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MOLECULAR IDENTIFICATION OF PYTHON SPECIES (SQUAMATA: PYTHONIDAE) FROM MIZORAM, NORTHEAST INDIA, WITH COMMENTS ON WILDLIFE TRAFFICKING

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Abstract

Pythonidae, an ancient group of Old World, wide-ranging, constrictor snakes, are known to contain a high degree of cryptic diversity. India harbours three python species, *Python molurus*, *P. bivittatus*, and *Malayopython reticulatus*. The former two species are not uncommon within their respective distribution range in India, but occurrence of the latter has only been confirmed in the Nicobar Islands, and there are two orphaned records from eastern West Bengal. We confirm the occurrence of *P. bivittatus* and *M. reticulatus* in Northeast India based on genetics using the mitochondrial cytochrome *b* gene and morphological characters. Our study reveals multiple lineages among *M. reticulatus*, corroborating previous studies, and further reveals the absence of a barcode gap between sequences submitted as *P. molurus* and *P. bivittatus* among the sampled DNA sequences, and an unexpected lineage of Northeast Indian *P. bivittatus* based on a sample divergent from the East and Southeast Asian populations that will need further systematic assessment.

Keywords: Burmese python, DNA barcoding, phylogenetics, reticulated python, wildlife forensic

Introduction

Snakes of the family Pythonidae, commonly referred to as pythons, are an ancient Old World snake lineage represented by medium to very large-sized constricting species (Schleip & O’Shea 2010). Globally, there are 38 extant

species in the family Pythonidae (Uetz *et al.* 2024) distributed from Africa, South and Southeast Asia to Australia, with the highest genetic diversity in Australia and New Guinea (Rawlings & Donnellan 2003). Currently, three species of pythons are recorded from India

(Whitaker & Captain 2008), namely, the Indian rock python, *Python molurus* (Linnaeus, 1758); the Burmese python, *P. bivittatus* Kuhl, 1820; and the Reticulated python, *Malayopython reticulatus* (Schneider, 1801). Among them, *P. molurus* is widely found throughout mainland India except for the islands (Whitaker & Captain 2008, Schleip & O'Shea 2010), and is likely present in Assam, Northeast India (Das & Bhattacharjee 2005). Bhupathy (1995) considered that *P. bivittatus* has a wide distribution throughout northeastern India, with an isolated population in Uttarakhand (Whitaker & Captain 2008). On the other hand, *M. reticulatus* is recorded only from the West Bengal cities of Baidyabati Hooghly and Kolkata in mainland India, but these records warrant further surveys to uncover whether there are natural or introduced populations in West Bengal (Mukherjee *et al.* 2012, Wallach *et al.* 2014, Kalki *et al.* 2018), while viable populations are believed to occur only in the Nicobar Archipelago (Whitaker & Captain 2008, Kalki *et al.* 2018, Prabakaran & Ramesh 2021). Furthermore, Kalki *et al.* (2018) and Prabakaran & Ramesh (2021) discussed the presence of *M. reticulatus* in mainland India based on preserved specimens alleged to have been collected from various Indian localities. These authors considered a specimen, bearing the locality "Calcutta, India", to be the possible first representative of this species in mainland India (see also Talukdar 1998). Recently, Lalremsanga & Lalronunga (2017) also reported the species based on a juvenile individual collected from the southern part of Mizoram. It remains to be determined if this specimen belongs to an isolated population still present in riparian areas of the southern part of West Bengal and Mizoram or if it results from a human-introduced specimen.

Due to their aesthetic and commercial values, various species of pythons are often smuggled from their places of origin to be sent overseas (Ciavaglia *et al.* 2015). *Python molurus* is listed in Appendix I while all other Pythonidae species are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). The IUCN Red List of Threatened Species listed *P. molurus* as Near Threatened (Aengals *et al.* 2021), *P. bivittatus* as Vulnerable (Stuart *et al.* 2012), and *M. reticulatus* as Least Concern (Stuart *et al.* 2018). In India, all members of the *Python* genus are protected and listed in Schedule I under the

Indian Wildlife (Protection) Amendment Act (2022). We aim here to confirm the identity and occurrence of pythons in Mizoram state, Northeast India, through DNA barcoding and molecular phylogenetics using mitochondrial cytochrome b (CYTB) marker.

