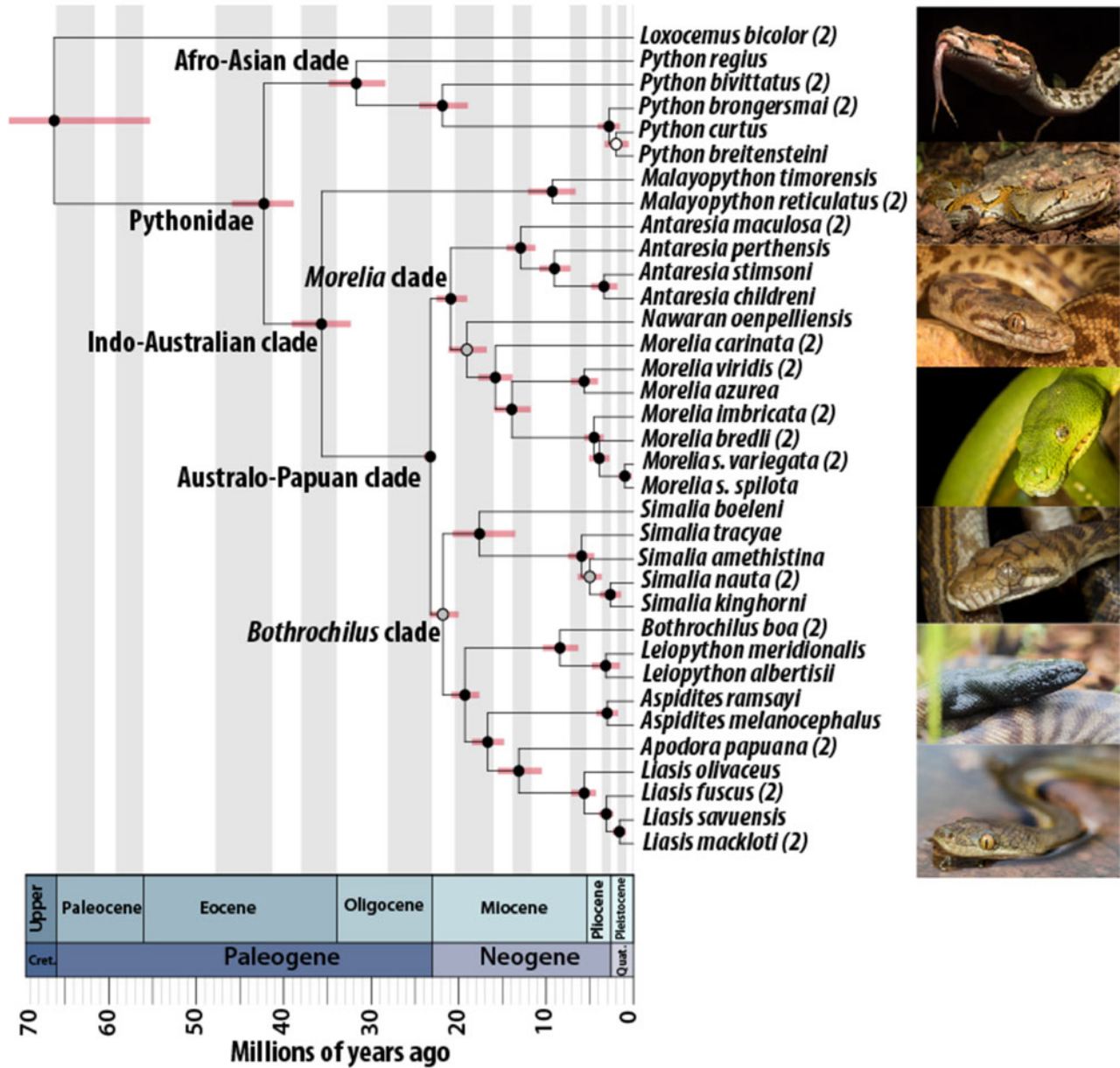
FIGURE 1. Fossil calibrated phylogeny estimated with StarBEAST2 and the 23 most informative loci. Node bars indicate the 95% High Posterior Density (HPD) for each node, and node balls indicate posterior support where black>0.95, gray: between 0.95 and 0.75 and white<0.75. Numbers in parenthesis indicate number of samples if more than one. Photos from top to bottom are: *Python molurus* (related to *P. bivittatus*), *Malayopython reticulatus*, *Antaresia stimsoni*, *Morelia viridis*, *Simalia kinghorni*, *Aspidites melanocephalus* and *Liasis fuscus*. All photographs by Damien Esquerré. The updated and revised taxonomy is shown here (Supplementary Appendices III and IV available on Dryad).

