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ORIGINAL ARTICLE

A widespread commensal loses its identity: suggested taxonomic revision for *Indotyphlops braminus* (Scolecophidia: Typhlopidae) based on molecular data

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Abstract
The widespread human commensal blindsnake species *Indotyphlops braminus* is currently the only known obligate parthenogenetic snake species. It is also known to be triploid. However, much of these data is from specimens collected outside India which is the native range of this species. Polyploidy and parthenogenesis are often associated with hybridization in amphibians and lizards. In this study, we generated nuclear and mitochondrial data from multiple *Indotyphlops* lineages from across peninsular India and investigated the possible hybrid origin of *I. braminus*. Species delimitation suggested three putative species, one of which was *I. pammeces* and the other two morphologically matched *I. braminus*. One of these was confined to the wet zone (high rainfall areas) while the other was largely distributed in the dry zone. There was wide discordance in the relationships between these lineages across markers and different tree building approaches suggesting past or ongoing geneflow. The statistical test for hybridization also implied geneflow across these three lineages. Furthermore, the dry zone *I. braminus* appears to be true *I. braminus* as the topotypic material falls within this clade. These results suggest that the widespread, commensal, and parthenogenetic *Indotyphlops* is a separate species from *I. braminus*, and further investigation is required to determine diagnostic morphological characters for a species description.

Keywords Typhlopidae · Hybridization · Phylogenetics · Parthenogenesis

Introduction

While interspecific hybridization and polyploidy is quite common in plants (Alix et al., 2017; Chen, 2010), for the longest time, interspecific hybridization in animals was considered of minor significance to evolution (Mayr, 1963; Schwenk et al., 2008). Hybridization not only contradicted the biological species concept but was thought to result in sterile offspring or hybrids, which could not persist in the population. Based on empirical evidence gathered between the 1950s and the 1980s, Harrison (1993) suggested that multiple evolutionary pathways were responsible for hybridization between species, and no one model could explain every case. One of the outcomes of interspecific hybridization is polyploidy with diverse unisexual modes of reproduction (Neaves & Baumann, 2011). Further, there is growing evidence of correlation between parthenogenesis and polyploidy in the animal kingdom (Bogart, 1980; Cole et al., 2014; Ghiselli et al., 2007; Saura et al., 1993; Schultz, 1969; Smith, 1971). In squamates, there are 10 reported cases of polyploid parthenogenetic lizard species (Bogart, 1980; Grismer et al., 2014; Hall, 1970; Kluge & Eckardt, 1969; Lowe & Wright, 1966; Pennock, 1965; Wright & Lowe, 1968). Parthenogenesis has been reported from over 80 animal taxa, including amphibians, reptiles, teleost fishes, and arthropods (Ghiselli et al., 2007; Neaves & Baumann, 2011; Saura et al., 1993; Schultz, 1969; Smith, 1971). All known cases of parthenogenesis in teleost fishes arose from interspecific hybridization. Most parthenogenetic lineages

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in amphibians, and all cases of parthenogenesis in lizards seem to have arisen from interspecific hybridization (Neaves & Baumann, 2011). Thus, there seem to be a lot of evidence linking parthenogenesis, polyploidy, and interspecific hybridization in these animal groups.

Obligate parthenogenesis is seen in many lizard species, but in snakes, it appears to be mostly facultative (Dawley & Bogart, 1989; Kearney et al., 2009; Neaves & Baumann, 2011; Sinclair et al., 2010; Sites et al., 2011). Except for one lizard species, *Lepidophyma flavimaculatum* (Sinclair et al., 2010), all obligate parthenogenetic lizards were a result of interspecific hybridization, mostly between sexual species (Cole et al., 1988; Neaves & Baumann, 2011). Many species of snakes switch to parthenogenesis when they have been in isolation for long periods of time, such as in zoos, and females do not encounter males (Booth & Schuett, 2016). Even in cases of facultative parthenogenesis, many of the clutches do not fully develop or develop with physical abnormalities (Booth & Schuett, 2016). Obligate parthenogenesis seems restricted to one lineage in snakes, *Indotyphlops braminus* (Daudin, 1803).

The genus *Indotyphlops* is distributed predominantly in the Indian subcontinent, Pakistan, Sri Lanka, parts of Southeast Asia, and Indonesia (Pyron & Wallach, 2014). It belongs to the subfamily Asiatyphlopinae and is sister to the typhlopid genera found in Southeast Asia and Australia (Pyron & Wallach, 2014; Vidal et al., 2010). The species *Indotyphlops braminus*, however, has a pan-tropical and subtropical distribution, and is the most widespread snake species, owing to human mediated dispersal through horticultural plant trade (Pyron & Wallach, 2014). It is also the only known snake to undergo obligate parthenogenesis (Booth & Schuett, 2016). Studies by McDowell (1974), Nussbaum (1980), and Ota et al. (1991) on populations of *I. braminus* from different regions of the world showed that there was an absence of males in these populations. This led them to propose that *I. braminus* could be an obligate parthenogen. Wynn et al. (1987) carried out the first karyotyping study of *I. braminus* from samples from Hawaii, the Seychelles, and South Florida, and found that its 42 chromosomes were best explained as 14 sets of triploid chromosomes. Ota et al. (1991) and Patawang et al. (2016) confirmed the triploid karyotype in samples from Ryuku archipelago, Taiwan and Saipan, and Thailand respectively. These studies suggested that *I. braminus* could be a result of one or more hybridization events between diploid parents, and the resulting triploid karyotype could be the reason for its obligate parthenogenesis. However, all these studies were based on samples collected outside India.

Our analysis showed that the published sequences of *Indotyphlops braminus* from outside India were nested within *Indotyphlops* cf. *braminus* from India suggesting that this species probably evolved in India and spread around the world (Pyron & Wallach, 2014; Sidharthan & Karanth, 2021; Wallach, 2009). Since previous studies speculated a hybrid origin for the species, and *I. braminus* evolved in India, here, we explore the hybrid origin hypothesis using molecular data from *Indotyphlops* sp. from India.

One of the indicators of hybridization is discordance in tree topology among different kinds of markers such as mitochondrial and nuclear markers (Funk & Omland, 2003). Furthermore, individual gene trees based on different nuclear markers might generate conflicting topologies (Mendes et al., 2016). The discordance indicates differing evolutionary histories for different genes—contrary to coalescent theory which assumes that all the genes sampled from a population would have been inherited from a common ancestor, resulting in similar gene genealogies (Wakeley, 2009). However, such patterns might also emerge due to incomplete lineage sorting (Maddison, 1997).

In this study, we carry out species delimitation to first delimit the species within this highly cryptic group of typhlopids. We then explore the possible hybrid origin of *Indotyphlops braminus* by comparing phylogenies based on different genetic markers, and different phylogenetic approaches, to detect discordance. We also use statistical testing to tease apart the various biological processes (chiefly hybridization vs. incomplete lineage sorting) that could result in mito-nuclear and gene tree-species tree discordances.

Materials and methods

Taxon sampling

The specimens used in this study were collected as part of a larger sampling carried out for a broader study to resolve the typhlopoid phylogeny (Sidharthan & Karanth, 2021). Table S1 in the supplementary materials lists the sampling locations for the specimens used in this study. Two *Indotyphlops* species distributed in peninsular India were targeted namely, *I. braminus* and *I. pammeces* (Günther, 1864).

Morphological data

All 32 specimens used for the mitochondrial data set were used for collecting morphological data. External morphological characters are patterns of scalation, meristic characters, and morphometric characters which were identified and used for diagnosis of species within the complex. All the morphological characters considered for the study are either characters defined in Wallach 1993, Wallach, 2009, and Wallach 2014 or their derivatives.

