



**- RESEARCH ARTICLE -**

**First records of the Brahminy blindsnake, *Indotyphlops braminus* (Daudin, 1803) (Squamata: Typhlopidae) from Malta with genetic and morphological evidence**

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**Abstract**

This publication reports the first two records of the Brahminy blindsnake *Indotyphlops braminus* (Daudin, 1803), representing a new alien species in Malta. This species, native to Indo-Malayan region, has over the years broadened its distribution through anthropogenic international transportation of goods. Its unique parthenogenic reproductive strategy increases its potential for fast population expansion, becoming invasive. The two specimens analysed in this study were found in May, 2020, and were identified through external morphology and genetic sequencing of 12S rRNA, 16S rRNA and COI genes. These sequences were compared to other genetic data available for *I. braminus* from other locations, where it was found that the mitochondrial DNA variation for this species is very low at a global scale.

**Keywords:**

*Indotyphlops braminus*, introduced species, reptiles, mtDNA

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**Introduction**

Anthropogenic activities are leading to an increasing rate of alien species introductions, with some becoming invasive and threatening native biodiversity (Hulme et al., 2009; Kark et al., 2009; Meshaka, 2011; Seebens et al., 2018). Within this scenario, reptiles are no exception, with numerous introductions being considered as either accidental with imported goods or else voluntary in association with the international pet trade industry (Lever, 2003; Meshaka, 2011; Borroto-Paez et al., 2015; Hulme, 2015; Silva-Rocha et al., 2015; Auliya et al., 2016; Capinha et al., 2017). Monitoring for these alien reptile species, especially when their occurrence is already

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known in neighbouring regions, could lead to early detection and can assist in better mitigation strategies to control their invasiveness (Liu et al., 2019).

The Brahminy blindsnake also known as the Flowerpot snake, *Indotyphlops braminus* (Daudin, 1803) is native to the Indo-Malayan region (Broadley & Wallach, 2009; Wallach, 2009; Hedges et al., 2014) and is considered as a species with low mobility and small ability to naturally disperse (Page et al., 2008; Mateo, 2013). Nonetheless, this species is probably the most widely distributed snake species in the world (Wallach, 2009; Bamford & Prendergast, 2017). Human-mediated introductions linked to the international plant trade (Page et al., 2008) have widened its distribution in more than 54 countries (Wallach, 2009; Global Invasive Species Database, 2018; Uetz et al., 2020) becoming almost globally distributed, with the history of many of these introductions being very often difficult to trace (Lever, 2003; Page et al., 2008; Hedges et al., 2014). Most of these introductions are recorded in tropical and subtropical regions, with *I. braminus* forming established populations and becoming a naturalized reptile in most of them (Lever, 2003; Global Invasive Species Database, 2018). Considered harmless to man, the Brahminy blindsnake represents one of the smallest snake species with a total body length rarely exceeding 190 mm (Wallach, 2009). This small subterranean snake lives in the soil, including potter plants, where it lives on small prey such as eggs, larvae and pupae of insects namely ants, termites and beetles (Page et al., 2008; Mizuno & Kojima, 2015). This fossorial non-venomous snake, like other typhlopids, rarely comes to the surface (Ehmann & Bamford, 1993; Bamford & Prendergast, 2017), and the habitat it lives in allows easy undetected transportation of specimens between different locations mostly between areas associated with plant nurseries, golf courses, compost deposits, agricultural farms and gardens including domestic gardens, botanical gardens and parks (Page et al. 2008; Urioste & Mateo, 2011; Mateo et al., 2011; Bamford & Prendergast, 2017; Zamora-Camacho, 2017; Snyder et al., 2019; Faraone et al., 2019). Moreover, the reproductive biology of this species facilitates its dispersion and potential for colonization. The karyotype of *I. braminus* indicates that this species is a triploid with all specimens being female and thus this unisexual snake reproduces only through parthenogenesis (Wynn et al., 1987; Kamosawa & Ota, 1996; Patawang et al., 2016). Consequently, once an individual reaches maturity, which occurs at a total length of 95 mm (Ota et al., 1991), that individual can lay eggs in clutches of up to 8 eggs, with larger females laying larger clutch sizes (Kamosawa & Ota, 1996; Ota et al., 1991). Therefore, one single mature female has the potential of founding and establishing a population. The timing of egg laying varies between locations. In subtropical areas, such as the Canary Islands, this takes place towards the end of the wet season (Mateo, 2013).