Material and Methods

We examined one preserved *M. reticulatus* (MZMU3327 from Belthai, Lunglei district) and six preserved specimens plus one live individual of *P. bivittatus* housed in the collection of the Departmental Museum of Zoology, Mizoram University (MZMU). Morphometric measurements were taken using Mitutoyo™ slide-calliper (model 505–671) to the nearest of 0.01 mm while the snout-vent length (SVL) and tail length (TaL) were taken using a measuring tape. We followed Dowling (1951) for counting ventrals (Ve), and the terminal, pointed scute was excluded when counting subcaudals (Sc). For DNA extraction, we sampled liver tissues from two ethanol-preserved specimens of *P. bivittatus* (MZMU1723 from ITI Veng, Aizawl District; MZMU2728 from Kawrthah, Mamit district); a fresh liver tissue sample of *P. reticulatus* was also obtained from a dead individual killed by local farmers on 6th July 2021 in New Laly, Siaha District, and the tissue sample was subsequently vouchered in the museum (MZMU2491).

Genomic DNA (gDNA) isolation from liver tissue was performed using DNeasy Blood and Tissue Kit (Qiagen™, Valencia, California, USA) following the procedure given by the manufacturer. The fragment of CYTB was amplified using forward (L14910) and reverse (H16064) primers (Burbrink *et al.* 2000) at the thermal regime of 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 49°C for 40 sec, 72°C for 30 sec, and with a final extension at 72°C for 5 min. The amplified PCR products were further processed for purification and sequencing using Sanger's dideoxy method at Barcode BioSciences, Bangalore, India. The newly generated DNA barcode data (CYTB and additional 16S rRNA) are deposited in GenBank repository (Benson *et al.* 2013) and received the following accession numbers, with the corresponding museum vouchers indicated in brackets: 16S: OP805382 [MZMU1723], OP805629 [MZMU2941]; CYTB: ON638922 [MZMU2728], OP086040 [MZMU2941].

For the molecular analyses, we utilized only CYTB due to the paucity of 16S rRNA sequence for the study taxa in the GenBank database

(Benson *et al.* 2013). We assembled the CYTB dataset (n=49; 1,114 bp) consisting of our newly generated and published sequences downloaded from the database, with *Python regius* (NC 007399) as an outgroup. We aligned the sequences by using the MUSCLE algorithm in MEGA 11 (Tamura *et al.* 2021) and partitioned them by codon positions. The best partitioning schemes and nucleotide substitution models were searched in PartitionFinder v2.1 (Lanfear *et al.* 2017). The Bayesian Inference (BI) phylogenetic tree was reconstructed in MrBayes v3.2.5 (Ronquist *et al.* 2012) using the optimal models selected under BIC score (TRNEF+G for CYTB pos1; F81+I for CYTB pos2; GTR+G for CYTB pos3). The MCMC (one cold and three hot chains) was run for 20 million generations by sampling every 5,000 generations and setting the burn-in to 25%. The branching supports in the BI tree are interpreted as Bayesian posterior probabilities (PP). Maximum likelihood (ML) tree was inferred in IQTREE webserver (Trifinopoulos *et al.* 2016) at 10,000 Ultrafast Bootstrap replicates (UFB) (Minh *et al.* 2013) utilizing the nucleotide substitution models selected under BIC score by ModelFinder (Kalyaanamoorthy *et al.* 2017) (TNe+G4 for CYTB pos1; F81+F+I for CYTB pos2; TIM2+F+G4 for CYTB pos3). The generated phylogenetic trees were annotated using Figtree v1.44 (Rambaut 2018). We estimated the uncorrected p-distance in MEGA 11 (Tamura *et al.* 2021), and the standardized matrix was utilized for Principal Coordinate Analysis (PCoA) (Gower 1966) to visualize the genetic differentiation across the study taxa.

Results

Phylogenetic relationships. The convergence diagnostic of our BI phylogenetic analysis showed an average Estimated Sample Size (ESS) ≥ 2500 and a Potential Scale Reduction Factor (PSRF) of 1.0. Our BI and ML phylogenetic inferences largely concurred in their topology (Fig. 1A), and both trees provide highly supported clades of *M. reticulatus* and *P. molurus* + *bivittatus* (PP=1.0; UFB=100), while the *P. bivittatus* sequence of Northeast India (ON638922) formed a highly supported (PP=1.0; UFB=100) distinct lineage from the other conspecific sequences from China, Vietnam, and Thailand. Notably, the three database sequences submitted as *P. molurus* from unspecified locations are clustered together to form a well-supported sub-clade (PP=0.90; UFB=84), and