Scale patterns

- Supralabial imbrication pattern (SIP) – arrangement of supralabial scales (scales on the upper lip); types found in genus Indotyphlops – T-III (when the 3rd supralabial scale overlaps the scale located posterodorsally to it – ocular scale, for Indotyphlops), T-V (when the 2nd and 3rd supralabial scales overlap scales posterodorsal to each – pre-ocular and ocular scales, respectively for Indotyphlops).
- Scale in contact with the inferior nasal suture (INS) – either pre-ocular (PO) or the 2nd supralabial scale (SL2) is in contact with the INS in snakes within the genus.
- Scale row reduction (SRR) – present or absent.

Meristic characters

- Longitudinal scale rows (LSRs) – number of scales around the body counted at the neck, at midbody and near the cloaca.
- Number of post ocular scales (PT).

Morphometric characters

- Total body length (L) – head-to-tail length of specimen; different from snout-to-vent length; a thread was spanned along the body of the specimen and then the segment was measured using a vernier calipers.
- Mid-body width (W).
- Body form (L/W) – ratio of total body length (L) to mid-body width (W).

Molecular data generation

Genomic DNA was extracted from the tissue samples using DNeasy (Qiagen™) blood and tissue kit. Partial sequences for one mitochondrial fragment – cytochrome *b* (Cyt *b*-839 bp) and seven nuclear fragments — brain-derived neurotrophic factor (BDNF ~ 630 bp), Amelogenin (AMEL ~ 361 bp), neurotrophin-3 (NT3 ~ 542 bp), recombination-activating gene 1 (RAG1 ~ 516 bp), oocyte maturation factor Mos (CMOS ~ 351 bp), Cullin-associated NEDD8-dissociated protein 1 (CAND1 ~ 854 bp), and zinc finger E-box-binding homeobox 2 (ZEB2 ~ 747 bp) — were amplified using polymerase chain reaction (PCR) and subsequently sequenced. Primer pairs for the nuclear markers used were from Vidal et al. (2010) and Miralles et al. (2018) and the primer pair for the mitochondrial marker was from Adalsteinsson et al. (2009). The PCR reactions were performed with the following conditions: initial denaturation at 94 °C for 3 min, followed by 40 cycles of (94 °C FOR 30 s, annealing temperature for 45 s, 72 °C for 2 min) and a final elongation at 72 °C for 10 min. PCR purification and sequencing were carried out by Medauxin Pvt. Ltd. (Bangalore, India) and Barcode Biosciences (Bangalore, India). For all the samples, complementary strands were sequenced and checked against each other to ensure accuracy.

For the Cyt *b*, sequences were generated for 38 samples, including 29 I. *braminus* and nine *I. pammeces*. Additionally, published sequences of *I. braminus* from Florida and Madagascar were also included. Nuclear markers were sequenced for 25 specimens, which included 19 *I. braminus* and six *I. pammeces.*

Published sequences for *Indotyphlops albiceps* (Boulenger, 1898) (from southeast Asia) for one mitochondrial and seven nuclear markers were downloaded from GenBank (accession numbers in supplementary Table S2), to use as the outgroup. The accession numbers for the sequences generated in this study are also provided in supplementary Table S2.

Individual gene sequences for all eight markers were aligned in MUSCLE (Edgar, 2004) using default parameters, implemented in Mega 6 (Tamura et al., 2013). All the markers are protein coding genes, and the sequenced fragments consisted only of coding regions. The alignment was then checked manually, and the translated amino acid alignment was checked to ensure the correct reading frame.

Species delimitation

Cytochrome *b* is a protein coding gene and hence, every third codon position in the gene is expected to evolve faster than the other two positions. Therefore, our sequence alignment was partitioned into three, as per codon positions and PartitionFinder (v2.1.1) (Guindon et al., 2010; Lanfear et al., 2012, 2016) was used to determine the most suitable model of sequence evolution using the Bayesian Inference Criterion and the greedy search algorithm (Lanfear et al., 2012).

The web server for IQ-Tree (http://iqtree.cibiv.univie.ac.at/) was used for the ML analysis. In IQ-Tree, Ultrafast Bootstrap Analysis was performed with 2000 bootstrap replicates and 1000 iterations, and Standard Bootstrap Analysis was carried out with 100 bootstrap replicates. Additionally, SH-aLRT-Test (Guindon et al., 2010) with 1000 bootstrap replicates was performed for assessing the reliability of internal branches of the resulting phylogenetic tree.

For identifying putative species, single-locus species delimitation models such as PTP and mPTP were used. The command line version for mPTP (Kapli et al., 2017) was used to run both the methods. The ML Tree built using IQ-Tree was provided as the input tree to the software. Taking the aid of the alignment using which the tree was built, mPTP searched for the pair of sequences which had the lowest p-distance. The branch length associated with this pair was used to calibrate the algorithm. Next, two MCMC (Markov Chain Monte Carlo) runs each with 1,000,000 generations and default burnin of one generation were performed for both the species delimitation models. Convergence of the MCMC chains was visually confirmed.

The multi-locus coalescent delimitation method integrated BPP (iBPP) v. 2.1.3 (Solís-Lemus et al., 2015) was used to validate the putative species delimited by PTP and mPTP. An extension of Bayesian Phylogenetics and Phylogeography (BPP; Zhang et al., 2011), iBPP allows the addition of continuous morphological trait data. The iBPP analysis was carried out using a guide tree based on the *BEAST species tree, and the seven nuclear markers and morphological trait data. The morphological traits used in this analysis were LSRs, SIP, INS, and SRR. The other morphometric and meristic measurements were not available for all the individuals used in the nuclear dataset. The species boundaries were defined based on the *BEAST and SVD quartet coalescent methods (described in the next section). The θ and τ priors used were G (2, 2000) and G (2, 20000), respectively. We used the "no variation" setting for locus rate, and used default settings for parameter tuning and scaling. The MCMC chains were run for 500,000 generations with parameters sampled every 10 generations and a burn-in of 50,000 generations. The algorithms 0 and 1 were both applied to a series of analyses with various combinations of ε, α, and m, to ensure robust results with different searching algorithms.

Phylogenetic analyses

The three putative species retrieved from the species delimitation analysis were selected for further phylogenetic analyses. Based on morphological data and distribution of the samples, these species were denoted as *Indotyphlops pammeces* (C in all the analyses), "wet zone *I. braminus*" (A in all the analyses), and "dry zone *I. braminus*" (B in all the analyses). *Indotyphlops pammeces* was identified based on morphological characters. The wet and dry zone *I. braminus* were identical for the morphological characters recorded and matched the published description for *I. braminus* (Daudin, 1803; Pyron & Wallach, 2014), therefore they were differentiated based on their distributions. The "wet zone *I. braminus*" samples were distributed exclusively to the wetter parts of Western Ghats, while the "dry zone *I. braminus*" samples were distributed in the drier parts of central and eastern peninsular India. All three putative species were subsampled to generate the nuclear dataset. *Indotyphlops albiceps* was used as the outgroup.

PartitionFinder 1.1.0 (Lanfear et al., 2012) was used to identify the best partitioning scheme and best-fit models, using the Bayesian Information Criteria, greedy search algorithm, and unlinked branch lengths (Supplementary Table S3). ML analysis was performed using IQ-TREE (Nguyen et al., 2015), implemented in the web server version (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al., 2016). Nodal support was assessed using ultrafast bootstrap for 2000 replicates and 1000 iterations.

Individual ML gene trees were built for the seven nuclear markers—AMEL, BDNF, CMOS, CAND1, NT3, RAG1, and ZEB2—using IQ-TREE (Nguyen et al., 2015), and nodal support assessed using ultrafast bootstrap for 2000 replicates and 1000 iterations.

The seven nuclear markers were concatenated in Mega 6 (Tamura et al., 2013) to form a dataset of 4002 bp. IQ-TREE (Nguyen et al., 2015) was used to build an ML tree for the concatenated dataset, with the same settings as above.