The herpetofauna of the Maltese archipelago is composed of 12 taxa and includes subspecies. Amongst these taxa, there is also the common chameleon, *Chamaeleo chamaeleon* which has been introduced in Malta in 1877 and subsequently became a naturalised local species (Lanfranco, 1955; Sultana & Falzon 1996; Baldacchino & Schembri 2002). All these species are legally protected by the Maltese Environment Protection Act and the Reptiles (Protection) Regulations of 1992 (S.L.540.02) (Legislation Malta, 2020). In the Maltese archipelago there are no records of Typhlopidae species, therefore the occurrence of blindsnake specimens in the wild deserved further investigation. This study utilized identification techniques that focus on both morphological and genetic characters. The latter are important as there are 274 species of blindsnake (ITIS, 2020), with some of them having a number of overlapping morphological characteristics, and therefore

genetic analyses, including DNA barcoding, help to back morphological data for accurate species identification.

### Material and methods

Two specimens of *I. braminus* were observed and collected from Marsa, Malta (Figure 1; 35°52'35.4"N 14°29'34.4"E) on 6<sup>th</sup> May, 2020 (Figure 2) and 8<sup>th</sup> May 2020 respectively. Both specimens died within a few days after capture. The specimens were identified down to species level following Wallach (2009) and subsequently stored in 100% ethanol as voucher specimens at the Conservation Biology Research Group collection at the University of Malta (CBRG-UM).



Figure 1. A map (Google Earth, 2020) of the Maltese Islands showing the location where the specimens of *Indotyphlops braminus* were found.

A small tissue sample was taken from one of the voucher specimens and used for total genomic DNA extraction via AccuPrep Genomic DNA Extraction Kit (Bioneer Inc., South Korea). PCR amplifications of: the 12S rRNA gene (12S) using the primers 12SAL and 12SBH (Kocher, 1989); the 16S rRNA gene (16S) using the primers 16Sar-L and 16Sbr-H (Palumbi et al., 1996); and the cytochrome c oxidase I gene (COI) using the primers jgLCO and jgHCO (Geller et al., 2013), were carried out following each respective amplification protocol. PCR products were purified and sequenced via ABI3730XL sequencer (Applied Biosystems) using each respective forward and reverse primer. Sequences were checked for consistency using Geneious v10 (Kearse et al., 2012) and were compared to other publically available sequences using BLASTn (NCBI, 2020). These sequences were aligned using Geneious v10 (Kearse et al., 2012). The smallest homologous sequence for each gene was used for comparative phylogenetic analyses by maximum-

likelihood which were conducted with MEGA v10 (Kumar et al. 2018) using 1000 bootstraps and the model of best fit as identified by jModelTest v2 (Darriba et al., 2012). The 12S rRNA tree was constructed using K2P (Kimura, 1980), while the 16S rRNA tree was constructed using the T92 (Tamura, 1992) and the COI gene tree was constructed using HKY+I (Hasegawa, Kishino & Yano, 1985), as they were identified as the model of best fit for each respective data set. The genetic data generated in this study was deposited in GenBank, under accession numbers MT659670, MT658780 and MT997278.



Figure 2. Photo of voucher specimen (total body length 15.1 cm).

