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Genetic confirmation of the *Indotyphlops braminus* complex (Serpentes: Typhlopidae) in Afghanistan, with a global "out-of-India" perspective on its introductions

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SUMMARY

The Brahminy blindsnake, *Indotyphlops braminus* (Daudin, 1803) complex, is a small, parthenogenetic typhlopid widely distributed across tropical and subtropical Asia and represented by numerous introduced populations worldwide. Although reported from neighbouring Pakistan and Iran, its occurrence in Afghanistan has remained unverified. During field surveys in Nangarhar Province, eastern Afghanistan, we collected a specimen morphologically consistent with *I. braminus* complex and generated mitochondrial DNA sequences (16S, COI, cytochrome b). Phylogenetic analyses, incorporating newly sequenced material from Pakistan, revealed that the Afghan and Pakistani samples form a distinct, well-supported clade within the *I. braminus* complex that is also known from southern India (*I.* cf. *braminus* II), and is genetically distinct from both *I. braminus sensu stricto* and the globally widespread introduced clade *I.* cf. *braminus* I. This constitutes the first genetically and morphologically confirmed record of *Indotyphlops* Hedges et al., 2014 from Afghanistan and the first published genetic data for the genus from Pakistan. Our findings extend the documented northwestern range limit of the *I. braminus* complex on the Indian subcontinent, with its occurrence in Afghanistan apparently associated with low-

elevation subtropical river corridors and limited northwards by the high-elevation and arid landscapes of the Hindu Kush region. In addition, our global synthesis of genotyped records provides an "out-of-India" perspective on the worldwide introductions within the complex.

INTRODUCTION

The snake fauna of Afghanistan is diverse, comprising over 30 species across seven families (Wagner et al., 2016; Jablonski et al., 2025). From a biogeographic perspective, these species can be broadly categorized into those with Palearctic affinities, for example, *Hemorrhois ravergieri* (Ménétries, 1832) or *Natrix tessellata* Laurenti, 1768 and those associated with the Oriental region, for example, *Fowlea piscator* (Schneider, 1799) or *Lycodon bicolor* (Nikolsky, 1903). In Afghanistan, and more broadly in Central Asia, these biogeographic elements converge, creating a unique setting for studying questions related to orographic barriers, ecological preferences, and historical dispersal patterns.

Although the biogeographic division is not absolute, general patterns are evident. Palearctic species typically inhabit the northern plains and (sub)mountainous regions of associated foothills and valleys of Hindu Kush, extending occasionally south- or eastward toward the Indus River, while Oriental taxa are mostly restricted to southeastern regions, i.e. provinces such as Nangarhar and Khost (Wagner et al., 2016), following riverine corridors of the Kabul and Kaitu Rivers (Jablonski et al., 2018). Some range overlap does occur in the region, particularly in ecotonal zones, for example, *Oligodon transcaspicus* (Nikolsky, 1902) or *Ptyas mucosa* (Linnaeus, 1758) that are present even in Turkmenistan or, *vice versa*, *Macrovipera lebetinus* (Linnaeus, 1758) in northern Pakistan and India (Sindaco et al., 2013; Jablonski and Masroor, 2020; Lee et al., 2023). Species with the predominant distribution in the Oriental biogeographic region are thus generally confined to the subtropical provinces of Afghanistan, which maintain biogeographic connectivity with the river valleys of northern Pakistan, corridors that have likely facilitated both their historical dispersal and continued presence under favourable climatic and environmental conditions (Mebert and Masroor, 2013; Jablonski et al., 2018, 2024).