The Bayesian clustering algorithm implemented in STRUCTURE v. 2.3.2 (Falush et al., 2003; Pritchard et al., 2000) was used to explore the number of genetic clusters within and shared haplotypes between the three putative species. The genotype matrix for the analysis was constructed by combining the allelic data for seven nuclear markers for 25 individuals spanning the three putative species. For sequences with polymorphic sites, the sequences were manually inspected for polymorphic sites, coded with nucleotide ambiguity codes, and the algorithm PHASE (Stephens et al., 2001) implemented in DnaSP v.5 (Librado & Rozas, 2009) was used to determine the gametic phases of alleles. For the structure analysis, the admixture ancestry model was used along with correlated allele frequencies with unlinked loci. A series of analysis were carried out for the number of assumed populations (K) ranging from 1 to 6, with 20 iterations for each K and a burn-in of one million followed by one million MCMC generations. The most likely number of genetic clusters was determined using the ΔK method (Evanno et al., 2005) which was calculated using STRUCTURE Harvester (Earl, 2012).

To visualize phylogenetic network relationships, the NeighborNet algorithm (Bryant & Moulton, 2004) was implemented in SplitsTree v. 4.15.1 (Huson & Bryant, 2006).

Since the individual gene trees were incongruent, two coalescent tree building methods were used-*BEAST (Heled et al., 2013) incorporated in BEAST v2.4.7 (Bouckaert et al., 2014), and SVD Quartets (Chifman & Kubatko, 2015).

The seven nuclear marker alignments were used for building the species tree in *BEAST (Heled et al., 2013). The xml input file was created in BEAUti v2.4.7 (Bouckaert et al., 2014). The best fitting partition scheme and models were set according to the PartitionFinder (Lanfear et al., 2012) results. An uncorrelated relaxed lognormal clock for each gene with default values for all the priors was used. For the species tree prior, the Yule model was implemented. The ploidy levels were set as diploid for all the genes as they are autosomal, nuclear genes. The tips were allocated a priori to the species groups dry zone *I. braminus*, wet zone *I. braminus*, *I. pammeces*, and outgroup (O) as discussed earlier in the section, according to the mptp results (see results Fig. 1). The analysis was run for 100 million generations, sampling every 5000 generations. Stationarity was assessed in Tracer v1.6 (Rambaut et al., 2013) by ensuring the effective sample size

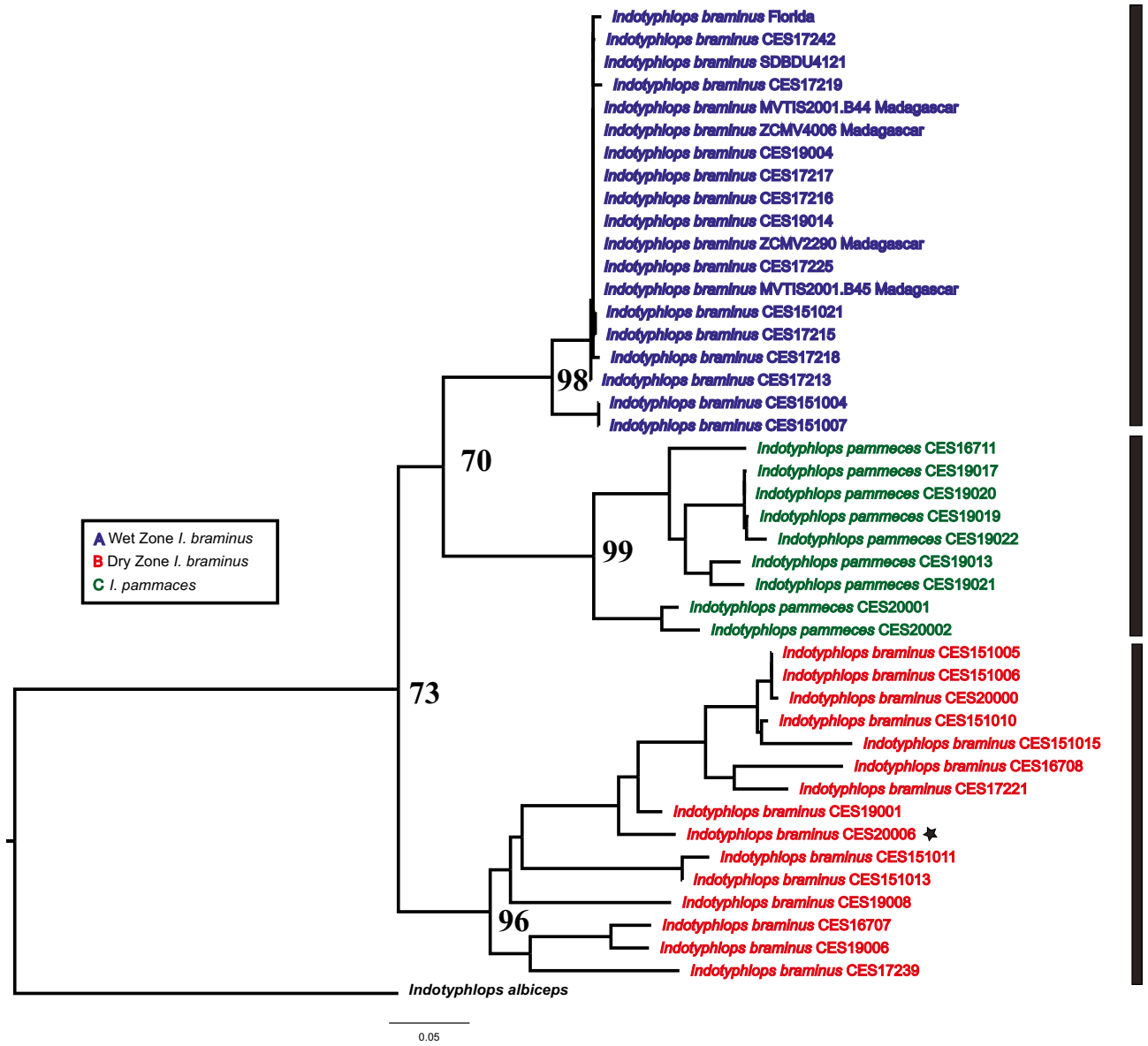

Fig. 1 Maximum likelihood phylogeny based on the mitochondrial marker (Cyt*b*-839 bp). Topotypic specimen for *Indotyphlops braminus* is indicated with a star (CES20006). The bars on the right show the three putative species according to the mPTP species delimitation analysis

(ESS) values were greater than 200. The first 25% trees were discarded as burn-in and the remainder were summarized using TreeAnnotator v2.4.8 (Drummond et al., 2012) to give the maximum clade credibility tree with median heights. The *BEAST analysis was repeated with all the same settings, but with the tips not allocated a priori to any of the species groups.

SVDQuartets is a single-site quartet method, which samples sets of four individuals, or quartets, from the dataset to produce the best tree for those four taxa and similarly, constructs a species tree from all sampled quartets. The tree with the lowest single value decomposition (SVD) score is chosen as the best tree. SVDQuartets was implemented in PAUP* v4a168 (Swofford, 2002). The analysis was run for 100,000 quartets, with the multispecies coalescent tree model and the taxa were not a priori assigned to any species groups. Bootstrap was calculated for 1000 replicates.