P. curtus group. There are two main clades of Australo-Papuan pythons, one we call the *Bothrochilus* clade comprising the genera *Apodora, Aspidites, Bothrochilus, Leiopython, Liasis* and *Simalia* and the other we call the *Morelia* clade comprising *Antaresia, Morelia* and *Nawaran* gen. nov.

The phylogenetic inference using the mitochondrial data largely agrees with the nuclear data, however, there are some important differences (Supplementary Fig. S1 available on Dryad). One area of discordance is in the placement of *Nawaran oenpelliensis*, as sister to *Simalia*, and the placement of *Simalia+Nawaran*, as sister to the remaining Australo-Papuan pythons. Additionally, the mitochondrial data place the clade comprising *Morelia carinata, M. viridis* and *M. azurea* as sister to a clade comprising *Antaresia* and the remaining *Morelia*, rendering *Morelia* paraphyletic. The mitochondrial data allows us to include the African species *Python sebae* and *P. anchietae*, which we did not have represented in the nuclear alignment. The mitochondrial data show that

these are sister species and together they form a sister clade to the remaining African species. *P. regius* is sister to both, making the African pythons monophyletic.

Dating

We performed several dating analyses using StarBEAST2 with different fossil calibrations and reached convergence using three of them simultaneously. We also performed an analysis with MCMCTree with the same three calibrations (see Supplementary Appendix II and Table S3 Supplementary for details). The divergence times estimated by MCMCTree are concordant with the StarBEAST2 analyses. We report the StarBEAST2 tree with three calibrations and use it for subsequent analyses. The results of the subsequent analyses did not change qualitatively when we used any other of the dated phylogenies. Loxocemidae diverged from Pythonidae between the late Cretaceous and early Paleogene, 66.25 Ma (95% HPD: 55.3-76.29). The initial divergence in extant pythons, between *Python* and the rest of the family, is dated at 42.27 Ma (95% HPD: 38.87-45.92) in the mid Eocene. *Malayopython* split from the Australo-Papuan clade in the early Eocene to late Oligocene, 35.69 Ma (95% HPD: 32.35-39.09). The Australo-Papuan clade diverged into the *Bothrochilus* and *Morelia* clades in the early Miocene, 23.21 Ma (95% HPD: 23-23.95). See Figure 1 and Supplementary Table S3 available on Dryad for details on divergence times.

Biogeographic History

Our biogeographic reconstruction supports an Asian origin of pythons. Setting *Python bivittatus* as Asian or Afro-Asian did not make a qualitative difference in the results results (Fig. 2, Supplementary Fig. S3 and Table S4 available on Dryad). The preferred biogeographic model was DEC+J+W and DEC+J for the analyses setting *Python bivittatus* as Asian and Afro-Asian, respectively (Supplementary Table S4 available on Dryad). The Likelihood-ratio tests also demonstrate the importance of the founder event (parameter J) in python dispersal (Supplementary Table S5 available on Dryad). Our analyses suggest that pythons originated in Asia in the Oligocene (Fig. 2), and from there they dispersed into Africa, and that the ancestor of the Indo-Australian clade lived in Wallacea in the early Miocene. The reticulated python (*Malayopython reticulatus*) dispersed widely through South-East Asia, crossing Wallace's line back from Wallacea, whereas the Australo-Papuan lineage dispersed to New Guinea in the mid Miocene. Two clades then separated, the *Bothrochilus* clade diversifying mostly in New Guinea and the *Morelia* clade diversifying mostly in Australia. The *Bothrochilus* clade had at least 5 subsequent dispersals into Australia (*Aspidites*, *Simalia kinghorni*, and three lineages of *Liasis*), and notably three lineages dispersing back to Wallacea during the Pliocene-Pleistocene, crossing Lydekker's line back (two