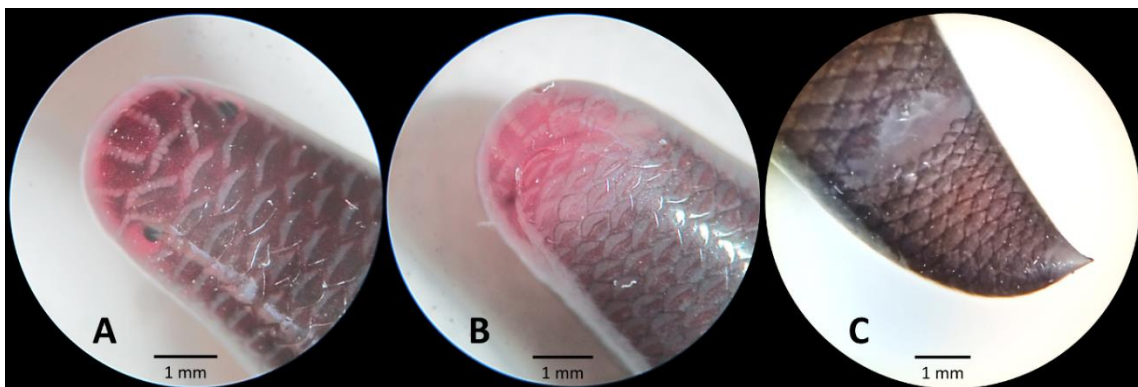


Figure 3. Close ups of the head area (A - dorsal view; B - ventral view) and tail area (C - ventral view).

Results

This study represents the first scientific records of a blindsnake in Malta, through the presence of two *I. braminus* specimens found at Marsa. These two specimens had a total length of 125 mm and 151 mm and a midbody diameter of 3.3 mm and 3.8 mm respectively, with a mean total length to midbody diameter ratio of 39. These specimens weighed 0.78 g and 1.15 g respectively. The external morphology of the two specimens matched the species description for *I. braminus* given by Wallach (2009) and Hedges et al. (2014). Both specimens had a rounded snout, with the head and tail scale patterns showing the expected arrangements described in Wallach (2009) (Figure 3) with 20 scale rows throughout the body. The scales were smooth and polished and their dorsal colour was very dark brown, while the ventral side was slightly paler with a triangular apical spot covering the anterior of each scale. The chin area, the lower snout, the cloacal region and the tip of the tail were much paler almost grey-cream in colour (Figure 2 and 3). Scientific field observations in the same area led to the confirmation of the presence of more specimens of similar body size, but these specimens were not collected.

The mtDNA sequences obtained from one of the voucher specimens were 401 bp, 506 bp and 581 bp for 12S rRNA, 16S rRNA and COI genes respectively. All sequences gave a 100% match to *I. braminus*. The 12S rRNA gene data matched completely with two sequences and differed by 1 bp from another two sequences, while the 16S rRNA gene data matched completely with almost all other *I. braminus* data from other geographical locations and the COI gene data matched with the most common haplotype found on GenBank and differed from a second haplotype by 1 bp. On BOLD the COI sequence classified our specimen under bin BOLD:AAI0608, which belongs to *I. braminus*. The mtDNA analyses and the maximum-likelihood phylogenetic trees (Figure 4) analysed in this study show that there is little global genetic variation for the studied genes of *I. braminus*.