Among these Oriental zoogeographic elements of the region, the Brahminy blind snake, *Indotyphlops braminus* (Daudin, 1803) complex (Typhlopidae) is of particular interest. This small, fossorial, snake is native to South Asia and possibly to other parts of Southeast Asian region (Wallach, 2020), has achieved a widespread global distribution (Wallach, 2021). It reproduces through obligate parthenogenesis and is frequently regarded as a potentially invasive species due to its ability to establish populations far outside its native range (e.g., Zavala & Arteaga, 2025). In Central and South Asia, the species is well-documented in Pakistan (Khan, 2006; Wallach, 2021), particularly in subtropical regions bordering the western Himalaya and Hindu Kush but unreported from dry Balochistan (Minton, 1966). On the other hand, the species has also been reported from neighbouring Iran, as a case of human-made introduction via the Persian Gulf (Afroosheh et al., 2010). In addition to *I. braminus*, two nominal *Indotyphlops* taxa have been described from northern Pakistan, *I. ahsanai* (Khan, 1999) and *I. madgemintonae* (Khan, 1999), both currently treated as *incertae sedis* (Wallach, 2000). Given the reported morphological differentiation and the lack of comparative molecular data, the diversity of these fossorial snakes in the region is likely underestimated.

A historical record of *I. braminus* complex from Afghanistan, based on a specimen collected by the "Boundary Commission" and reported by Sclater (1891) (ZSI 12896), suggests its possible presence in the country. However, Wagner et al. (2016), in their checklist of the herpetofauna of Afghanistan, note "no specific locality known," and, considering the historical borders, the specimen might have been collected outside present-day Afghanistan. More recently, Wallach (2021) mentioning without any details the species from the Afghan provinces of Kandahar, Helmand, Nimruz, and Nuristan. However, these records remain ambiguous, and the species has not been independently verified despite the former research in south-eastern Afghan provinces (e.g. Král, 1969; Masroor, pers. data). Here, we address this gap by formally confirming the presence of the genus *Indotyphlops* Hedges, Marion, Lipp, Marin & Vidal, 2014 and the *I. braminus* complex in Afghanistan, and by providing the first phylogeographic context for populations at the northernmost margin of the Indian subcontinent.

MATERIALS AND METHODS

During field investigations on the species and genetic diversity of herpetofauna in Nangarhar Province, we collected a subadult individual of the genus *Indotyphlops* (family Typhlopidae) on 25 September 2024 at 8:45 PM in Darūntah (WGS84: 34.474° N, 70.368° E; 603 m a.s.l.; Fig. 1), in the campus of the Nangarhar University. Based on preliminary morphological characteristics, the specimen was tentatively identified as *I. braminus* complex (Fig. 1A-D). Because these snakes are primarily nocturnal, the specimen was encountered actively moving on the ground surface. Later that night, an additional adult individual was observed at the same locality. The collected specimen was preserved in 70% ethanol, and a tissue sample was stored separately in 96% ethanol for genetic analysis. The specimen is deposited in the Comenius University Herpetological Collection of the first author under the voucher number CUHC 13596. For the further external examination, the specimen was photographed by a Zeiss Axio-Zoom V-16 stereomicroscope.

In addition to the Afghan material, we included two tissue samples from closely related populations in Pakistan to improve regional coverage and genetic connectivity: one from Kallar Kahar, Chakwal (sample CUHC 7874; 32.769° N, 72.706° E; 613 m a.s.l.) and one from Isakhel (CUHC 9531; 33.006° N, 71.705° E; 214 m a.s.l.).