Simalia and at least one *Liasis*). The *Morelia* clade had at least three independent dispersals into New Guinea (*Antaresia maculosa*, *Morelia spilota* and *Morelia viridis/azurea*).

Phenotypic Evolution

The two most important PCs of head shape phenotypic variation primarily represent a broadening of the head along PC1 and a thinning of the snout along PC2. The two most important PCs of body shape phenotypic variation primarily represent increasing body girth and shortening of the tail along PC1 and a reduction in head size, thickening of the neck and body elongation along PC2. Loadings and proportion of variance explained by each PC are in Supplementary Table S6 available on Dryad. For both head and body shape, morphological space occupied by the Australo-Papuan radiation is remarkably broader than Afro-Asian pythons (Fig. 3). This is supported by our measures of morphovolume with Australo-Papuan pythons having increased morphological diversity compared to Afro-Asian pythons (head shape: 0.009 vs 0.001, W = 10000, $P < 2.2e^{-16}$; body shape: 0.236 vs 0.037, W = 10000, $P < 2.2e^{-16}$) (Fig. 3). Similarly, Australo-Papuan pythons have diversified into more diverse microhabitats. While the terrestrial *Python* and semi-arboreal *Malayopython* have a more conserved and generalist ecology, Australo-Papuan pythons also have evolved specialized habitat exemplified by some highly arboreal *Morelia*, semi-aquatic *Liasis* and semi-fossorial *Aspidites*.

We detected a remarkable difference between the Afro-Asian pythons and the Australo-Papuan python clade in head shape adaptive phenotype shifts. No adaptive phenotypic shifts were detected in the Afro-Asian pythons, whereas 12 shifts where detected in the Australo-Papuan pythons, and two of these were convergent (the semi-arboreal *Simalia* and *Morelia*) (Fig. 3). Four shifts were detected in all of the sampled trees from the posterior distribution for body shape and none of them were convergent. Two of these shifts were inferred among the African lineage (in the stocky *Python regius* and the *P. curtus* group) and two in the Australo-Papuan clade (in the extremely elongated *Nawaran oenpelliensis* and the similarly elongated but proportionally larger headed *Simalia amethistina* group) (Fig. 3 and Supplementary Fig. S4 available on Dryad).