JML test for hybridization

To distinguish between the two possible causes of this discordance, deep coalescence and hybridization, we carried out a statistical test for hybridization using JML (Joly, 2012). JML works on the principle that in cases of hybridization, the genetic distance between two individuals of two different species in a gene tree will be lower than the genetic distance

Table 1 Scale patterns, and meristic and morphometric measurements for the three putative species. Clades A, B, and C are the three clades retrieved in the mitochondrial tree, with a sample size of 13, 11, and 8 respectively

Clade	Putative species	Scale patterns			Meristic			Morphometric		
		INS	SIP	SRR	LSR	PT	TSR	L	W	L/W
A	Wet zone *I. braminus*	PO	T-III	No	20	1	290–321	63.47–163.31	1.29–3.6	38.56–51.84
B	Dry zone *I. braminus*	PO	T-III	No	20	1	300–352	63.93–173.35	1.17–3	45.46–56.95
C	*I. pammeces*	SL2	T-III	No	20	1	278–316	64.25–151.25	1.98–2.87	37.16–54.42

between those two species in a species tree since the hybridization event would have happened after the speciation event. JML uses predictive posterior checking to test for the null model which assumes coalescence. It uses the species trees derived from *BEAST to model gene coalescence for each marker. The individual gene trees in conjunction with the appropriate substitution models (from PartitionFinder) are used to simulate sequences for the terminal nodes in seq-gen 1.3.2 (Rambaut & Grassly, 1997). This simulation is carried out for all the species trees in the posterior distribution. For each marker, it then calculates the expected genetic distance between species (given coalescent model) from the simulated sequences, which is given in the "Distribution" output file. JML also uses the alignment for each nuclear marker to calculate the observed genetic distance between species. It then compares the expected genetic distance distribution to the observed genetic distance to calculate the probability value (*P*-value). The *p*-value is calculated as follows:

$$P \text{ value} = \frac{\text{number of times expected gen. dist of species tree} \leq \text{observed gen. dist}}{\text{Total number of simulations}}$$

JML calculates the *P*-value for each species pair and presents it in the "Probabilities" output file.

We used JML to test for hybridization for the seven nuclear markers. For each marker, the alignment was provided in phylip format. The control file was coded accordingly for each marker, with the appropriate model of sequence evolution retrieved from PartitionFinder. The heredity scalar value was set to 2 since these are autosomal nuclear markers. The mutation rate value was procured from the log file from the *BEAST run for each marker. The species trees from *BEAST, consisting of 20,001 trees, was provided as the input file.

Results

Morphological data

Individuals from clades A and B had identical scale patterns with the preocular scale in contact with the inferior nasal suture, a supralabial imbrication pattern of T-III and no scale row reductions. The meristic and morphometric measurements of the individuals in these two clades were also similar or had a large overlap in the ranges (see Table 1). Based on these sets of characters, both clades A and B were identified as *Indotyphlops braminus*. Clade C was identified as *Indotyphlops pammeces* based on the combination of characters, most importantly, the inferior nasal suture being in contact with the second supralabial scale. Figures 2 and 3 show dorsal and lateral views, respectively, of the head scales of representative specimens from the three putative species.

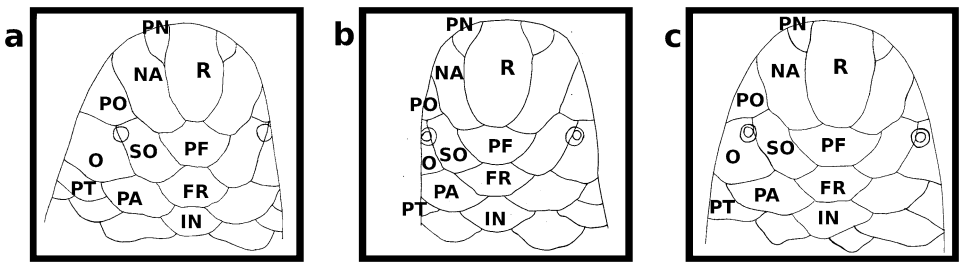

Fig. 2 Dorsal view of the head of specimens. **a** CES17213 (wet zone *I. braminus*), **b** CES151013 (dry zone *I. braminus*), **c** CES16711 (*I. pammeces*). R = rostral, PF = frontal, FT = post-frontal, IN = inter-parietal, PN = pre-nasal, NA = nasal, SO = supra-ocular, PA = parietal, PO = pre-ocular, O = ocular, PT = post-ocular. Except R, PF, FT, and IN, all these scales are present in pairs in bilaterally symmetrical positions

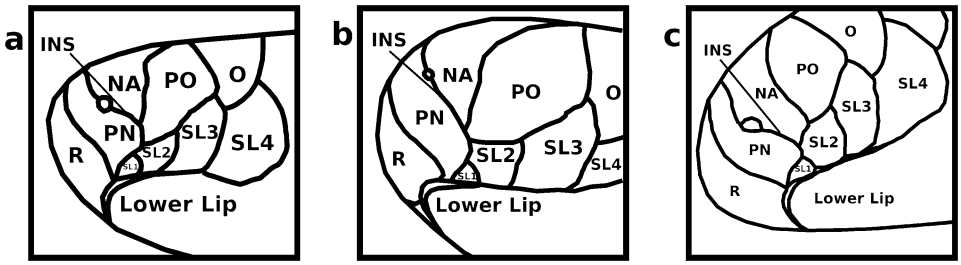

Fig. 3 Lateral view of the head of specimens. **a** CES17213 (wet zone *I. braminus*), **b** CES151013 (dry zone *I. braminus*), **c** CES16711 (*I. pammeces*). R = rostral, PN = pre-nasal, NA = nasal, PO = pre-ocular, SL 1–4 = Supralabial scales 1–4

Species delimitation

PTP estimated 24 species, whereas mPTP estimated 3 putative species (Fig. 1). The likelihood score for the multi-coalescent model (mPTP) is higher than the score for the single-coalescent model (PTP) and both the scores are higher than the score for the null model (that all the specimens belong to a single species). The average support value (ASV) for the estimate provided by mPTP is nearly 81% which means 81% of the MCMC samples (each sample being a delimitation estimate) show the delimitation pattern that has been given as the output. However, the estimate for PTP had a low ASV support of nearly 44%. Therefore, evidently the mPTP three species model fits our data better. Figure 4 shows the distributions of the three putative species, plotted on an annual precipitation layer (BIOCLIM 12) available at https://www.worldclim.org/data/bioclim.html. The map was prepared using the opensource QGIS v. 3.2 (QGIS.org, 2022).

The independent iBPP runs with multiple combinations for algorithms 0 and 1 were congruent and had similar likelihood values. Algorithm 1 with $\alpha = 1$ and $m = 0.5$ had the best likelihood, and recovered the three lineages provided in the guide tree with high statistical support (PP = 1).