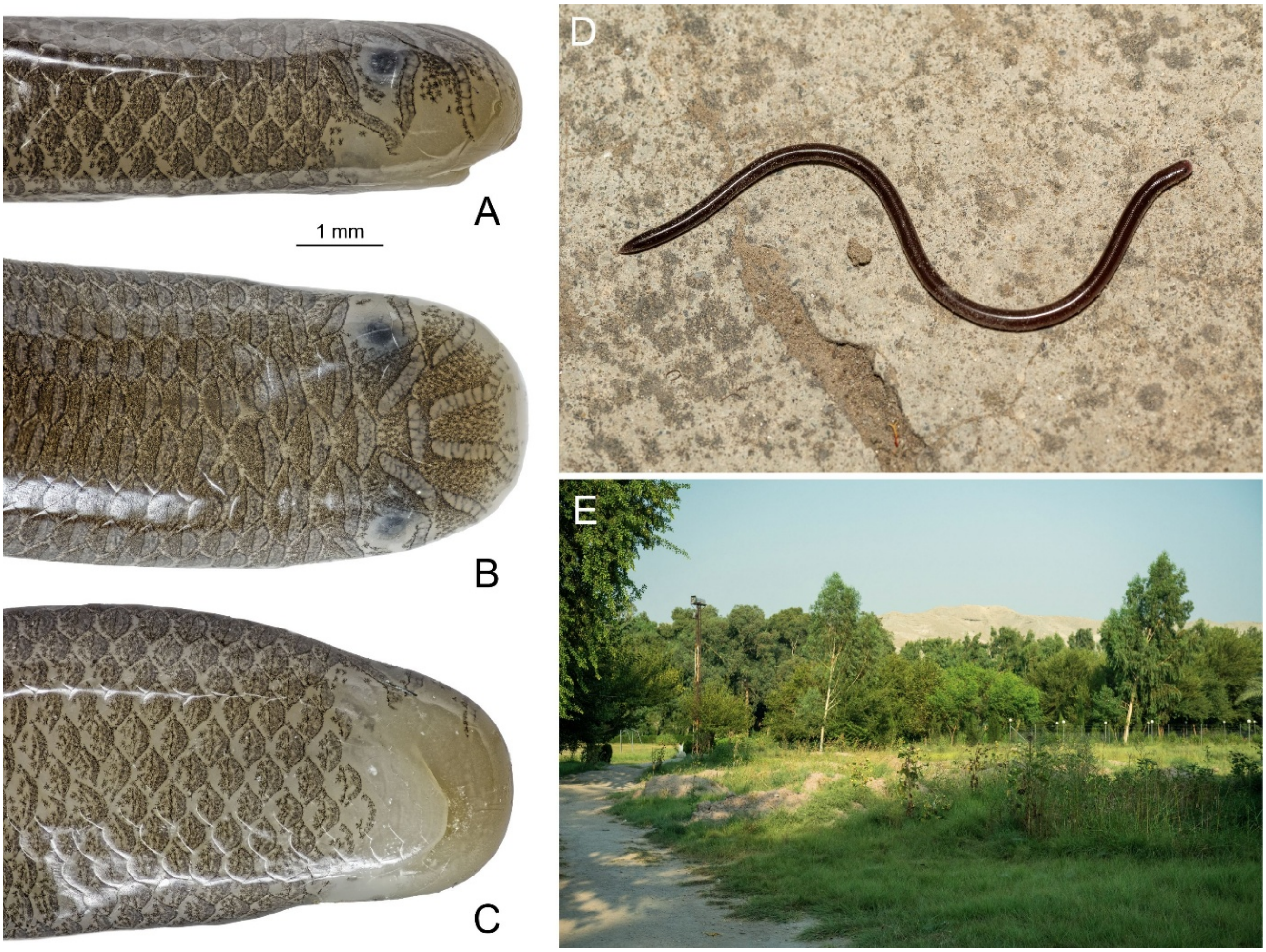

Figure 1. Head and body morphology and habitat of the *Indotyphlops braminus* complex (specimen CUHC 13596) collected in Darūntah, Nangarhar Province, Afghanistan. (A–C) Lateral, dorsal and ventral views of the head, showing diagnostic scale arrangement and pigmentation patterns. (D) Entire specimen in life. (E) Habitat at the collection site an irrigated peri-urban garden area in the Darūntah, Jalalabad area, characterized by scattered vegetation and proximity to cultivated land in the campus of the Nangarhar University.