nested within the major clade of *P. bivittatus* in forming a polytomy alongside the Northeast Indian *P. bivittatus* specimen (ON638922); contrarily, the sequence submitted as *P. molurus* (U69854) is nested among East and Southeast Asian *P. bivittatus*, which warrants an investigation on the taxonomic status of this specimen. Although the specimens' geographical origins and morphological data will be imperative for precise identification, particularly between *P. bivittatus* and *P. molurus*, our molecular inferences suggest that all the GenBank sequences submitted as *P. molurus* included in this study (unknown sample locations; Table S1) can be referred to as *P. bivittatus*. *Malayopython reticulatus* also contains a high degree of lineage diversification corroborating previous studies (e.g., Auliya *et al.* 2002): the basal lineage comprised the subspecies *M. reticulatus saputrai* Auliya, Mausfeld, Schmitz, and Böhme, 2002 from the islands of Saleyar and Sulawesi (Indonesia) plus a single sequence of *M. reticulatus* from Southeast Asia (MF576213) (PP=0.73; UFB=89); the second lineage accommodated the sequences of *M. reticulatus* from Sangihe Archipelago, Indonesia (AY014891) and other samples from unspecified localities in Southeast Asia (MF576180; MF576206–11) (PP=1.0; UFB=88); the third lineage depicts polytomy and the subspecies *M. reticulatus jampeanus* Auliya, Mausfeld, Schmitz, and Böhme, 2002 is also forming a moderately supported sub-clade (PP=0.70; UFB=95). The Northeast Indian sample (OP086040) is also nested within another sub-clade and clustered with the other samples from China (NC042397) plus unspecified localities from Southeast Asia (MF576183; MF576200–205) (PP=0.74; UFB=82).

Genetic divergence. The ordination of the standardized p-distance also depicted distinct clustering of *M. reticulatus* with four marginally separated clusters among them. The sequence of the Northeast Indian *P. bivittatus* is placed distantly from the conspecific sequences while the sequences submitted as *P. molurus* are clustered adjacent to the East and Southeast Asian *P. bivittatus* samples (Fig. 1B). The uncorrected p-distance also showed the absence of a barcode gap between *P. bivittatus* and the sequences submitted as *P. molurus*; the Northeast Indian sample is genetically closer to the database sequences submitted as *P. molurus* by showing genetic divergence from 2.3% (AY099983; GQ225654; U69853) to 5.6%

(U69854) while it ranged from 4.7% (JX401161–3) to 5.0% (JX401139; JX401142–3) with those sequences submitted as *P. bivittatus* (Table S2). Our sequence of *M. reticulatus* is also showing minimal intraspecific genetic divergence (0.6%) with the sample from Java (Indonesia) (AY014888) and the highest (5.4%) with the sample from an unspecified location in Southeast Asia (MF576210) (Table S2).

Morphology. The Mizoram specimen of *M. reticulatus* (MZMU3327) agrees well morphologically with the diagnostic characteristics of *M. r. reticulatus* *vide* Auliya *et al.* (2002). Although, it has only 62 mid-body scale rows (vs. 68–78) (Fig. 2A; Table 1). For *P. bivittatus*, the Mizoram specimens also agree well with the species diagnostic features *vide* Jacobs *et al.* (2009), although they show higher

Ve counts (252–294 vs. 242–275) (see Fig. 2B; Table 2); they are also distinct from *P. molurus* in having suboculars (vs. absent in *P. molurus*) (Schleip & O’Shea 2010).

Distributional records. We documented *P. bivittatus* from four districts in the central and northern part of Mizoram (Kolasib, Mamit, Aizawl, and Serchhip) at elevations ranging from 87–870 m a.s.l, which falls within the elevational range given in the existing literature (below 1,500 m; Das & Das 2017). We documented *M. reticulatus* from the three southern districts of Mizoram (Lunglei, Lawngtlai, and Siaha) at elevations ranging from 96–1,013 m a.s.l., and these records from Mizoram bridge the known ranges in the adjacent countries of Bangladesh (Chittagong and Sylhet Hills) in the west and Myanmar in the east (Kalki *et al.* 2018; Fig. 1C).

Table 1. Comparative morphological data for *Malayopython reticulatus* from the population in Mizoram of this study, Northeast India, along with other published data. Bilateral values of the parameters are given as left/right.