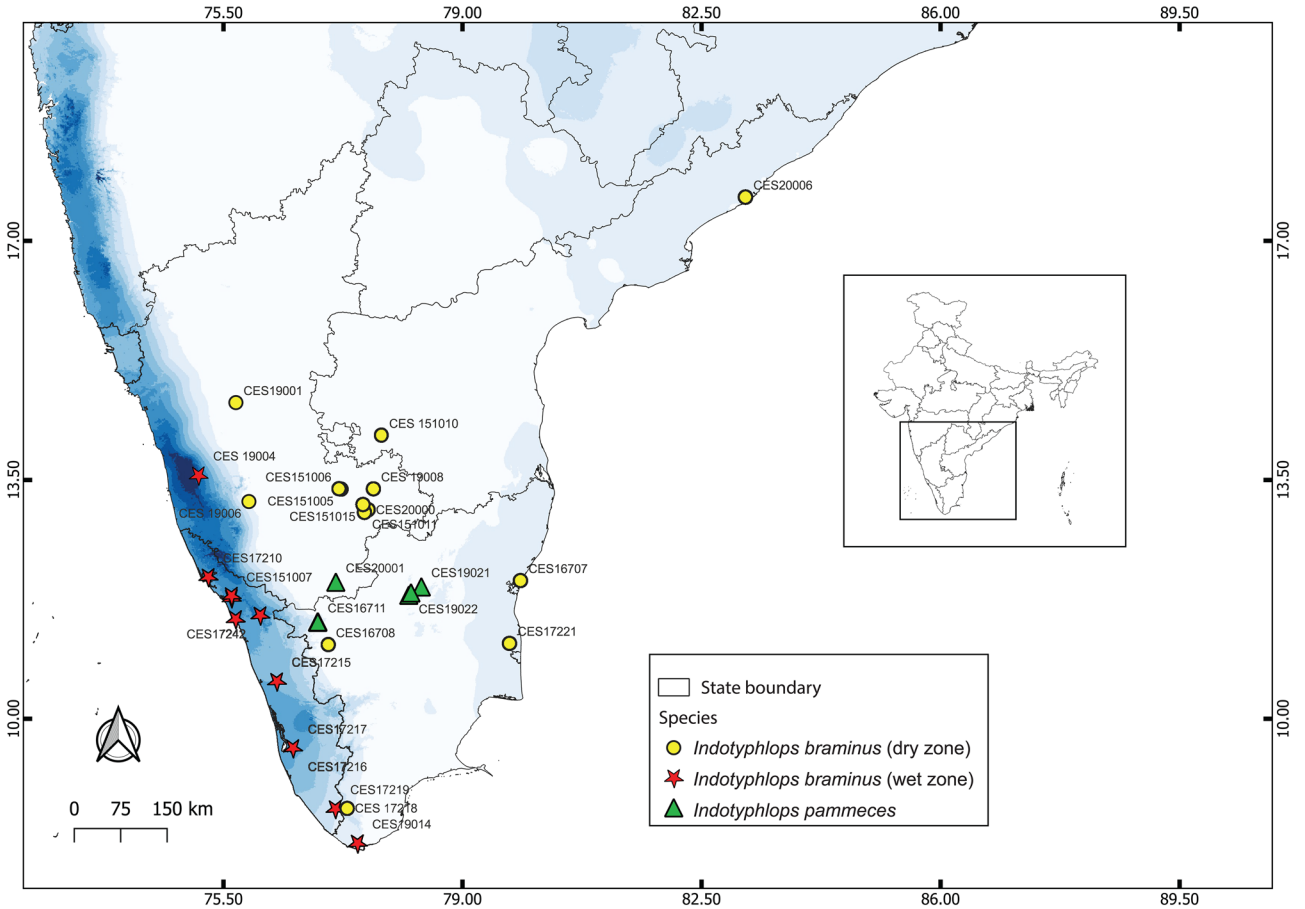

Fig. 4 The distributions of the three putative species, plotted on an annual precipitation layer (BIOCLIM 12) to indicate the wet zone

Phylogenetic analyses

In the ML phylogeny based on the mitochondrial marker (Cyt *b*-839 bp), the published sequences for *Indotyphlops braminus* individuals from outside the Indian subregion (Madagascar and Florida) are nested within the wet zone *I. braminus* (A) clade, which shows very low variation (Fig. 1). The wet zone *I. braminus* (A) clade is sister to *Indotyphlops pammeces* (C) clade, and the dry zone *I. braminus* (B) clade is sister to both A and C, with high support. The support for the monophyly of A, B, and C were also high such as 99, 97, and 100, respectively.

In the ML phylogeny based on the concatenated nuclear dataset (seven markers, 4002 bp) (Fig. 5), the wet zone *I. braminus* (A) clade and dry zone *I. braminus* (B) clade were not monophyletic and A is nested within B. The *Indotyphlops pammeces* clade is sister to clades A and B. The support for the monophyly of clade C and clades A + B is high: 96 and 92, respectively.

The Bayesian structure analysis retrieved three populations or genetic clusters (average of log probability of data = − 725.63, standard deviation = 29.11, $\Delta K = 3.628$ for $K = 3$). The dry zone *I. braminus* samples were assigned to two populations, with two individuals (CES16708 and CES19001) showing an admixture of the two populations (PP < 0.80). The wet zone *I. braminus* samples were all assigned, with high posterior probability (PP > 0.95), to one of the dry zone *I. braminus*

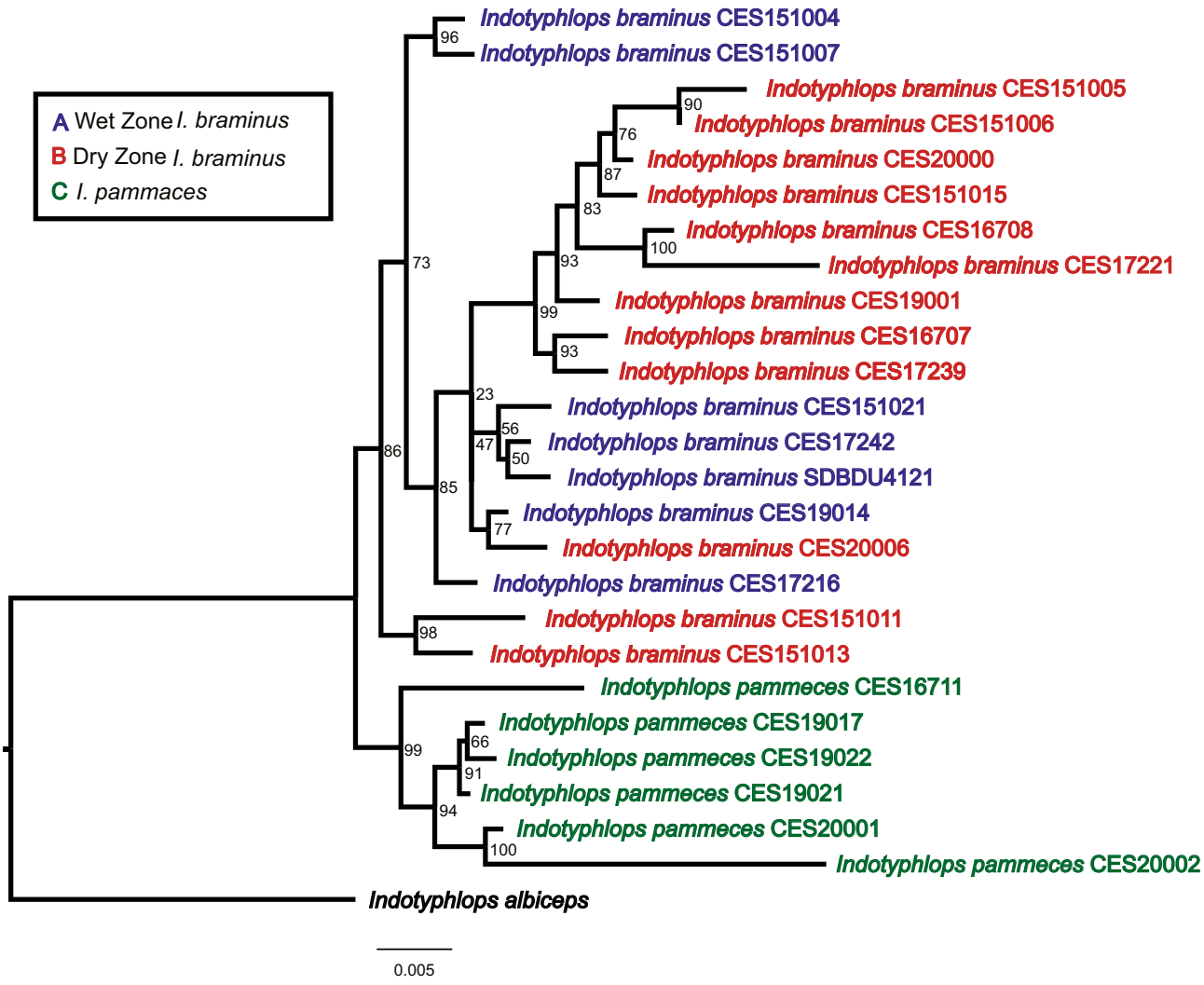

Fig. 5 Maximum likelihood phylogeny based on the concatenated dataset of seven nuclear markers (AMEL, BDNF, CMOS, CAND1, NT3, RAG1. and ZEB2-4002 bp)