The ability to accurately recover the correct (generating) process is essential to any study that seeks to explain observations using models. We find consistent support for the accurate assignment and preference of generating models in our simulation study (Supplementary Fig. S5 available on Dryad). This provides evidence that even with our comparatively small phylogeny of pythons, we can accurately infer the process or patterns dictating phenotypic evolution. Despite warnings about the behavior of OU models, we also find relatively low false positive rates for single and multi-peak OU models.
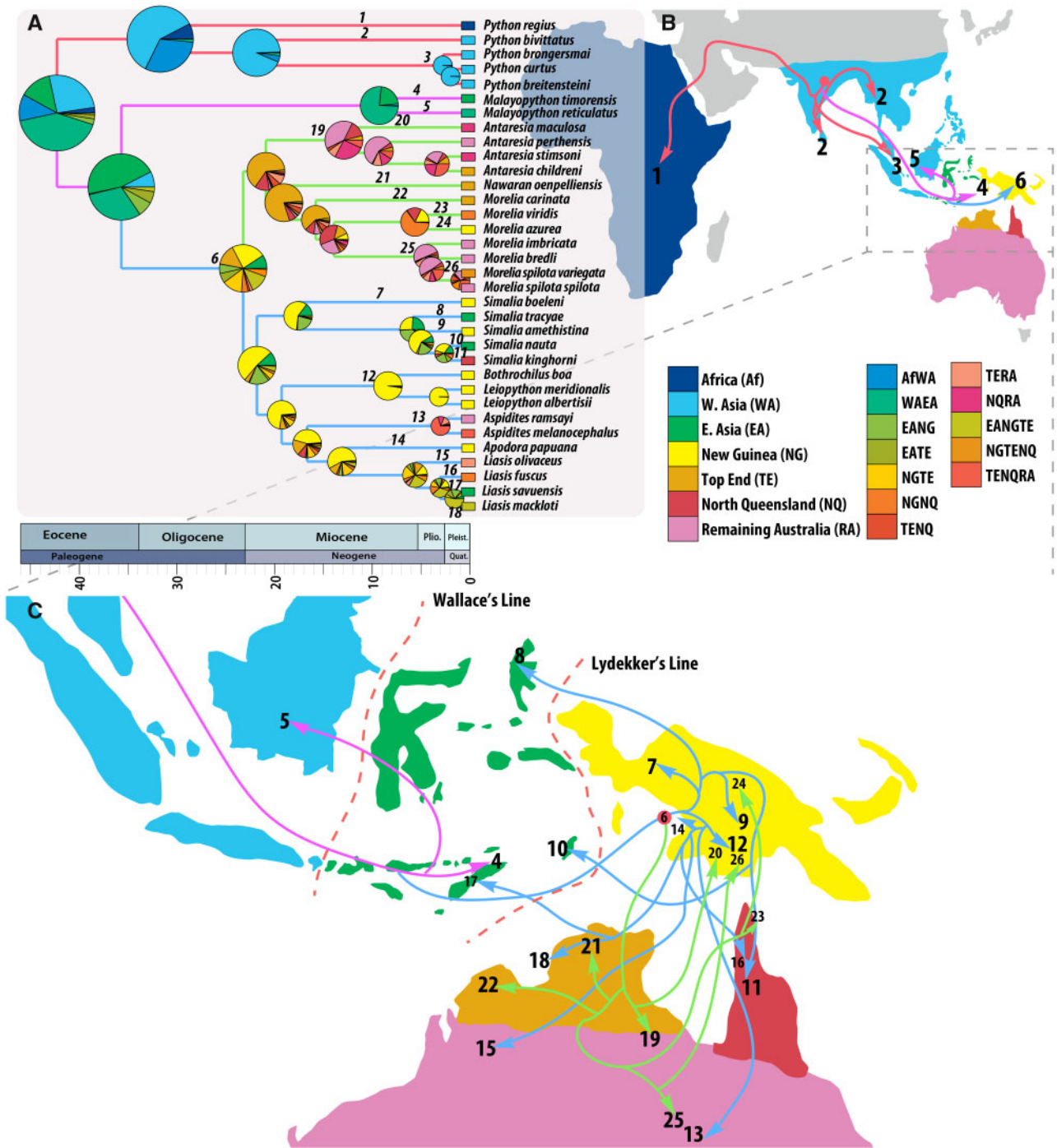
FIGURE 2. A: Biogeographic history reconstruction of pythons using BioGeoBEARS under the preferred DEC+J model. Area codes on tips of the phylogeny and pie charts correspond to the legend. B: Lines on the maps depict the phylogeny and possible path of dispersal of pythons. Numbers at the tips correspond to clades and taxa numbered in the phylogeny. C: A detailed dispersal hypothesis for the Australo-Papuan clade. This is based on the most likely node ancestral areas inferred from BioGeoBEARS but given high uncertainty in many of them the figure should only be taken as a general biogeographic pattern. Land connections between Australia and New Guinea have been frequent over the past, therefore we do not want to imply overwater dispersal between these two areas.

