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# Is there only one species of flowerpot snake around the world? Phylogenetic position of a specimen from Italy (Ischia) (Serpentes: Typhlopidae)

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## SUMMARY

The flowerpot blindsnake (*Indotyphlops braminus*), native to India, nowadays holds the widest global distribution among the snakes, due to passive transport by humans. Previous genetic analysis showed that two geographically separated mtDNA clades occur in India, one confined to the wetter parts of Western Ghats (“wet zone” clade), while the other distributed in the drier parts of central and eastern peninsular India (“dry zone” clade). All the so far studied flowerpot specimens found all over the world belong to the “wet zone” clade and are characterized by a very low mtDNA diversity. In this paper, the phylogenetic position, based on two mtDNA markers (16S and cytb) was assessed for one specimen of flowerpot blindsnake found in Ischia Island (Italy), one of the two localities where the species has been introduced in the country. The results showed an elevated divergence between the Ischia specimen and all other specimens from different worldwide non-native location (7.9% for 16S and 14.1 % for cytb). Moreover, the specimen unequivocally clusters within the Indian “dry zone” clade. In literature it has been suggested that the “dry zone” clade represents the nominal *I. braminus*, while the other clade represents a different and yet unnamed species. In this perspective, our results indicate that the Ischia specimen would be the first true *I. braminus* found outside its range of origin. Finally, the results of this study reinforce the necessity to continue studying the flowerpot snake populations outside their range of origin also from a chromosomal and morphological point of view.

## INTRODUCTION

The flowerpot blindsnake *Indotyphlops braminus* (Daudin, 1803) (Serpentes Typhlopidae) is a small subterranean blind snake, originating from the Indo-Malayan region, with adult approximately 100–130 mm long, 3–4 mm in diameter, and weigh less than 1 g (Wallach 2020). Flowerpot blindsnake holds the widest distribution on the globe among the snakes (Rato et al. 2015, Bamford and Prendergast 2017), being found in several sites in Asia, Oceania, Africa, Americas and Europe (Kamosawa and Ota 1996, Rato et al. 2015, Paolino et al. 2019, Faraone et al. 2019, Vella et al. 2020). Furthermore, *I. braminus* is the only known snake species with parthenogenic reproduction and no males are known (McDowell 1974, Wallach 2009). Previous 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 (McDowell 1974, Nussbaum 1980, Wynn et al. 1987, Ota et al. 1991, Patawang et al. 2016).

The wide diffusion of the flowerpot blindsnake in the world is due to the fact that it takes refuge in pot plants (from which it takes the name), and it is indirectly transported by humans via the main trade routes (Bamford and Prendergast 2017). In addition, its unique type of reproduction would allow the establishment of viable populations even with a single translocated individual (Buřič et al. 2016; Feria and Faulkes 2011).

The great propensity for passive transport has led to a series of studies on *I. braminus* aimed at highlighting the origin of the introduced populations (e.g. Rato et al. 2015). These studies had a limitation: until recent times, there were no information on the genetic structure of the source populations in India (Wickramasinghe et al. 2022). Regardless of the very low mtDNA diversity found all over the world (Rato et al. 2015, Wickramasinghe et al. 2022), a recent phylogenetic study (Sidharthan

et al. 2023), based on both mitochondrial and nuclear markers, have found an unexpected genetic diversity within *I. braminus* from India with two well supported allopatric clades. One of these is confined exclusively to the wetter parts of Western Ghats, while the other is largely distributed in the drier parts of central and eastern peninsular India.

Although these two lineages are morphologically identical, based on lepidosis, the phylogenetic analysis suggested that they could belong to two different species (Sidharthan et al. 2023). The study also confirmed the “dry zone” clade as the nominal *I. braminus*. In fact, one of the samples in this clade was topotypic (Vizagapatam [Visakhapatnam]). Conversely, the “wet zone” clade of the Western Ghats, needs further taxonomic revision and possibly a specific status (Sidharthan et al. 2023).

The situation is complicated by a third species, *I. pammeces* (Günther, 1864), which is closely related to the other two. Phylogenetic analysis showed admixture among all the three species and indicated that hybridization between *I. pammeces* and members of the “dry zone” clade may have originated the “wet zone” clade which, therefore, is probably the source population of the triploid and parthenogenic specimens found worldwide (Sidharthan et al. 2023).