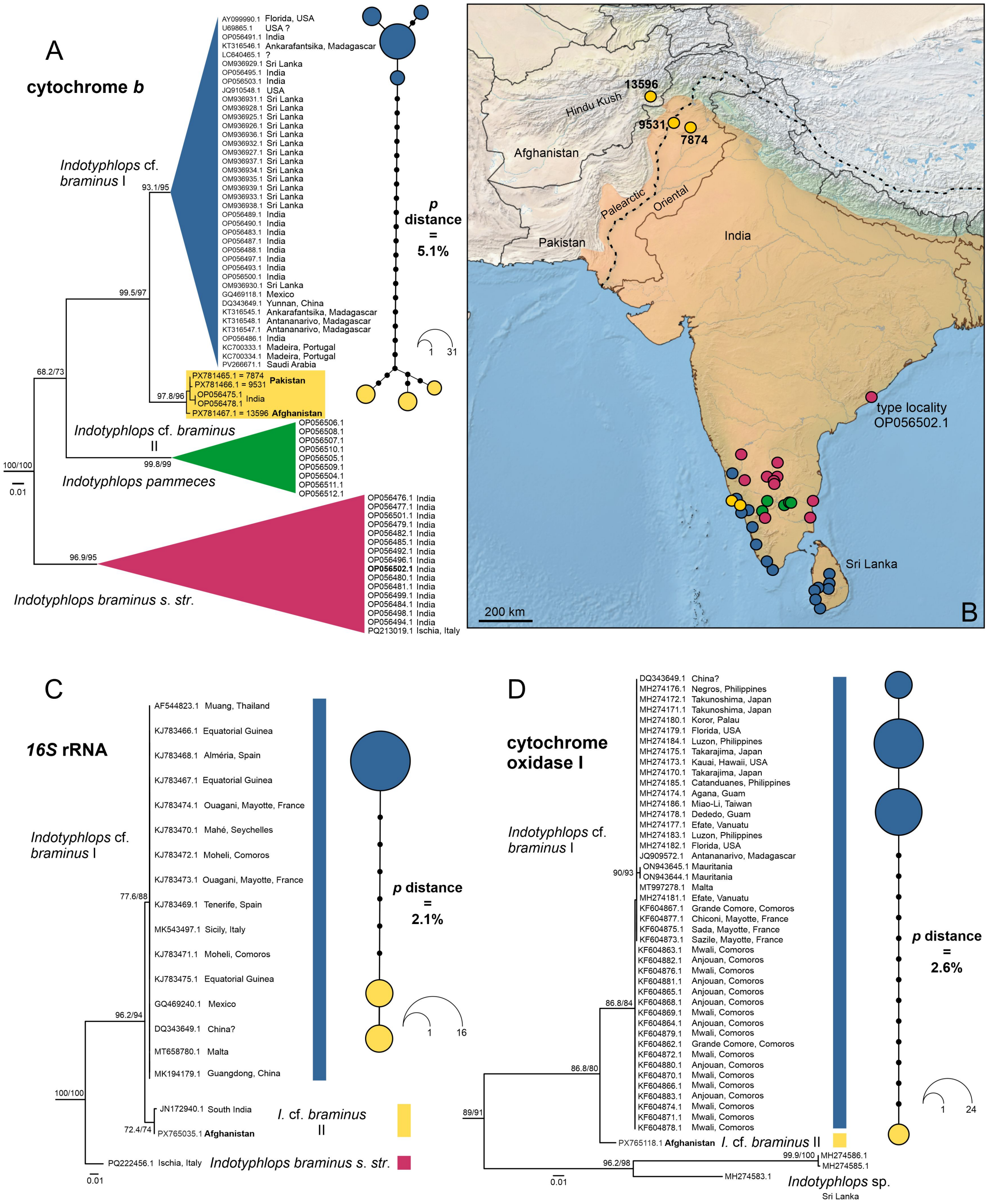

Figure 2. Mitochondrial phylogenetic placement and regional distribution of the *Indotyphlops braminus* complex in South Asia, with newly genotyped samples from Afghanistan and Pakistan. (A) Maximum-likelihood (ML) phylogeny inferred from cytochrome b. Branch support is given as SH-aLRT/UFBoot (>60%). The Afghan (CUHC 13596) and Pakistani samples (CUHC 7874, CUHC 9531) cluster with sequences from southern India forming a distinct, well-supported clade (*I.* cf. *braminus* II) within the complex, separated from *I.* cf. *braminus* I, *I. pammeces*, and *I. braminus sensu stricto*. The cyt b haplotype network summarizes divergence between *I.* cf. *braminus* I and II and its uncorrected p distance; circle sizes are proportional to haplotype frequency and hatch marks denote mutational steps. (B) Distribution of genotyped populations in South Asia based on cyt b. Coloured circles correspond to clades in panel A; the type locality sample is indicated (OP056502). The orange shading depicts the approximate range of the *I. braminus* complex in South Asia, and the dashed line marks the approximate boundary between the Palearctic and Oriental biogeographic realms. (C) ML tree and the haplotype network based on 16S sequences showing placement of the Afghan sample within *I.* cf. *braminus* II. (D) ML tree and the haplotype network based on COI sequences showing the same pattern.