Somewhat surprisingly, the false positive rate for the *l1ou* model is the lowest of the four models fitted. This method is designed around a flexible framework of OU models (of which BM is a special class, α=0), which should incorporate both BM and single-peak OU models, however this does not appear in our simulated model fittings, suggesting the *l1ou* model is instead selected against in these instances.

Head Shape

Body Shape

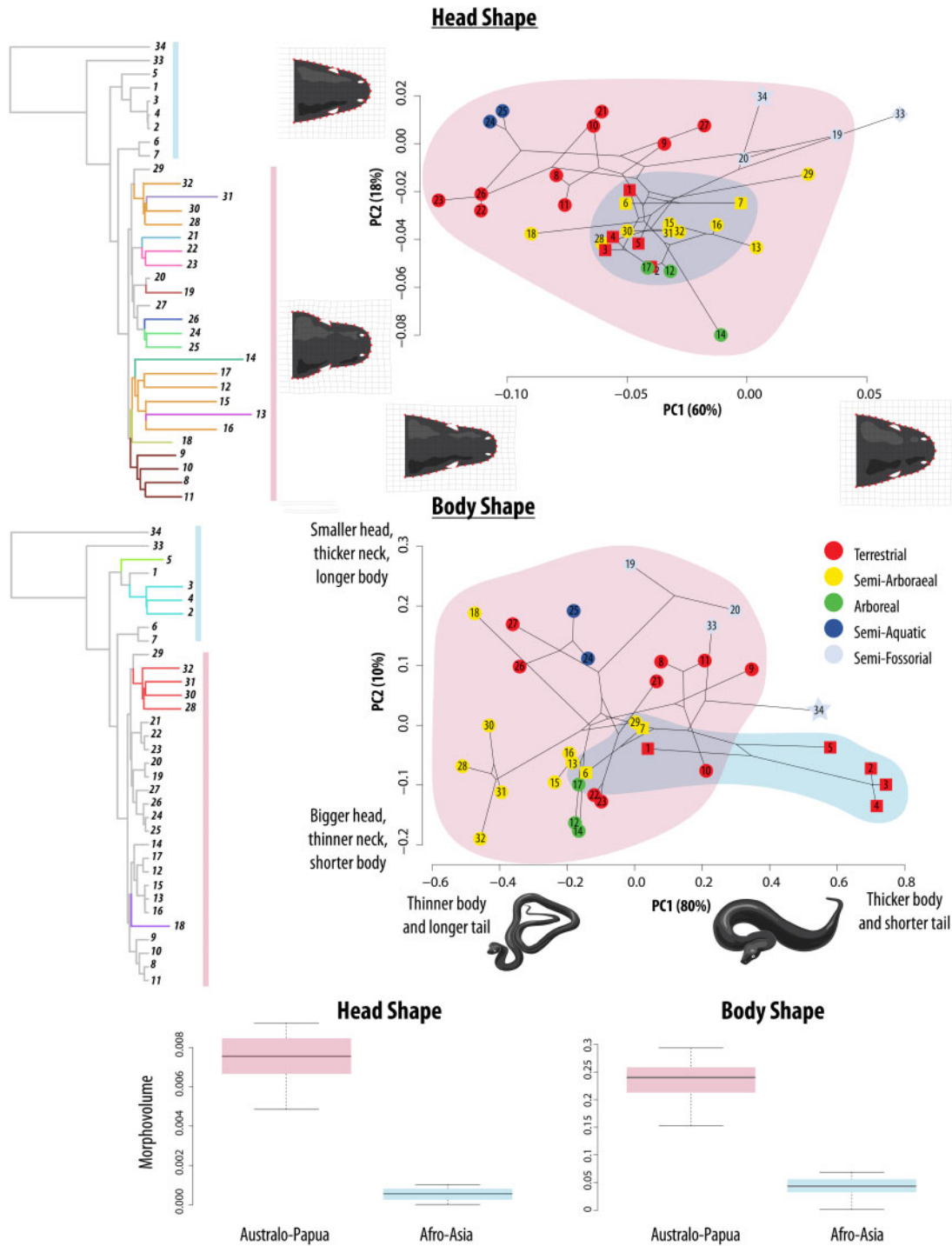**Head Shape**

Body Shape

FIGURE 3.   Morphological evolution of head shape (above) and body shape (below). The trees on the left represent the phenotypic evolutionary shifts detected by *l1ou* which are colored (ochre colored regimes in head shape for *Simalia* and *Morelia* are convergent). Branch lengths on these trees are proportional to the relative number of times that a shift was recovered in the random posterior sample. On the right are phylomorphospace reconstructions. For head shape, at the extremes of the X (PC1) and Y (PC2) axis there are deformation grids displaying the deformation from the mean shape to the maximum and minimum of the respective PC. Similarly, for body shape, text descriptions describe what high and low values of either PC represent. On both plots, stars represent *Xenopeltis*, diamonds *Loxocemus*, squares *Python* and *Malayopython* and circles the Australo-Papuan pythons. Numbers on each point indicate the species according to Table S2, in brief: 1-5: *Python*, 6-7: *Malayopython*, 8-11: *Antaresia*, 12-17: *Morelia*, 18: *Nawaran*, 21: *Bothrochilus*, 19-20: *Aspidites*, 22-23: *Leiopython*, 24-26: *Liasis*, 27: *Apodora*, 28-32: *Simalia*, 33: *Loxocemus*, 34: *Xenopeltis*. The morphospace occupied by the Afro-Asian *Python* and *Malayopython* is shaded in blue and by the Australo-Papuan pythons is shaded in red (also depicted by the bars on the phylogeny on the left), to show the increase in phenotypic diversity after colonizing Australasia. Each dot is colored according to ecological guild as in the legend on the right. Bottom panel: Comparison of morphovolume, measured as convex hull volume for head shape and body shape, between Afro-Asian and Australo-Papuan pythons.