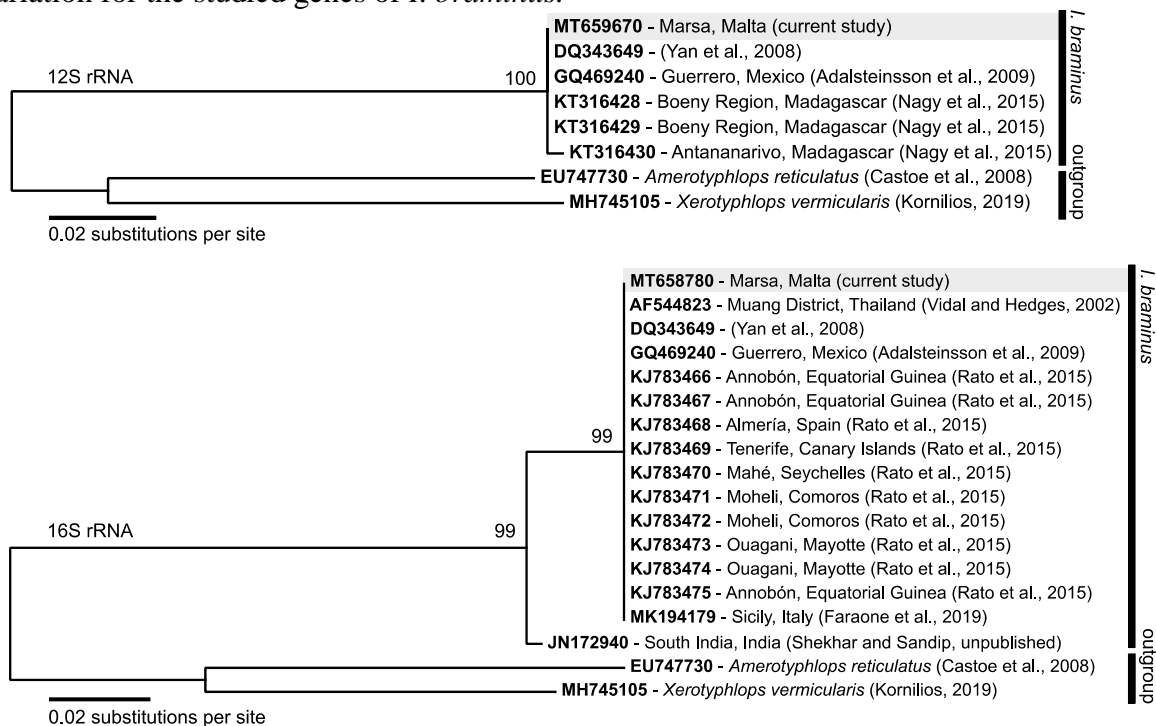


Figure 4. (continues on following page)