The taxonomic diversity in the source area in India indicates some potentially interesting genetic variation also in the introduced populations and encourages additional sequencing effort for *I. braminus* from across its global range. In this paper, the phylogenetic position of one specimen of flowerpot blindsnake found in Ischia Island (Italy) was assessed based on two mtDNA markers.

## MATERIALS AND METHODS

Genomic DNA was extracted from a tissue sample of the specimen collected in Ischia

(Paolino et al. 2019; deposited at Museum of Comparative Anatomy “Giovanni Battista Grassi”, University of Rome “La Sapienza” voucher number AC2078) using the universal extraction protocol described by Aljanabi and Martinez (1997), consisting in incubation at 56° C with proteinase K and precipitation with isopropanol. We amplified two mtDNA fragments, the 16S rRNA gene (16S) and the cytochrome b gene (cytb) were amplified. We choose the two markers in order to maximize comparative analysis with available sequences in GenBank.

For the 16S gene, we obtained sequences using the primers 16SA-L (light chain; 59-CGC CTG TTT ATC AAA AAC AT-39) and 16SB-H (heavy chain; 59-CCG GTC TGA ACT CAG ATC ACG T-39) (Palumbi et al. 1991). We performed the PCR cycling procedure as follows: 90 sec denaturing step at 94° C, 34 cycles of denaturation for 90 sec at 95° C, primer annealing for 60 sec at 50° C, extension for 90 sec at 72° C and final 7-min elongation step at 72° C. We amplified the cytb with primers L14910 and H16064 (Burbrink et al., 2000). The PCR cycling procedure was only slightly modified with respect to Burbrink et al. (2000) as follows: 7-min denaturing step at 94° C followed by 40 cycles of denaturing for 40 sec at 94° C, primer annealing for 30 sec at 48° C, and elongation for 1 min at 72° C, with a final 7-min elongation step at 72°C.

We purified the templates with the Sure Clean (Bioline) purification kit and then sequenced by Macrogen© (www.macrogen.com). The obtained electropherograms were checked for ambiguous positions and manually edited with Chromas 1.45 (Technelysium Pty Ltd, Tewantia, Queensland, Australia) (GenBank accession numbers: PQ222456 and PQ213019).

Two alignments were built separately for the two mtDNA markers. Concatenated sequences were not used due to the unavailability to obtain in GenBank specimens that had been sequenced for both markers. The

alignment for 16S (385 bp) included 16 sequences of *I. braminus* downloaded from GenBank plus other sequences belonging to other Leptotyphlopidae used as outgroup, i.e., *Tricheilostoma bicolor* (Jan, 1860), *Rhinoleptus koniagui* (Villiers, 1956), *Myriopholis blanfordi* (Boulenger, 1890) and *M. narirostris* (Peters, 1867). (Vidal and Hedges 2002, Adalsteinsson et al. 2009, Rato et al. 2015, Li et al. 2020). The alignment (783 bp) for cytb included 63 sequences belonging to *Indophyplops* including *I. braminus*, *I. pammeces* plus sequences of *I. albiceps* as outgroup (Slowinski and Lawson 2002, Yan and Zhou 2008, Adalsteinsson et al. 2009, Marin et al. 2013, Pyron et al. 2013, Nagy et al. 2015, Sidharthan et al. 2023, Wickramasinghe et al. 2022).

We aligned sequences for the two datasets with MUSCLE (Edgar 2004) in MEGA (version 11.0.13, Tamura et al. 2021).

To infer phylogenetic relationships for both the alignment, we built maximum likelihood (ML) and Bayesian phylogenetic trees. We choose model of molecular evolution among 54 evolutionary models using the software ModelGenerator v85 (Keane et al. 2006). Accordingly, for cytb the analyses were run under the assumption of a HKY model of sequence evolution considering a gamma rate of substitutions (+G). For 16S the chosen model was the time reversible model with invariable sites (GTR+I). For both alignments ML analyses were 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. The Bayesian tree was obtained with the software MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003). We performed two independent runs (5,000,000 generation sampling every 1000 generations). The 10,000 retained topologies were used to obtain a consensus tree after the first 25% of topologies were discarded.

Genetic divergence (p-distance) among species/clades were calculated as net between group mean for both cytb and 16S genes with p-distance with MEGA 11. Mean genetic divergences within group were also calculated with the same software.