DISCUSSION

Pythons are a diverse and iconic group of reptiles. Here, we resolve their controversial phylogenetic and biogeographic history, revise their taxonomy, and demonstrate that most of the ecomorphological diversity in the group evolved both quickly and recently after invading Australo-Papua. Pythons originated in Asia 38 to 45 ma and then dispersed into the Australo-Papuan region around 23 ma. The ecological opportunity available in this region allowed pythons to diverge eco-morphologically into the extremely disparate set of species we observe today. For several million years pythons in Africa and Asia remained terrestrial or semi-arboreal, with a conserved morphology. Then, in a relatively short time in Australo-Papua, pythons exploded in morphological diversity, coupled with an exploration of novel ecological niches, ranging from the fully arboreal *Morelia viridis* to the semi-fossorial *Aspidites*, from the semi-aquatic *Liasis* to the ground dwelling *Antaresia*.

Phylogeny and Biogeography

Our study confirms previous hypotheses on the phylogenetic history of pythons and their closest relatives (Lawson et al. 2004; Noonan and Chippindale 2006; Rawlings et al. 2008; Pyron et al. 2013; Reynolds et al. 2014) using a larger data set than previously available. Our estimate of the divergence between Loxocemidae and Pythonidae agrees with previous studies that have dated the divergence to around 55-76 Ma between the Cretaceous and Paleogene (Vidal et al. 2009). While modern pythons are an exclusively Old World group, with no fossils found elsewhere (Head 2015), *Loxocemus* represents a single species found in Mesoamerica and the only known fossil loxocemids are from the Eocene and Oligocene in North America (Smith 2013). Xenopeltids (the sister group to loxocemids and pythonids) are South-East Asian, and since pythons also have an Asian origin, it therefore seems most likely that loxocemids represent a unique dispersal to North America in the Paleogene.