Total genomic DNA was extracted using the E.Z.N.A.® Tissue DNA Kit following the manufacturer's protocol. We amplified three commonly used markers, i.e. 16S rRNA (16S), cytochrome oxidase I (COI), and cytochrome b (cyt b), using primers and PCR conditions from Palumbi et al. (1991) for 16S (16Sar-L and 16Sbr-H), Nagy et al. (2012) for COI (RepCOI-F and RepCOI-R), and Burbrink et al. (2000) for cyt b (H16064 and L14910). These loci were selected because they are widely applied in reptile DNA barcoding and are well represented in GenBank, including extensive coverage for *I. braminus* complex (see accession numbers in Fig. 1 and Appendices 1–3).

In addition to our newly generated sequences, we compiled and compared available, georeferenced published (and one unpublished) sequences (16S, COI, cyt b) from Campbell (1997), Slowinski & Lawson (2002), Vidal & Hedges (2002), Yan et al. (2008), Adalsteinsson et al. (2009), Shekhar & Sandip (2011; unpublished), Hawlitschek et al. (2013), Jesus et al. (2013), Marin et al. (2013), Nagy et al. (2012, 2015), Rato et al. (2015), Faraone et al. (2019), Li et al. (2020), Vella et al. (2020), Kambayashi et al. (2022), Mulcahy et al. (2022), Velo-Antón et al. (2022), Wickramasinghe et al. (2022), Sidharthan et al. (2023), Castiglia et al. (2024), and Liz et al. (2025). These data were used to assess (i) the phylogeographic placement of the analysed samples from Afghanistan and Pakistan, and (ii) the clade(s) that have colonized different regions worldwide. The complete dataset of analysed sequences and their sources is provided in Appendices 1–3. Newly generated sequences have been deposited in GenBank under the following accession numbers: PX765035 (16S), PX765118 (COI), and PX781465-67 (cyt b).

Phylogenetic relationships were inferred separately for the mitochondrial markers using maximum likelihood (ML) in IQ-TREE v3.0.1 (Nguyen et al., 2015) via W-IQ-TREE (Trifinopoulos et al., 2016). Alignments comprised 24 sequences (484 bp) for 16S, 50 sequences (655 bp) for COI and 72 sequences (1,141 bp) for cyt b including outgroups (Appendix 1-3). For each dataset, the best-fit substitution model was selected using ModelFinder (Kalyaanamoorthy et al., 2017). Nodal support was assessed using both the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010) and ultrafast bootstrap (UFBoot; Minh et al., 2013), each with 1,000 replicates. To reduce potential overestimation of UFBoot support, the BNNI optimization step was enabled. For protein-coding loci (cyt b and COI), datasets were partitioned by codon position (1st, 2nd, and 3rd positions). Partition-specific models were selected with ModelFinder, and the optimal partitioning scheme was allowed to be merged based on the data (ModelFinder + MERGE). The 16S dataset was analysed unpartitioned under the best-fit model selected by ModelFinder. Branch supports are reported as SH-aLRT (%) / UFBoot (%), respectively.

Using Hapsolutely v0.2.3 (Vences et al. 2024), with the implemented TCS algorithm (Templeton et al. 1992), we reconstructed haplotype networks for the 16S, COI, and cyt b, focusing on the clade comprising samples from Afghanistan and Pakistan and its sister clade, to visualize their genealogical relationships. To minimise artefacts caused by missing data, very short cyt b sequences were excluded from the network dataset compared to the tree dataset. Between-clade genetic distance (uncorrected p-distance) were calculated using DnaSP 6.00 (Rozas et al. 2017).