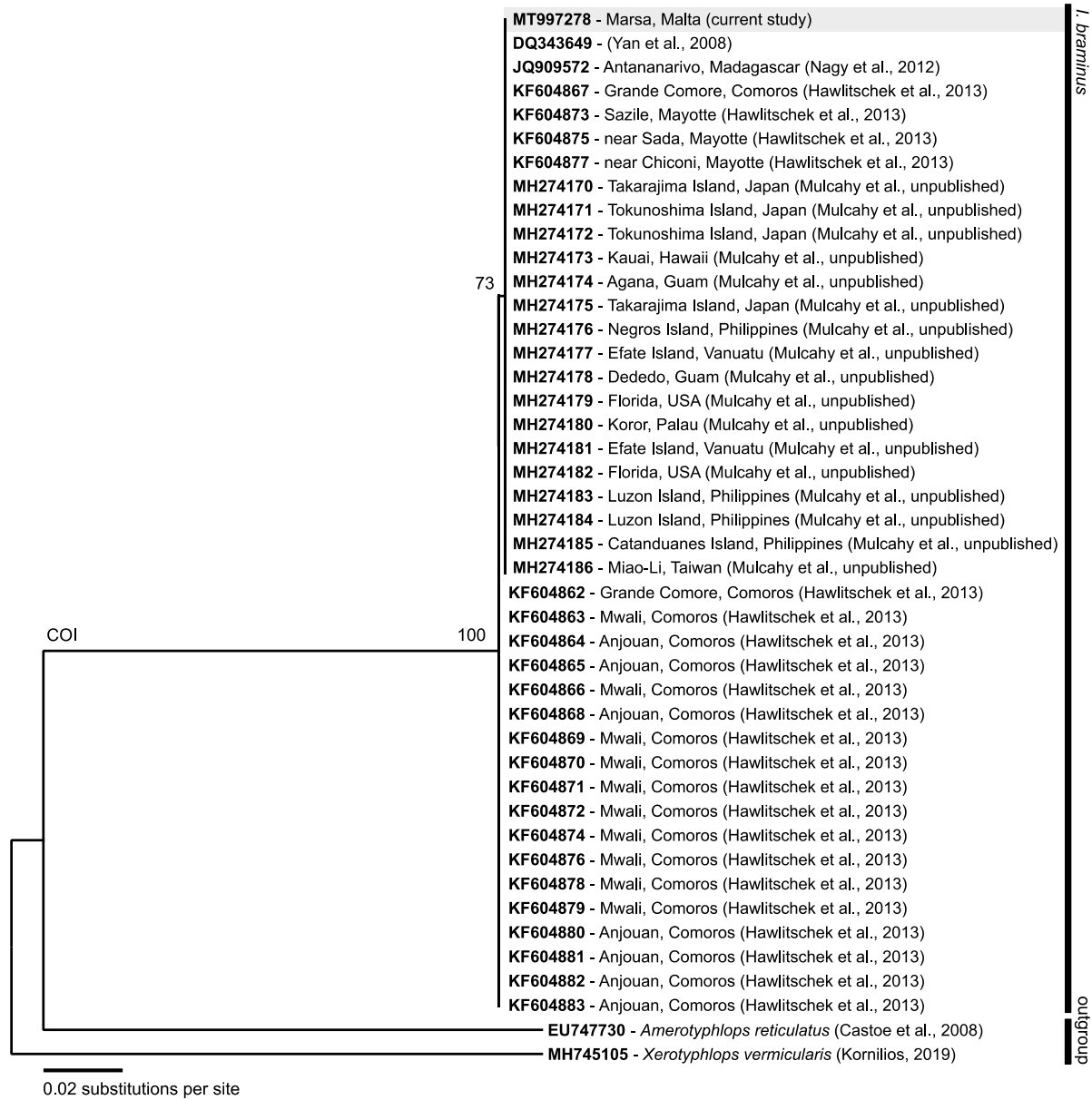


Figure 4. Maximum-likelihood phylogenetic trees for the 12S rRNA, 16S rRNA and COI genes utilizing the shortest homologous sequence for each respective dataset (340 bp, 361 bp and 581 bp) using the substitution models described in the methodology. Bootstrap values are shown at their respective nodes. The accession numbers, country of origin and respective references are included on the right. Two other Typhlopidae species were used as outgroup.

### Discussion

A number of human-mediated introductions have led *I. braminus* to increase its distribution globally (Wallach, 2009; Bamford & Prendergast, 2017). In Mediterranean countries, the species has been recorded in Egypt (Baha El Din, 1996, 2001), Libya (Joger et al., 2008), mainland Spain (Mateo, 2013; Rato et al., 2015; Zamora- Camacho, 2017), the Balearic Islands (Mateo, 2013) and more recently on two Italian islands, Sicily (Faraone et al., 2019) and Ischia (Paolino et al., 2019),

with the current study confirming its first occurrence on the island of Malta. Being a small sized snake with a tubular-shaped body, smooth scales and subterranean mode of life, this species to be easily mistaken for earthworms (Wallach, 2009), delaying its early detection of introductions by stakeholders and by citizen-scientists. Additionally, as with other species outside their native range, species identification has to be cautiously tackled, especially given that there are 274 blindsnake species in the family Typhlopidae with 23 species belonging to the genus *Indotyphlops* (ITIS, 2020). For this purpose, morphological identification is best to be backed by genetic identification which has been proven to provide accurate species identification for reptile species (Hawlitschek et al., 2013). In this study the species was morphologically and genetically identified using three mitochondrial DNA genes. Global analyses of the latter have shown that this species exhibits very little mitochondrial DNA variation, so results cannot assist in identifying the population of origin, while the multiple introduction events known to have occurred in different countries make it even more difficult to deduce colonization patterns (Rato et al., 2015).