African and Asian *Python* species are sister to all other pythons (the Indo-Australian clade), and the South-East Asian reticulated and Timor pythons (*Malayopython*) are sister to the Australo-Papuan pythons, supporting what has been found in previous studies (Rawlings et al. 2008; Pyron et al. 2013; Reynolds et al. 2014). Our results also support previous findings that there are two main clades of Australo-Papuan pythons (Pyron et al. 2013; Reynolds et al. 2014). Furthermore, our phylogenomic scale data allowed us to address the phylogenetic relationships of the previously challenging Oenpelli python (*Nawaran oenpelliensis*). This species does not belong to *Simalia*, as inferred from the mitochondrial phylogeny (Pyron et al. 2013; Reynolds et al. 2014), but instead is a member of the *Morelia* clade along with *Morelia* and *Antaresia*. The relationship between these three lineages is difficult to resolve, which we attribute to rapid diversification

events or to past introgression. Nevertheless, the Oenpelli python is an independent and morphologically distinct lineage, which is why we have allocated it to a new monotypic genus, *Nawaran*. Details of the phylogenetic and taxonomic implications of our study can be found in Supplementary Appendices III and IV available on Dryad. Previous studies have supported either an Australasian or Gondwanan origin of pythons, mainly due to the morphology-based inference that the Australian endemic *Aspidites* was sister to all other pythons (Kluge 1993; Scanlon 2001). Our phylogenetic and biogeographic analyses support the hypothesis that pythons originated in Asia (McDowell 1975; Cadle 1987; Underwood and Stimson 1990; Rawlings et al. 2008). The presence of python fossils in Europe, the Middle East and Africa from the Miocene onward (Hoffstetter 1964; Rage 1984; 1997), and the connection of Africa to Eurasia through the Middle East in the middle Miocene (Gheerbrant and Rage 2006) supports either a migration from Asia or an origin in Africa or Europe. Much older pythonid-like fossils have also been well documented from the middle Eocene in Europe (Szyndlar 1994; Rage 1997), but given their age and a 30 Ma gap in python fossil records between the middle Eocene and early Miocene (see Supplementary Appendix II available on Dryad), we argue these belong to a stem lineage that populated Europe. This could translate into pythons having an origin in Europe or Africa rather than Asia. The massive extinction often called the "Grande Coupure" during the Eocene-Oligocene transition was possibly responsible for killing off this ancient pythonid lineage (Prothero 1994; Duffaud and Rage 1997), and the presence of Miocene pythonid fossils in Europe is likely from recolonization by crown pythons (Szyndlar and Böhme 1993).

A lineage of pythons, likely in the late Oligocene, migrated to the Malay Archipelago, and then into Australo-Papua, crossing Lydekker's line (Oliver and Hugall 2017). Once there, they divided into two main lineages, one diversified in New Guinea (the *Bothrochilus* clade) and another in Australia (the *Morelia* clade). It is interesting to note that at least three python lineages crossed Lydekker's line back into the Indonesian islands, which is known to be difficult for animals and illustrates the ability of these snakes to disperse overwater. The migration of animal groups from Asia to the Australo-Papuan region in the Miocene is now known to have occurred in a number of groups, providing the opportunity for the rapid diversification of species and phenotypes once they reached Australia. Elapid snakes (Keogh 1998; Lee et al. 2016), varanid lizards (Vidal et al. 2012), agamid lizards (Hugall and Lee 2004) and indeed most squamate reptile radiations in Australo-Papua share this same history and pattern of diversification (Oliver and Hugall 2017). While some have suggested that this wave of reptile colonization happened before the collision of the Australian and Asian plates (Vidal et al. 2012), 25 Ma (Hall 2002), our data suggests this dispersal of pythons happened more

recently, likely just after the collision of the plates, around 23 Ma (Oliver and Hugall 2017). A likely scenario for dispersal is island hopping, as pythons are in general good swimmers (Walls 1998). For example, *M. reticulatus* is sometimes found in the open ocean and was among the first vertebrates to recolonize Krakatoa island after its devastating volcanic eruption (Thornton 1997).