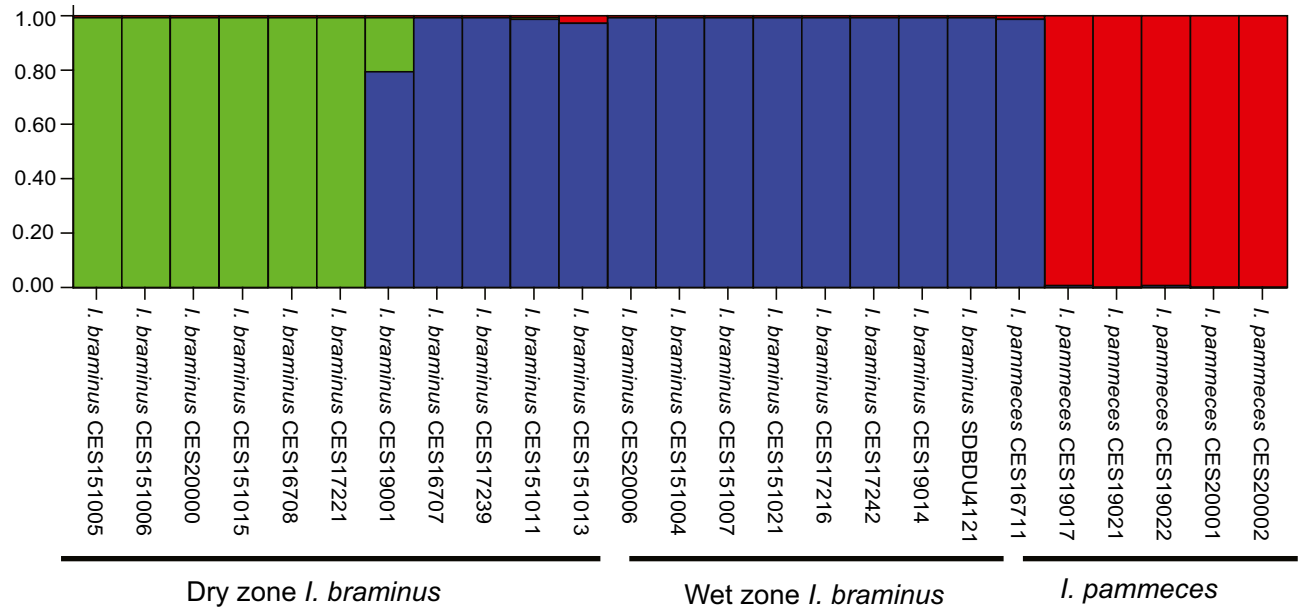

Fig. 6 Bayesian assignment of the 25 individuals to three populations based on the, using STRUCTURE. The individual ids are indicated below each bar, and the y-axis is the posterior probability. The three putative species are indicated below the individual ids

populations (Fig. 6). *I. pammeces* samples belonged largely to the third population, with the exception of one (CES16711), which was assigned to one of the dry zone *I. braminus* populations.

The neighbor network built using SplitsTree (Fig. 7) retrieved three clusters, largely congruent with the three putative species, except for one dry zone *I. braminus* individual (CES20000), which was placed with the wet zone *I. braminus* cluster, and two individuals (CES151011 and CES151013), which were placed between *I. pammeces* and the dry zone *I. braminus*. The network did not depict a tree-like pattern of evolution, and individuals of the three putative species formed a complex network with multiple branches connected across the three major clusters.

The coalescent species tree built in SVD Quartets (Fig. 8), retrieves the wet zone *I. braminus* (A) clade as a monophyletic group, nested within the dry zone *I. braminus* (B) clade. *Indotyphlops pammeces* clade is sister to clades A and B. The support for the monophyly of clades C and (A + B) is high. The coalescent species tree built in *BEAST, where the tips were assigned a priori to species groups, showed the same topology as the SVD Quartets results. A was sister to B, and C was sister to A + B. However, in the coalescent *BEAST species tree, where the tips were not assigned a priori to a species group, two of the dry zone *I. braminus* individuals were sister to *I. pammeces* (Fig. 9).

The ML trees for individual nuclear genes all showed discordant topologies (See Fig. S1a to g). None of the three putative species were retrieved as monophyletic for any of the genes except ZEB6, where *Indotyphlops pammeces* was retrieved as a monophyletic group, but the relationships within the clade were still unresolved.

JML test for hybridization

The results from the JML runs for each nuclear marker are given in Table 2. The *P*-value for each species pair is given in the column labeled "probability."

All seven genes showed low *p*-values for the null model for different species pairs. For AMEL, the *p*-value was very low (0.0099) between species groups C and A (henceforth referred to in C-A format). It was relatively low (0.1609) for C-B as well as for B-A (0.2178). For BDNF, the *p*-value was very low between C-A (0.0414) and C-B (0.0542) and relatively low for B-A. CAND1 and CMOS, like BDNF, showed low probability values for the null model, for C-A and C-B and relatively low probability values for B-A. NT3, RAG1, and ZEB6 showed relatively low probability values for one or more species groups, as seen in Table 2). In comparison, the probability values are very high for Outgroup-A,

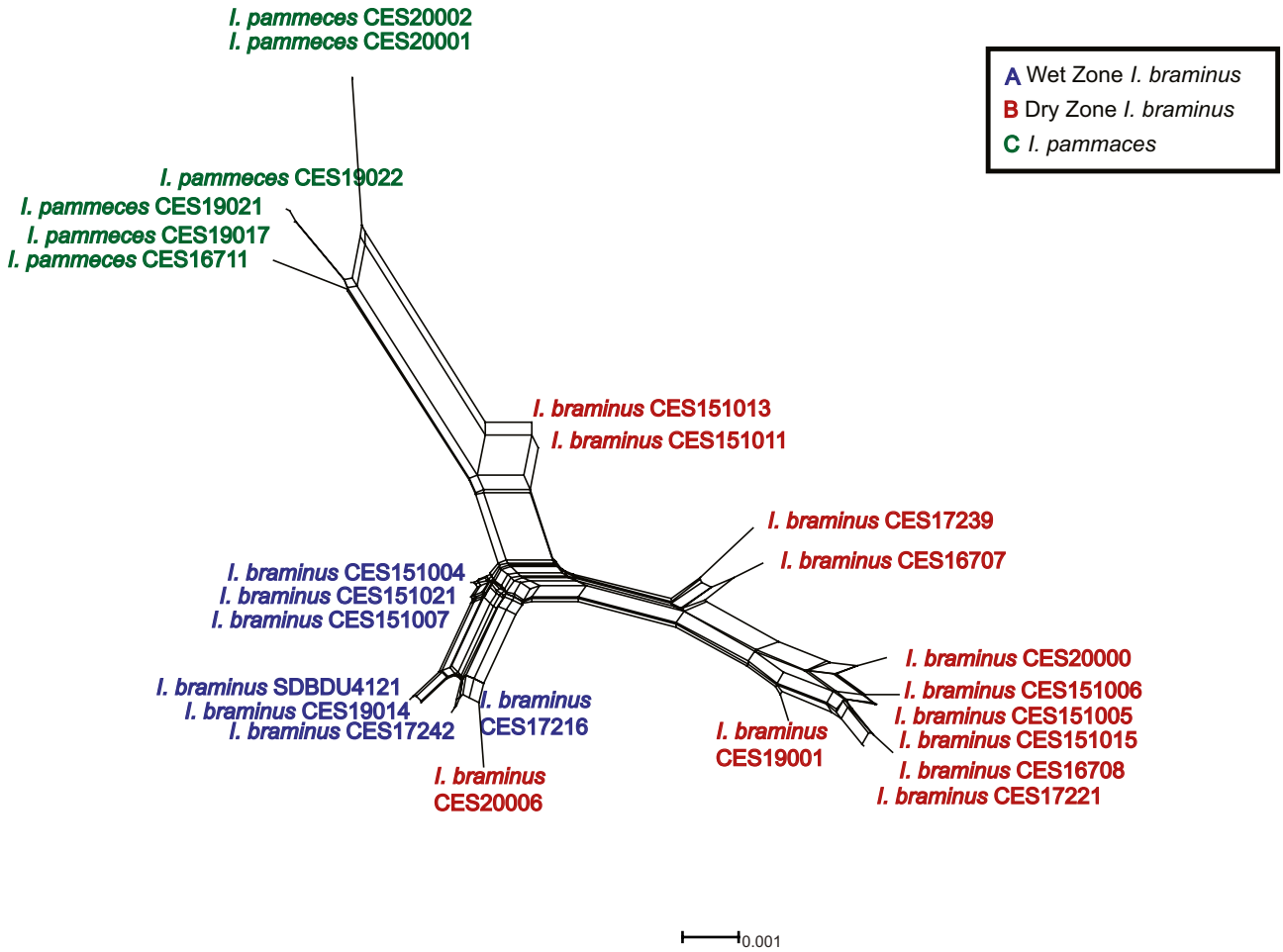

Fig. 7 Neighbor network built using SplitsTree, based on the nuclear dataset consisting of seven markers (AMEL, BDNF, CMOS, CAND1, NT3, RAG1, and ZEB2-4002 bp)

Outgroup-B, and Outgroup-C which are all non-hybridizing species group pairs, since the outgroup is from a separate biogeographical area and does not have overlapping distribution for hybridization to occur.