The specimens found in this study were collected from a public space at the periphery of Malta's largest outdoor sports facility. This area, which covers close to 0.4 km<sup>2</sup> (Figure 1), is mostly turfed grounds and includes a golf course, a habitat that is already known to have the right humid conditions required for sustaining populations of this blindsnake (Urioste & Mateo, 2011). The area where these snakes were found was composed of loose soil and organic debris, and was thriving on ants, one of the main components of this snake's diet (Page et al., 2008). Given that the total length in juveniles range between 61 mm and 119 mm, while sexually mature adults range between 95 mm and 179 mm (Ota, 1991), then the specimens collected can be classified as adults. Although we did not record the presence of eggs or hatchlings, the presence of specimens of different lengths, and given that this species reproduces only through parthenogenesis (Wynn et al., 1987), then it is very likely that *I. braminus* has established itself in the area under study. The occurrence of this species in Malta indicates the possible accidental importation through translocation of planting media. It is unlikely that the soil in the exact area of sampling was recently imported, so this blindsnake has either been present in the area undetected for years, or else it has been imported to the nearby grounds where it expanded its range. Plants, turf and other landscaping supplies are imported to Malta from nearby countries, such as Italy (Planning Authority, 2002; The Malta Independent, 2016). Locally, the importation of plant material and soil is regulated by the Plant Quarantine Act (ERA, 2018; Legislation Malta, 2020) to avoid the introduction of pests, however this species being fossorial and its close resemblance to earthworms makes it difficult to detect. This indicates that better monitoring systems for the detection of alien fossorial species is required to ensure that imported soil is free of non-native species.

In dry countries like Malta, artificial ecosystems such as those found in plant nurseries, gardens and sport grounds create the right warm and relatively wet tropical habitat required for this species to survive (Ota et al., 1991). These areas are usually watered all year round creating a sufficiently humid environment as required for the survival and establishment of this species, consequently these areas have to be monitored for possible additional introductions of this species. Recent records of this species on the Canary Islands, Madeira, coastal areas of Spain, Balearic Islands, Sicily and Ischia have found the presence of *I. braminus* in artificial habitats close to the sea possibly due to an increased level of humidity and temperature stability (Urioste & Mateo,

2011; Jesus et al., 2013; Mateo, 2013; Zamora-Camacho, 2017; Faraone et al., 2019; Paulino et al., 2019).

In Malta, native reptiles are larger in size than *I. braminus* and are not known to occupy the same habitat therefore no direct habitat competition between this alien snake and the native local herpetofauna is expected. Nonetheless, a precautionary approach has to be adopted to investigate its potential impacts both in Malta and in the numerous locations around the world where this species has now established itself. In the meantime, due to its reported diet preferences (Page et al., 2008; Bamford & Prendergast, 2017), this introduced species can affect the invertebrate community in the area where it is found through predation and so might compete with local predators for the same prey species. Additionally, as with any introduced species, this species has to be evaluated for its own pathogens and their possible transmission to native vertebrate species (Field & Horrocks, 2011; Young et al., 2016; Scalera, 2019).

### Conclusion

The detection of an established population of *I. braminus* in Malta adds on and highlights this species' recent expanding range across various Mediterranean countries. Better monitoring of this species at European level has to be carried out as a precautionary measure to ensure minimal impact on native biodiversity, including that to other small fossorial European herpetofauna, such as the Eurasian blindsnake, *Xerotyphlops vermicularis* (Merrem, 1820); the Iberian worm lizard, *Blanus cinereus* (Vandelli, 1797); and the Maria's worm lizard, *Blanus mariaae* Albert & Fernández, 2009 (Speybroeck et al., 2010). A better control strategy on exotic species importations should be in place to prevent costly eradication management of invasive species. Application of risk analysis on any potential bio-invasion associated with pet, crop, plant, tree species importation and their associated pathogens and pests has been increasingly advocated due to globalisation of trade in biological resources. For this purpose, in Malta, the National Biodiversity Strategy and Action Plan (NBSAP) 2012-2020 highlights four measures (BI1 to BI4) which are aimed to prevent the introduction of alien species that may be invasive and also have early detection and control mechanisms in place to control their invasiveness (ERA, 2012). However, due to its habitat preferences and also its reproductive biology, extra care must be taken to detect the Brahminy blindsnake which can easily go unnoticed and establish populations wherever it is unintentionally transported to.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethical approval:** For this type of study formal consent is not required.



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