Ecological Opportunity

Ecological opportunity is often associated with islands (Pinto et al. 2008), but continents that have been isolated from particular groups of taxa also can provide ample ecological opportunity (Derryberry et al. 2011). The power of ecological release resulting from the invasion of a new environment can be observed in the contemporary examples of successful biological invasions (Keane and Crawley 2002; Broennimann et al. 2007; Vellend et al. 2007). Adaptive radiation has long been associated with ecological opportunity (Yoder et al. 2010), and pythons provide a prime example of such association. In terms of speciation, adaptive radiation caused by ecological opportunity is predicted to show an early-burst of diversification (Schluter 2000; Gavrilets and Vose 2005; Rabosky and Lovette 2008). However, the modest species richness of extant pythons limits our statistical power to confidently address diversification processes. Ecological opportunity in pythons had a profound effect on expanding their morphological diversification. The appearance of directional selection or relaxation of stabilizing selection with newly available ecological niches (Roughgarden 1972; Johnson and Barton 2005; Yoder et al. 2010) promotes phenotypic variation (Harmon et al. 2003; Nosil and Reimchen 2005). Morphological diversity has been long associated with the appearance of new ecological niches and thus represents a proxy for ecological diversity in both neontological (Ricklefs and Travis 1980; Roy and Foote 1997; Yoder et al. 2010; Mahler et al. 2010) and paleontological studies (Gould 1989; Erwin 2001). The clear expansion of morphological space happened in pythons after they crossed Lydekker's line, supporting the notion that invading this new land provided ample ecological opportunity to diversify. This pattern of initial diversification of morphological disparity partitioned among clades has been identified as a key component of adaptive radiation (Harmon et al. 2003; Burbrink and Pyron 2009).

That pythons in Asia and Africa did not diversity to the same extent as their counterparts in Australo-Papua is explained possibly by competition from large snakes in the extinct family Madtsoiidae that inhabited northern Africa during the late Eocene (Rio and Mannion 2017). While pythons in Australia also co-existed with the large madtsoiid snakes (Scanlon and Lee 2000; Scanlon 2003; 2006; Palci et al. 2018), it is difficult to establish the degree of competition that could have existed between pythons and madtsoiids without knowing the extent of range and ecological overlap. The specific factors that provided the ecological opportunity for pythons in Australo-Papua remain to be properly identified. Africa and Asia also have extremely diverse biotas, but pythons either failed to adaptively radiate or have faced much greater extinction rates in these regions.

Taxonomy

The phylogenetic results presented in this paper have a series of taxonomic implications, which are detailed in Supplementary Appendix III available on Dryad. The most important though, is the finding that the Oenpelli python represents a unique lineage and we therefore describe a new genus for this species below:

Nawaran gen. nov. Esquerré, Donnellan, Brennan, Lemmon, Lemmon, Zaher, Grazziotin & Keogh

Python Daudin 1803

Nyctophilopython Wells & Wellington 1985

Morelia Gray 1842

Etymology: Nawaran is the traditional name of the snake given by the people of Arnhem Land, where *Nawaran oenpelliensis* lives.

Type species: *Python oenpelliensis* Gow 1977

Diagnosis and definition: A lineage endemic to northern Australia that underwent a rapid divergence from *Antaresia* and *Morelia*, and showing intermediate traits between the two. They are slender and long snakes, with adults up to over 4 meters in total length, larger than any *Morelia* and especially the dwarfed *Antaresia*. Pale brownish to olive coloration, with longitudinal rows of large dark blotches. Enlarged nasal, internasal, anterior pre-frontals, supraocular and frontal symmetrical head shields. The posterior prefrontals are usually several smaller asymmetrical scales. In *Morelia* head scales are smaller and more divided, in *Antaresia* and *Simalia* there are no small divided posterior prefrontals. Heat pits present on rostral, first three supralabials and posterior infralabial scales. They are the only pythons with over 400 ventral scales. Along with *Aspidites*, this is one of the two python genera that are endemic to Australia.

Contents: *Nawaran oenpelliensis* (Gow 1977)

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