## RESULTS

In the cytb alignment, all sequences belonging to *Indotyphlops braminus* and *I. pammeces* fall in a well-supported clade (PP = 1). Within this clade, three well supported lineages can be found. The first lineage (PP = 1; ML = 73%) includes sequences of *I. braminus* belonging to the dry zone of India. The only sequence of this clade, not included in this geographic region, is the new sequence obtained from the specimen we collected in Ischia (Fig. 1).

The other two clades are sisters even if the support for their relationship remains low (PP = 0.58; ML = 68%). One of these clades (PP = 1; ML = 99%) includes sequences belonging to *I. pammeces* while the other (PP = 1; ML = 99%) includes all the other sequences attributed to *I. braminus*. These includes specimens from the wet zone of India as well as all the other specimens found all over the world specifically from Florida, Madagascar, Sri Lanka, China and Mexico (Fig. 1).

Net between-group mean genetic divergences among the three main lineages range from 8% (“wet zone” vs “dry zone” clades of *I. braminus*) to 10.4% (*I. pammeces* vs “wet zone” clade). It is worth of note that intraclade mean divergence is much higher in *I. braminus* from the dry zone (13%) than within *I. braminus* from the wet zone (2%). The studied specimen diverged by a mean genetic divergence 14.1% respect to all the other specimens found outside India.

The results obtained with 16S are coherent with those obtained with cytb (Fig. 1). In fact, the sequences of *I. braminus* fall in two lineages. The first lineage corresponds to the “wet zone” clade and includes two haplotypes.

One haplotype is shared by geographically distant populations, including the Comoros, Guinea, Spain, the Seychelles, Thailand, Sicily (Italy), Mexico, and China, while the other haplotype was found in southern India. The other lineage is composed by the unique sequence of *I. braminus* from Ischia. Thus, it corresponds to the “dry zone” clade identified with cytb. The divergences of the two lineages at 16S is 7.9%.

## DISCUSSION

Sidharthan et al. (2023) study showed that all the populations, so far identified by DNA, outside the native range of *Indotyphlops braminus*, belong to only one of the two lineages present in the source area, i.e., to the “wet zone” lineage. It also appears evident that, through the analysis and integration of nuclear and mitochondrial information, this lineage would derive from hybridization events, presumably occurred in India, between *I. pammeces* and members of the other lineage, the “dry zone” one (Sidharthan et al. 2023). This hybridization event would result in polyploidy and parthenogenesis to overcome the mitotic and meiotic instability due to the presence of non-homologous chromosomes in the germline (Mezzasalma et al. 2023). In adding, the very low mtDNA genetic diversity, which is found both in the native range and in the introduced populations, would be linked with this particular type of uniparental reproduction (Moritz et al. 1992).

The individual from Italy (Ischia) studied in this study, surprisingly, reveals that it does not belong to the “wet zone” lineage but to the “dry zone” one. At the best of our knowledge, it represents the first occurrence record of this mitochondrial lineage outside its range of origin. This find has some important consequences. From a taxonomic point of view, Sidharthan et al. (2023) suggest that the two lineages “wet zone” and “dry zone” belong to two different species. Therefore, if this interpretation was correct, the Ischia specimen would be the first *I. braminus* found outside its range of origin.