Discussion

One of the assumptions of the coalescent theory is that genes sampled from a population were all derived from a common ancestor (Wakeley, 2009). In such a case, the gene trees from various genetic markers would all show the same relationships between species. Discordance between gene trees indicates a deviation from the coalescent model of evolution. This could be explained primarily by two other biological processes — incomplete lineage sorting and hybridization. In our study, discordances were observed between various phylogenetic trees. The mitochondrial ML tree was discordant with the nuclear concatenated ML tree. The relationships between the three species groups A, B, and C were different in both trees with the mitochondrial tree showing B and sister to A + C, and the concatenated nuclear ML tree showing A nested within B and sister to C. The mitochondrial ML tree was also discordant with the nuclear coalescent ML tree. The nuclear coalescent tree also retrieved A nested within B and sister to C. We also observed discordance between the nuclear concatenated ML tree and the nuclear coalescent ML tree. The concatenated ML tree did not retrieve the clades A and B as monophyletic groups, while the coalescent ML tree retrieved A as a monophyletic group, nested within B. The discordance between the coalescent and concatenated trees for the four nuclear markers indicates different evolutionary histories for different genes. As expected, the individual gene trees also show discordance, with only two markers retrieving one out of the three species as a monophyletic group.

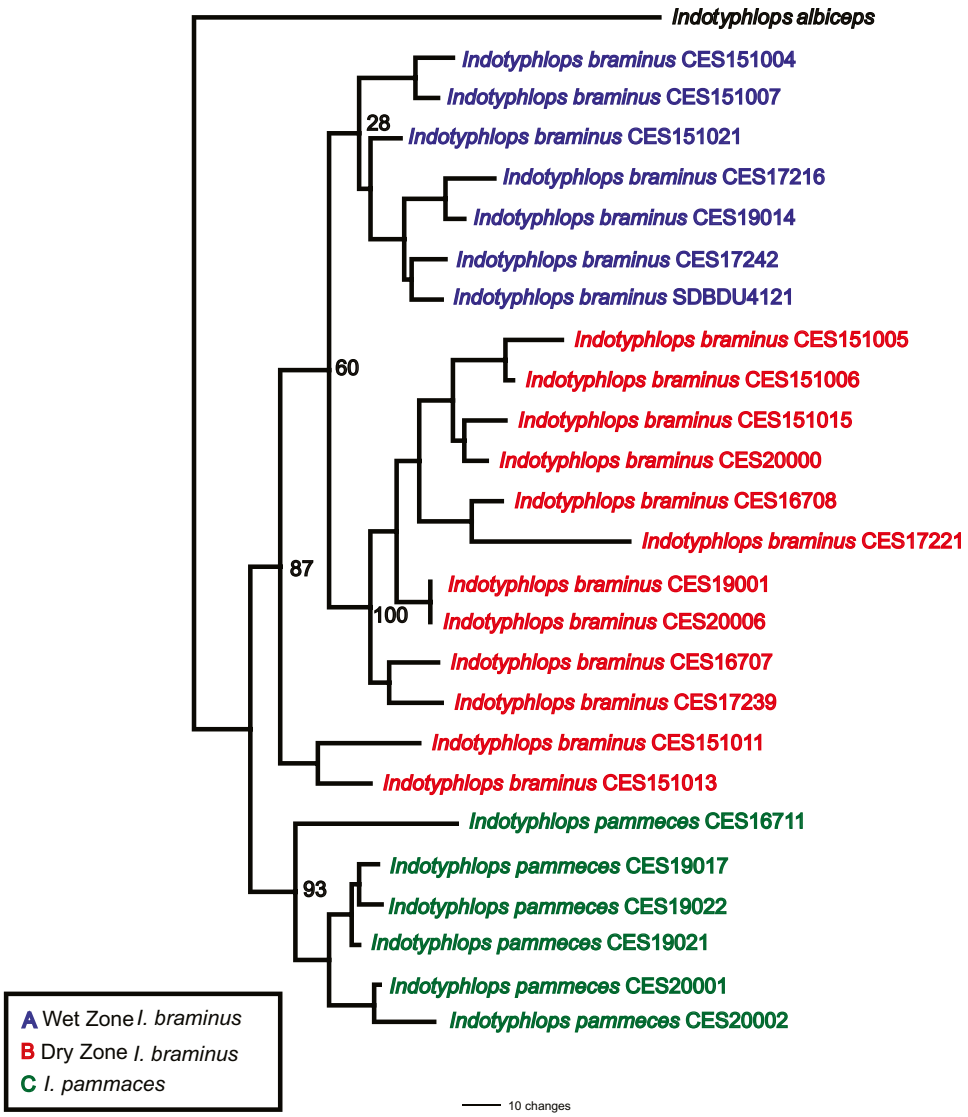

Fig. 8 Coalescent species tree built in SVD Quartets using seven nuclear markers (AMEL, BDNF, CMOS, CAND1, NT3, RAG1, and ZEB2-4002 bp)

The genetic structure suggests ongoing gene flow between *I. pammeces* and one of the populations of *I. braminus*. The wet zone *I. braminus* appears to be genetically a subset of the dry zone *I. braminus*. This pattern suggests that the wet zone *I. braminus* could have originated from hybridization between *I. pammeces* and one of the populations of dry zone *I. braminus.* The network, which was based on sequence data, indicated admixture between all three species.

The JML results also show low probabilities for the coalescent model for various species pairs for different markers. A low *P*-value for a species pair indicates that the null model (coalescent model) does not explain the genetic distance between species in the species tree being higher than the genetic distance between individuals of those two species pairs in the gene tree (based on the alignment). Thus, deep coalescence/ILS can be ruled out. The alternate explanations could point towards hybridization, convergent evolution, or gene duplication. Since multiple nuclear markers were exhibiting discordant topologies, convergent evolution was ruled out as a possible cause. The possibility of multiple markers being under selection, to evolve independently in multiple individuals across species groups, was low. Horizontal gene transfer was ruled out as it generally occurs in bacteria. Since many of these markers had been used in large scale squamate phylogenies, the presence of orthologs for these markers would have been known, and it does not seem to be the case.