To visualize mitochondrial clades in a geographic context and illustrate their distribution, we prepared distribution maps in QGIS 3.44 (Solothurn; 2025; https://qgis.org/). Distances shown by arrows in Fig. 3 were calculated as great-circle (geodesic) distances on the WGS84 ellipsoid. We measured distances from a single reference point in India, defined as the (unweighted) geographic centroid of the genotyped Indian localities, to the most distant introduced (non-native) genotyped localities (i.e., maximum centroid-to-locality distances). Distances (km) were calculated in QGIS and rounded to the nearest 10 km. These distances are intended as an illustrative measure of spread and do not imply a single source population or a reconstructed introduction route.

RESULTS

The newly sequenced cytochrome b samples from Pakistan (CUHC 7874, 9531) and Afghanistan (CUHC 13596) formed a distinct, well-supported clade within the *I. braminus* complex, here referred to as *I.* cf. *braminus* II (Fig. 2A). These sequences clustered with two previously published sequences from southern India (OP056475, OP056478), corresponding to the "Wet Zone *I. braminus*" clade of Sidharthan et al. (2023). The cyt b dataset, which is the most densely sampled of the analysed loci, recovered four well-supported clades separated by substantial uncorrected genetic distances, ranging from 5.1% between *I.* cf. *braminus* I and *I.* cf. *braminus* II to 14.4% between *I. braminus s. str.* and *I.* cf. *braminus* II. The number of mutation steps between haplotypes of the *I.* cf. *braminus* I and II is 23. The clade comprising the newly sequenced populations is thus clearly distinct from both the southern Indian *I. braminus s. str.* clade and *I.* cf. *braminus* I.

The 16S rRNA and COI analyses corroborated this pattern by placing the Afghan sample (CUHC 13596) within *I.* cf. *braminus* II and closest to sequences from southern India (JN172940; Fig. 2C, D), with comparatively lower divergence between *I.* cf. *braminus* I and II (16S: 2.1%; COI: 2.6%). Finally, our reassignment of all available sequences to the recovered mitochondrial clades indicates that most genotyped introduced populations worldwide belong to *I.* cf. *braminus* I rather than to *I. braminus s. str.* Records of *I.* cf. *braminus* I span Southeast Asia, west Pacific and Oceania, Madagascar and the Comoros, parts of western Africa including several Atlantic islands (Madeira, Tenerife), the Mediterranean, the Arabian Peninsula, and the Americas (including Hawaii), consistent with a single globally widespread introduced clade (Fig. 3). The only genotyped, introduced population of *I. braminus s. str.* is known from Ischia, Italy (see Castiglia et al. 2024).

Biogeographically, the newly analysed Afghan and Pakistani samples represent the north-westernmost confirmed records of the *I. braminus* complex in the Indian subcontinent (Fig. 1B) and the first genetically and morphologically verified record of this complex for Afghanistan. The phylogenetic position of the *I.* cf. *braminus* II clade, together with its observed genetic distinctiveness, suggests that it may represent a distinct taxonomic entity within this species complex. However, in the absence of a detailed taxonomic evaluation with other populations, its formal status remains unresolved. The head scalation of the Afghan specimen is shown in Fig. 1A–C.

DISCUSSION

There are few published herpetological data from Nangarhar Province, Afghanistan (e.g., Král, 1969, as summarized in Wagner et al., 2016; see also Jablonski et al. 2019, 2025). Our study therefore provides a valuable addition to the regional herpetofauna and delivers the first genetically confirmed record of the genus *Indotyphlops* from Afghanistan. At the same time, it represents the first published genetic data for the genus from Pakistan. These findings substantially expand the geographic coverage of molecular data for *Indotyphlops* in South Asia (cf. Wickramasinghe et al. 2022; Sidharthan et al. 2023) and, by re-analysing all available sequences, reveal a coherent phylogeographic signal underlying global introductions within the *I. braminus* complex. This framework can support future work on taxonomy, phylogeography, and invasion history in the region and beyond.