Subspecies	<i>M. r. reticulatus</i>		<i>M. r. jampeanus</i>	<i>M. r. saputrai</i>
Population	Mizoram, India	SE Asia mainland, Greater and Lesser Sundas	Tanahjampea Island, Indonesia	Selayar Island, SW and SE Sulawesi, Indonesia
Museum vouchers	MZMU 3327	–	–	–
Sex	F	Sex pooled	Sex pooled	Sex pooled
Head length	43.38	–	–	–
Head width	23.32	–	–	–
Head depth	18.76	–	–	–
Eye Diameter (horizontal)	6.56	–	–	–
Snout to nostril	6.12	–	–	–
Snout to eye	17.60	–	–	–
Nostril to eye	10.36	–	–	–
Internarial distance	9.20	–	–	–
Interorbital distance	11.90	–	–	–
Snout–vent length	1020	–	–	–
Tail length	165	–	–	–
Ventrals	317	304–325	290–301	332–334
Subcaudals	93	–	–	–
Supralabials	12/14	–	–	–
SUP contact orbit	7	–	–	–
Infralabials	20/22	–	–	–
Dorsal scale rows (anterior)	59	–	–	–
Dorsal scale rows (midbody)	62	68–78	64–68	72–81
Dorsal scale rows (posterior)	44	–	–	–
Postoculars	2/2	–	–	–
Suboculars	Absent	–	–	–
Preoculars	2/2	–	–	–
Cloacal scale	Single	–	–	–
Source	This study	Boulenger (1893), Mertens (1928), Taylor (1965), Auliya & Abel (2000), Auliya <i>et al.</i> (2002)	Auliya <i>et al.</i> (2002)	Kopstein (1935), Auliya <i>et al.</i> (2002)

Table 2. Comparative morphological data of *Python bivittatus* from the population in Mizoram of this study, Northeast India, along with other published data

Subspecies	<i>P. b. bivittatus</i>							<i>P. b. progschai</i>				
	Mizoram, India							Sulawesi, Indonesia	Hainan, China	Yuan-kiang, Hoihow, Fukien	General data	Sulawesi
Museum vouchers	MZMU 1723	MZMU 2728	MZMU 2774	MZMU 2885	MZM U 2886	MZM U 2887	Live specimen	–	–	–	–	–
Sex	F	F	–	F	M	F	M	pooled	pooled	pooled	pooled	pooled
Head length	42.5	51.0	99.2	31.2	41.6	41.9	38.4	–	–	–	–	–
Head width	28.2	32.3	62.2	21.1	25.0	23.8	27.2	–	–	–	–	–
Head depth	15.6	23.1	45.6	13.3	14.4	12.3	13.8	–	–	–	–	–
Eye Diameter	5.0	4.6	8.3	5.0	6.4	5.6	4.7	–	–	–	–	–
Snout to nostril	8.4	7.5	13.9	6.9	6.0	5.1	8.9	–	–	–	–	–
Snout to eye	15.3	11.6	37.1	14.4	20.2	16.7	14.3	–	–	–	–	–
Nostril to eye	9.3	10.4	25.6	9.0	12.2	11.5	9.5	–	–	–	–	–
Internarial distance	11.3	6.6	11.6	6.3	8.5	9.3	5.4	–	–	–	–	–
Interorbital distance	13.0	14.5	34.2	13.3	16.0	15.8	13.1	–	–	–	–	–
Snout–vent length	876	992	–	730	452	955	845	655–1830	–	–	–	–
Tail length	115	150	–	105	90	150	130	72–225	–	–	–	72–240
Ventrals	257	260	–	260	253	294	252	252–262	255–262	255–259	242–275	252–262
Subcaudals	65	67	–	63	72	66	70	59–67	65–71	61–65	58–75	59–67
Supralabials	11/11	11/12	11/11	11/11	11/11	11/11	12/12	11–13	11–12	13	10–13	11–13
SUP contact orbit	–	–	–	–	–	–	–	–	–	–	–	–
Infralabials	17/20	18/19	18/19	19/20	20/20	19/20	19/18	17–19	–	–	16–22	17–19
Dorsal scale rows	58-66-44	56-66-43	?-?-?	58-70-50	55-63-42	55-67-?	59-69-?	?- (61–69)-?	?- (65–72)-?	?-69-?	?-(60–75)-?	?-(61–69)-?
Postoculars	4/4	4/4	4/4	4/4	4/4	4/4	4/4	–	–	–	2–4	–
Suboculars	Present	Present	Present	Present	Present	Present	Present	–	–	–	1–3	–
Preoculars	2/2	2/2	2/2	2/2	2/2	2/2	2/2	–	–	–	2–3	–
Cloacal scale	Single	Single	–	Single	Single	Single	Single	–	–	–	–	–