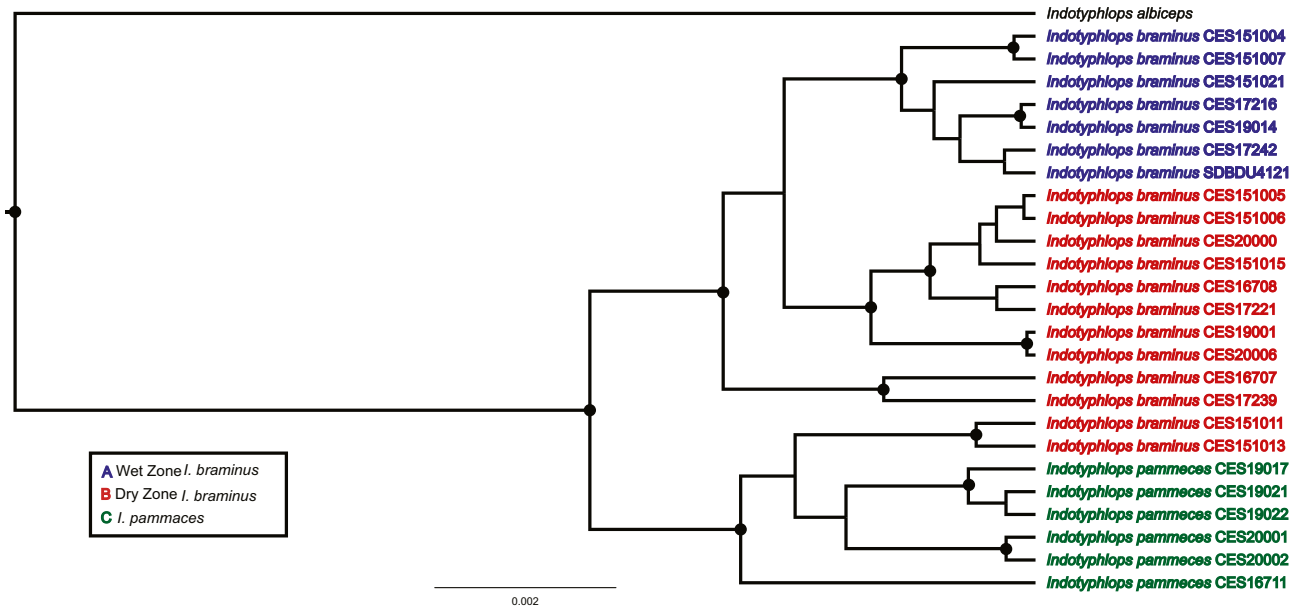

Fig. 9 Coalescent species tree built using seven nuclear markers (AMEL, BDNF, CMOS, CAND1, NT3, RAG1, and ZEB2) in *BEAST. The nodes with posterior probability ≥ 0.8 are indicated with a solid black circle

All seven markers showed low support for the coalescent or null model for one or more species pairs. AMEL results gave very low support for the null model for C-A, and relatively low support for C-B and B-A. This indicates a very high probability that hybridization explains why the genetic distance between individuals of species C and A in the AMEL gene tree is lower than the genetic distance between species C and A in the species tree. The low p-values for C-B and B-A also point towards some introgression between C and B. BDNF JML results also show a similarly high support for C-A and C-B, as do CAND1 and CMOS genes. NT3 and ZEB6 results point towards hybridization being the likely explanation for the lower genetic distance between B and A in the gene tree versus the species tree. The results for RAG1 also support the conclusion that introgression and hybridization cannot be ruled out for any of the species pairs. The JML results, along with the discordance between the individual gene trees, the mito-nuclear discordance, and the concatenated vs coalescent tree discordance, indicate the possibility of *Indotyphlops pammeces* and the dry zone *Indotyphlops braminus* being either or both parents in one or more hybridization events that resulted in the evolution of wet zone *I. braminus*. In the mitochondrial tree, the specimens from outside the Indian subregion fall within the wet zone *I. braminus* clade, thus this lineage is likely to be a triploid and parthenogenic.

Since there are two putative species, representing two distinct clades which look like *I. braminus*, identifying the true *Indotyphlops braminus* becomes necessary for further taxonomic actions. *Indotyphlops braminus* was originally described by Daudin (1803), from Vizagapatam (now Visakhapatnam, Andhra Pradesh, India) based on an illustration (Plate XLIII) from Russell, 1976). One of the samples (CES 20006) in this study is topotypic (Visakhapatnam) and falls in the dry zone *Indotyphlops braminus* clade (B), confirming the dry zone clade as the true *Indotyphlops braminus*. Conversely, the hybrid species (Clade A) seems confined to the wet zones of the Western Ghats, warranting further taxonomic revision and specific status. Wallach (2020) recently erected a new genus *Virgotyphlops* to accommodate this cosmopolitan, hybrid species (*I. braminus*), courtesy of its obligate parthenogenetic mode of reproduction. However, Fretty and Dubois (2021) deemed this action invalid and showed that this nomen *Virgotyphlops*is nomenclaturally unavailable. Our results also favor a disagreement to the assignment of the species into a new genus, since it is nested well within the Indian *Indotyphlops* clade. However, a revision of the species name is in order, for which a more detailed study of the external and perhaps internal morphology needs to be carried out.

Table 2 *P*-values for each species pair, for each nuclear marker. The species are A (wet zone *I. braminus*), B (dry zone *I. braminus*), and C (*Indotyphlops pammeces*)

Gene	Species pairs	Observed genetic distance	Probability
AMEL	B-A	0	0.217889
	C-A	0	0.0099495
	C-B	0.00277008	0.160942
	Outgroup-A	0.0470914	0.927904
	Outgroup-B	0.0443213	0.898855
	Outgroup-C	0.0443213	0.888306
BDNF	B-A	0.0031746	0.188491
	C-A	0.00793651	0.0414479
	C-B	0.00793651	0.0542973
	Outgroup-A	0.031746	0.619919
	Outgroup-B	0.031746	0.640768
	Outgroup-C	0.0349206	0.729714
CAND1	B-A	0.00117096	0.218789
	C-A	0.00117096	0.00824959
	C-B	0.00234192	0.0612969
	Outgroup-A	0.132319	1
	Outgroup-B	0.133489	1
	Outgroup-C	0.131148	1
CMOS	B-A	0	0.217739
	C-A	0	0.0101995
	C-B	0	0.0146993
	Outgroup-A	0.102564	0.9999
	Outgroup-B	0.102564	0.99995
	Outgroup-C	0.0968661	0.99965
NT3	B-A	0.00369004	0.283986
	C-A	0.0276753	0.780061
	C-B	0.0239852	0.721364
	Outgroup-A	0.0904059	0.99925
	Outgroup-B	0.0922509	0.99925
	Outgroup-C	0.0922509	0.9993
RAG1	B-A	0.00387597	0.323384
	C-A	0.0155039	0.318134
	C-B	0.0135659	0.283986
	Outgroup-A	0.0523256	0.960452
	Outgroup-B	0.0542636	0.968352
	Outgroup-C	0.0523256	0.959202
ZEB6	B-A	0	0.217539
	C-A	0.00668449	0.494825
	C-B	0.00667659	0.566572
	Outgroup-A	0.0160428	0.948453
	Outgroup-B	0.0173797	0.971041
	Outgroup-C	0.0227273	0.911684

Conclusion

Mito-nuclear discordance, discordance between nuclear gene trees, and between the trees built using concatenated and coalescent approaches, as well as JML analysis support the possible hybrid origins of this widespread species. As seen with other groups, the one or more hybridization events could have resulted in the triploid karyotype, subsequently resulting in a parthenogenetic form of reproduction due to the polyploidy. However, since two putative species within the groups had morphological characters identical to the described *Indotyphlops braminus*, we used a topotypic specimen to identify the true *Indotyphlops braminus.* Since the widespread, triploid and possibly parthenogenetic species was not the true *I. braminus*, a taxonomic revision is required for this species. In this regard, it would also be interesting to determine the karyotype and sex ratio of the *Indotyphlops* species of peninsular India.

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Author contribution Chinta Sidharthan and K. Praveen Karanth contributed equally to the study conception and design. Material preparation, molecular data generation, and large parts of the analysis were performed by CS. Species delimitation and morphological data collection were carried out by Pragyadeep Roy. Sample collection was carried out by CS, KPK, and SN. SN built the distribution map. The first draft of the manuscript was written largely by CS, with certain section of methods and results written by PR. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets used in this study are currently available from the corresponding author on reasonable request.

Declarations

Competing Interest The authors declare no competing interests.

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