New Afghan and Pakistani sequences form a distinct, taxonomically unassigned mitochondrial clade, marked in the study Sidharthan et al. (2023) as a part of the "Wet Zone *I. braminus*". Genetically, this clade is distinct, sister to the globally widespread, genetically depauperate clade (*I.* cf. *braminus* I), and together they form a mitochondrial clade that is sister to *I. pammeces*. In Pakistan, specimens reported as *I. braminus* have been recorded from multiple regions, including Sindh, Punjab, and parts of Khyber Pakhtunkhwa (e.g., Minton, 1966; Khan, 2006; Masroor, 2012), however their taxonomic affiliation in the light of genetic data is unknown. These records, however, follow the subtropical lowland corridors along the Indus River, showing an Oriental biogeographic affinity. In contrast, the species is absent from the arid deserts of Balochistan and from the montane region of Gilgit–Baltistan, consistent with climatic and ecological constraints and an apparent preference for moist subtropical habitats with suitable soil conditions and vegetation cover. In Afghanistan and Pakistan, the distribution of *Indotyphlops* thus likely reflects the availability of suitable low-elevation, comparatively humid habitats and soils along subtropical riverine corridors, whereas extensive high-elevation and xeric areas are unsuitable. The Hindu Kush (and adjacent ranges) is therefore likely to limit further northward occurrence through a combination of orographic and climatic constraints, consistent with the general restriction of many Oriental-affiliated lowland species on the Indian subcontinent (e.g., *Bungarus*, *Fowlea*, *Myriopholis*; cf. Wagner et al. 2016).

The new record from Nangarhar Province in south-eastern Afghanistan supports this pattern. The locality lies within the Kabul River basin, a lowland corridor with a subtropical climate and riparian vegetation, which likely facilitated the species' natural historical west- northward dispersal from the Indus basin via favourable ecological corridors. However, accidental human-mediated introduction from Pakistan or India to Afghanistan through various forms of material exchange cannot be excluded. A similar introduction scenario is expected for other Middle Eastern countries and Iran where the species has been reported. However, genetic evidence is currently available only from Saudi Arabia (Fig. 3), whereas records from the remaining countries lack comparative genetic data (e.g. Afroosheh et al., 2010).