MOLECULAR IDENTIFICATION OF PYTHON SPECIES

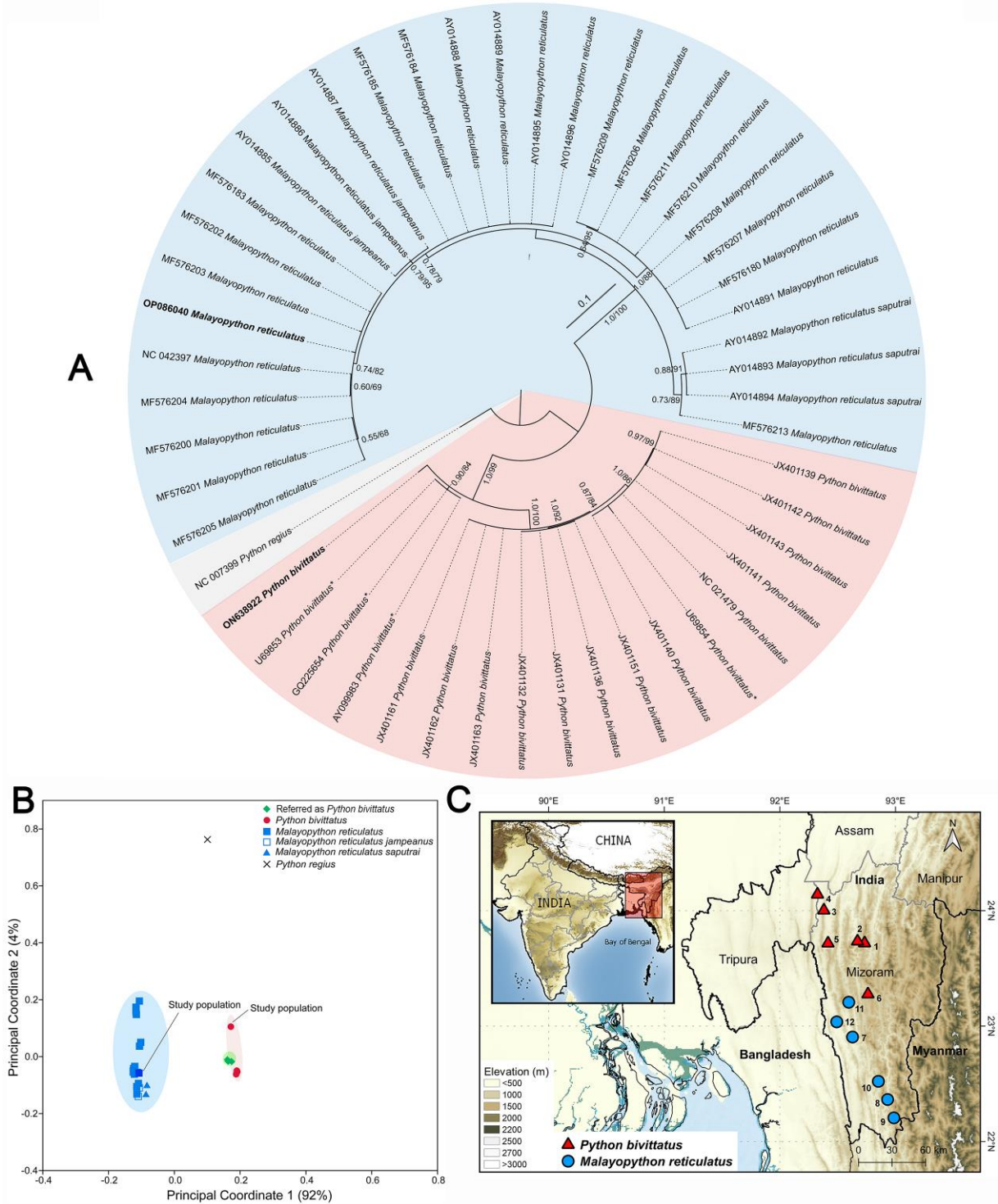


Figure 1. (A) Bayesian inference (BI) phylogram based on partial cytochrome b sequence. The lineage of *Malayopython reticulatus* is shaded in blue, *Python bivittatus* in red, and the outgroup in light grey. The sequences generated in this work are given in bold, and the sequences submitted as *P. molurus* which we referred to as *P. bivittatus* are indicated by asterisks. The branch support from the BI and Maximum Likelihood phylogenetic trees are given as Posterior probability/Ultrafast Bootstrap values. (B) Ordination of standardized p-distance estimated using cytochrome b sequence among *P. bivittatus*, *P. regius*, and *Malayopython reticulatus* along the first and second Principal Coordinate (PCo) axes. The total variance captured by PCo1 and PCo2 are 92% and 4%, respectively. (C) Map showing the distributional localities of *P. bivittatus* (red triangles) and *M. reticulatus* (blue circles) from Mizoram, India: ¹ITI, ²Mizoram University Road, ³Kawrthah, ⁴Zawlnuam, ⁵Damparengpui, ⁶Thenzawl, ⁷Belthei, ⁸New Laty, ⁹Palak National Wetland, ¹⁰Mampui, ¹¹Thenhlum, ¹²Tuikawi.

Detailed distributional records of the Mizoram specimens with the corresponding museum vouchers and the photographic documents for the uncollected individuals are given in Table S3 and Fig. S1, respectively. We are convinced that *P. bivittatus* is not rare and that it is widely distributed in the northern and the central part of Mizoram, while the *M. reticulatus* population appears to inhabit the southern part of Mizoram State. Therefore, they do not seem to co-exist syntopically in this state. Similarly, isolated populations of *P. bivittatus* along the Nepalese borders and Northeast India (Assam) exist sympatrically, but not syntopically with *P. molurus* as they avoid interbreeding with each other in occupying different habitat types (see Barker & Barker 2008, Schleip & O'Shea 2010). Nevertheless, Hunter *et al.* (2018) observed possible hybridization between the two species among the invasive populations in the USA, and they even speculated that the introgression likely occurred before their invasion.

Wildlife trafficking. In recent years, Mizoram appears to be one of the important routes for smuggling out Indian native animals. For example, a total of 194 juvenile Indian star tortoises (*Geochelone elegans*), a Vulnerable species under the IUCN Red List of Threatened Species (Choudhury *et al.