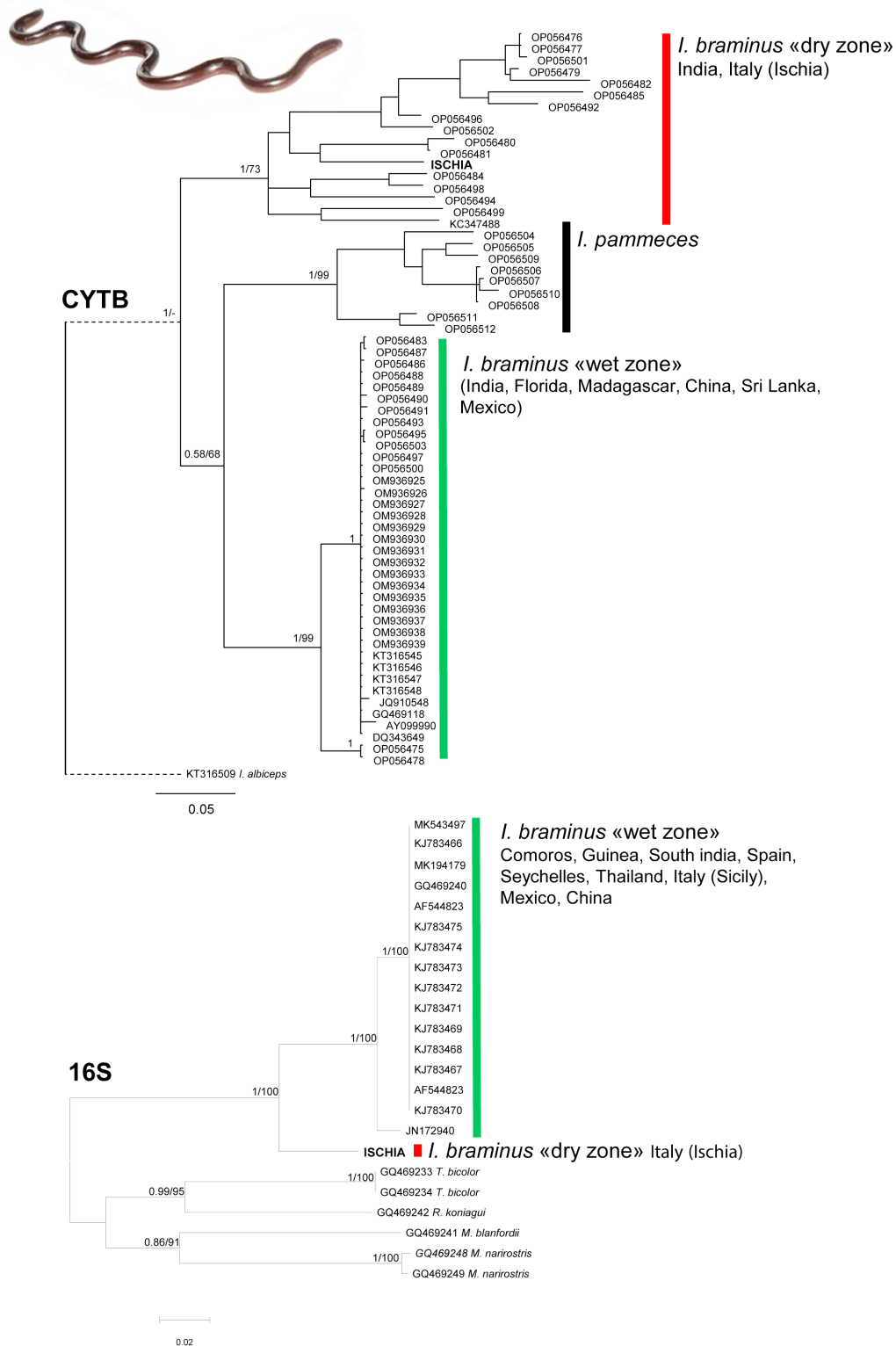


Figure 1. Phylogenetic position of the Ischia specimen. Above: Cytochrome b, CYTB, (783 bp) Maximum Likelihood (ML) phylogenetic tree including 63 sequences belonging to *Indotyphlops braminus*, *I. pammeceus* plus sequences of *I. albiceps* as outgroup.; below: 16S rDNA gene (385bp) ML phylogenetic tree including 16 sequences of *I. braminus* and sequences belonging to other Leptotyphlopidae (i.e., *Tricheilostoma bicolor*, *Rhinoleptus koniagui*, *Myriopholis blanfordii*, *M. narirostris*) used as outgroup. Support to each node is indicated, only for more basal nodes, as BI posterior probability/ML bootstrap values.

Furthermore, leaving nomenclatural considerations aside, one can expect a different ecological value of the specimens belonging to the two lineages, whose distribution in the source area is marked by very different bioclimatic conditions (Sidharthan et al. 2023). These differences might have consequences on the adaptive capabilities, and therefore on the distribution of the two lineages, even outside the range of origin (Conyers and Roy 2021).

Finally, according to Sidharthan et al. (2023), it is not possible to confirm the parthenogenetic and triploid nature of the members of the “dry lineage”. In fact, all the chromosomal studies carried out so far have been performed on members of the “wet lineage” (Sidharthan et al. 2023) and the dry lineage lack of chromosomal and reproductive characterization. It must also be considered that the related species *I. pammeces* has amphimictic reproduction and it has been even confused with *I. braminus* in the past (Wallach 2009) contributing to complicate the situation.

The results of this study reinforce the need to continue studying the flowerpot snake populations located outside their range of origin also from a chromosomal and morphological point of view.

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