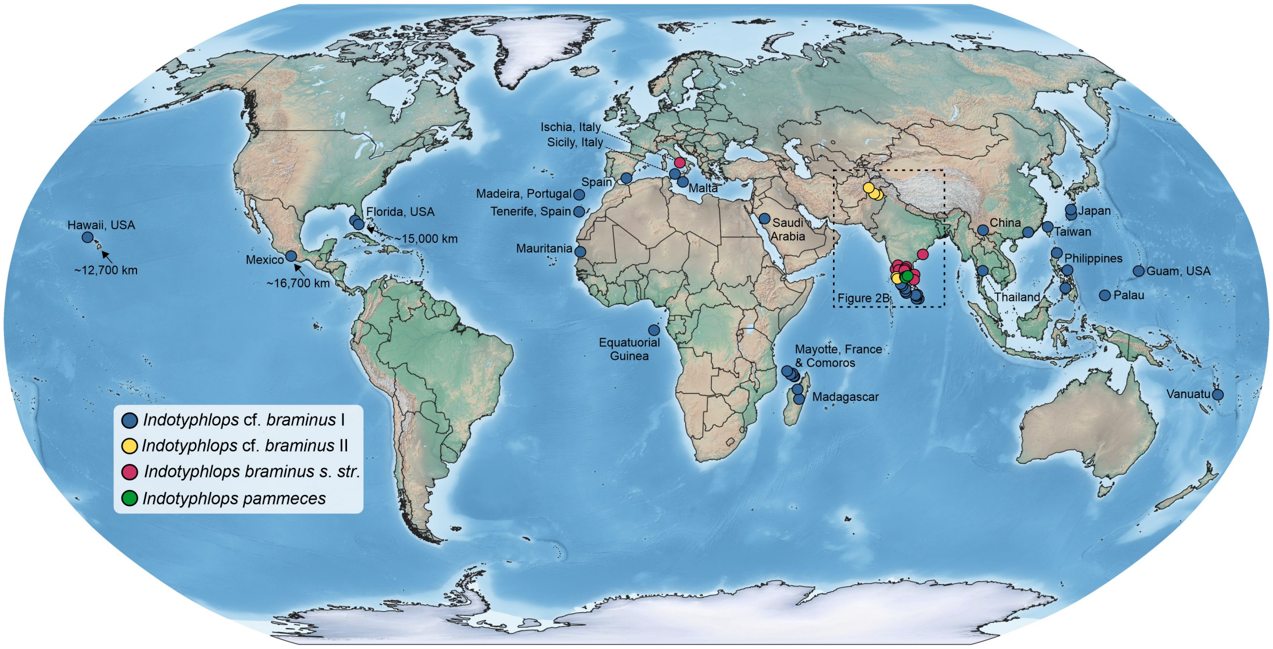

Figure 3. Global distribution of genotyped samples (16S, COI, cyt b) of the *Indotyphlops braminus* complex included in the phylogenetic analyses (Fig. 2). Circles indicate sampling localities; colours correspond to the major clades recovered in Figure 2, highlighting the phylogeographic "out-of-India" scenario. Arrows (with distances in km) indicate the geographically most distant localities (Florida, Hawaii, Mexico) of genotyped *I. braminus* complex populations (all belonging to *I.* cf. *braminus* I) relative to the putative source area in the southern Indian subcontinent.

Although the *I. braminus* complex (type locality: Visakhapatnam, India; GenBank OP056502; Sidharthan et al., 2023) is well known for parthenogenetic reproduction and an exceptional capacity for colonization, its taxonomy and phylogeography remain poorly resolved. The complex is probably widespread across tropical and subtropical South Asia, with particularly high mitochondrial diversity reported from southern India (Sidharthan et al., 2023). In that study, three major mitochondrial clade were identified: *I. braminus s. str.*, *I. pammeces*, and a third, taxonomically unassigned clade (here as *I.* cf. *braminus* I) that is more closely related to *I. pammeces* than to *I. braminus s. str.* Here, by re-analysing available data and adding evidence from additional mitochondrial markers (cf. Vella et al., 2020), we defined four clades in the complex and corroborate the distinctiveness and broad geographic footprint of *I.* cf. *braminus* I. This clade includes samples from southern India and Sri Lanka as well as numerous introduced populations across distant regions (Fig. 3), including localities up to ~16,700 km from the putative source area in the Indian subcontinent (e.g., Mexico), consistent with a high capacity for long-distance, human-mediated dispersal and supporting an "out-of-India" scenario similar to that inferred for other taxa with strong introduction potential (e.g. Agarwal et al., 2019; Dufresnes et al., 2025).

However, *I.* cf. *braminus* I is not the single clade involved in introductions. Castiglia et al. (2024) reported a population from Ischia Island (Italy) that clusters with *I. braminus s. str.*, suggesting that at least two major clades of the complex have been translocated to different parts of the world, including Europe (cf. Rato et al., 2015; Faraone et al., 2019; Vella et al., 2020). This underscores the need for a taxonomic re-evaluation of the complex to determine which species are represented in introduced populations, and for additional genotyping of regions that remain unsampled (e.g. Zavala & Arteaga, 2025). Ultimately, such a framework can provide a comparative baseline for inferring the timing, geographic pathways, and genetic diversity underlying the global, historical or recent spread of individual clades.

Further sampling across the northern Indian subcontinent, particularly in underrepresented areas, is essential to resolve the taxonomic status of *I.* cf. *braminus* II and other dubious taxa described from northern Pakistan (see Wallach, 2000). Such data will be crucial for understanding the evolutionary history and taxonomy of this morphologically conservative genus of fossorial snakes.

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AUTHOR CONTRIBUTIONS

Conceptualization: DJ; Data curation: DJ; Formal analysis: DJ; Investigation: all authors; Methodology: DJ, RM; Visualization: DJ; Writing – original draft: DJ; Writing – review and editing: all authors.

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