* 2020), were seized by Mizoram state forest officials on 5th November 2021 (Margaret Lalramchhani, pers. comm.), and a sub-adult individual was also seized from Tiau River (Indo-Myanmar border) on 3rd April 2022 (Lalruatfela pers. comm.). To a greater extent, Mizoram can be considered an active route for illegal wildlife trafficking to and from Southeast Asia, Africa, or even New World countries as evidenced by the multiple cases of seized exotic animals from lower to higher vertebrates including exotic reptiles such as pythons, anaconda, crocodiles, and leopard tortoise (see [Link 1](#), [Link 2](#)). There were even some sightings/captures of free-ranging exotic animals in Mizoram state, for instance, a single individual Green iguana (*Iguana iguana*) was seen in the forest within the area of Aizawl Zoological Park (Aizawl district) on 24 May 2022 (Vishal Santra pers. comm.); another individual was captured from Chhingchhip village (Serchhip district) on 14th April 2023 (Zirsangliana Varte pers. comm.), and another captured under a bridge on the shoreline of Tlawng river at Sihhmui (Aizawl district) (see [Link 3](#)). A Red eared-slider (*Trachemys scripta elegans*) was also recorded in

the wild from Chawlhmun, Aizawl, Mizoram (see Hmar *et al.* 2021).

Discussion

Given that the majority of the database sequences of *P. molurus* from the Indian subcontinent are of unusually short fragments, and also considering the possible hybridization between the two species among invasive populations in the USA (Hunter *et al.* 2018), we excluded those shorter sequences as well as those originating from the USA in our analyses. Consequently, we suggest further work to generate more DNA data of *P. molurus* and *P. bivittatus* from native populations to obtain a more resolved phylogenetic signal between them and to assess the events of putative introgression/hybridization of the two species in their native home range as previously hypothesized by Hunter *et al.* (2018). Molecular identification is considered a useful approach even in the field of forensic wildlife identification because animals often die during the smuggling process, so morphological identification of the dead animal remains often becomes difficult. Furthermore, different python species are quite similar phenotypically, thereby baffling their identification using phenetic characters alone (see Ciavaglia *et al.* 2015). In conclusion, we suggest extensive work to acquire more genetic data on pythons from their natural home range that will be critical not only for the conservation and integrity of the native populations but also for the identification and ongoing investigations of the seized animals (dead or alive) to enforce international and national legislation (Ciavaglia *et al.* 2015).

Author contributions

All the authors contributed equally.

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Research permits

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