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Uncover the underground : distribution and population status of blind snakes and Bogadek's burrowing lizards

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UNCOVER THE UNDERGROUND: DISTRIBUTION AND POPULATION STATUS OF BLIND SNAKES AND BOGADEK'S BURROWING LIZARDS

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UNCOVER THE UNDERGROUND:
DISTRIBUTION AND POPULATION STATUS OF
BLIND SNAKES AND BOGADEK'S BURROWING LIZARDS

by
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ABSTRACT

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by

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Globally, 28% of the reptiles are fossorial. Fossorial reptiles, which perform important ecological functions, such as being ecosystem engineers, top predators and bioindicators, are indispensable members of the soil ecosystem. However, the ecology of fossorial reptiles is relatively little known because of their cryptic behavior and rarity. In Hong Kong, there are four species of fossorial reptile, three of which are rare and of potential conservation concern. Among the four fossorial reptile species that occur in Hong Kong, *Indotyphlops lazelli* and *Dibamus bogadeki*, are endemic to Hong Kong and are globally threatened species. On the other hand, the distribution of *Indotyphlops albiceps* is disjunct with no record between Myanmar and Hong Kong, necessitating a study to clarify the taxonomy status of different populations. Besides anecdotal sighting records, no systematic ecological studies have been conducted on fossorial reptiles—basic information, including distribution, population status and basic ecology, is largely lacking.

In view of the conservation concern of fossorial reptiles, existence of knowledge gap and potential threats, a study on the distribution and population status of local fossorial reptiles is essential. In this study, firstly, systematic field surveys using three sampling techniques (quadrat search, artificial refuges and opportunistic search) were conducted in four sites (Pok Fu Lam, Lung Fu Shan, Lady Clementi's Ride and Sunshine Island) to evaluate the effectiveness of sampling methods for detecting fossorial reptiles. Only *Indotyphlops braminus* and *I. albiceps* were detected in the field surveys. I found that artificial refuge is suitable for longer-term study with sufficient manpower, while active search is cost-effective method for detection of fossorial reptiles and suitable for both long-term and short-term studies.

The encounter correlates significantly with a set of environmental factors, including ambient humidity, gradient and canopy cover. Secondly, phylogenetic analysis was carried out to clarify the taxonomic status and phylogenetic relationship among the three target blind snake species. My results consolidated that *I. lazelli* is a genetically valid species, and suggested that *I. albiceps* is a potential new species.

This study provides insights into optimising surveys on fossorial reptiles in ecological impact assessment and ecological studies, advices to inform conservation actions for the threatened species (including *D. bogadeki* and *I. lazelli*), and suggestions on further studies to fill the vast knowledge gaps of the ecology of fossorial reptiles.

DECLARATION

I declare that this is an original work based primarily on my own research, and I warrant that all citations of previous research, published or unpublished, have been duly acknowledged.

SIGNED

(CHAN Man Ho)

Date: 5 th September, 2023.

CERTIFICATE OF APPROVAL OF THESIS

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER 1 - INTRODUCTION

1.1 Introduction of fossorial reptiles

Among the 1.5 million described living species, around 23% are soil animals (Decaëns et al., 2006). Soil-dwelling animals can be categorised into two major groups, depending on how the organisms utilise soil. The first group refers to species that are primarily epigeous, using soil, leaf litter and wood as refuges, while the second group consists of strictly fossorial species that spend most of their lifetime underground with burrowing ability (Martín et al., 2021b; Measey, 2006; Wu et al., 2015). For reptiles, 28% (2209 out of 7879) are fossorial (Measey, 2006). To adapt to the underground environment, fossorial reptiles often share similar morphological features, such as rudimentary eyes, scale modification for streamlining, small skull, elongated bodies, absence of limbs and reduced sensory system (Foureaux et al., 2010; Jackson & Reno, 1975; Lee, 1998; Maritz & Alexander, 2009; Navas et al., 2004; Roscito & Rodrigues, 2010; Webb et al., 2000).

1.2 Evolutionary history

Fossorial reptiles include members from the Order Squamata (snakes and lizards) (Measey, 2006). Among reptile group, snakes have the highest diversity (>4000 extant species), which is divided into two infraorders Scolecophidia and Alethinophidia (Integrated Taxonomic Information System, 2022; Uetz & Hallermann, 2022). The members of Scolecophidia are commonly known as "worm snakes", all are fossorial species with small body size and reduced eyes (Vidal et al., 2010). They often have a limited gape size, feeding mainly on small prey, such as ants and termites (Cundall & Greene, 2000; Vidal & David, 2004). The members of

Alethinophidia refers to “typical snakes” (Cundall & Greene, 2000; Vidal & David, 2004), having enlarged gape (macrostomate) that allow them to swallow large prey (Moon et al., 2019). However, some members of Alethinophidia, namely Families Aniliidae and the Uropeltida, who lack “macrostomate”, are fossorial species as well (Miralles et al., 2018).

Scolecophidia contains 457 species that are distributed on all continents except Antarctica (Uetz & Hallermann, 2022). In general, their evolutionary and dispersal history are driven by continental drift and ancient ocean currents (Marin et al., 2013a). Originating from Gondwana, scolecophidians are traditionally divided into three families: Anomalepididae, Leptotyphlopidae and Typhlopidae, with different distributions (Adalsteinsson et al., 2009; Vidal et al., 2010). For Typhlopidae (blind snakes), Vidal et al. (2010) proposed that Typhlopidae originated from East Gondwana and the group was further divided into Gerrhopilidae, Xenotyphlopidae and Typhlopidae. The five clades initially diverged in the Middle Jurassic age (~150 mega annum [Ma]), paralleling the radiation of their major food sources (ants and termites) (Vidal et al., 2010). However, with the discovery of the oldest fossil blind snake, *Boipeba tayasuensis* (Fachini et al., 2020), and molecular evidence (Pyron & Wallach, 2014), other scientists suggested that Typhlopoidea may have a West Gondwana origin.

The discovery of fossil, combined with findings from molecular studies, provides important information to reconstruct the trait evolution for fossorial lifestyles and trace the burrowing origin of reptiles (Ebel et al., 2020; Miralles et al., 2018). Extant blind snakes are much smaller than their ancestors, and this may be attributed to the selection pressure during the Cretaceous-

Paleogene (K/Pg) extinction event, which favoured small-sized, cryptic animals (Fachini et al., 2020). Environmental conditions, such as flooding, could induce isolation that promoted allopatric speciation (Tiatragul et al., 2022). However, after dispersal to a new environment, the extreme conditions, in which organisms are restricted to a number of adaptations, may impose stabilising selection on the morphological traits of fossorial reptiles (Nevo, 2001). Therefore, in spite of their wide distributions and diversity, blind snakes across the world still possess similar morphological traits that many species complexes are yet to be investigated and resolved. Hopefully, with advancement in methods in molecular study, cryptic species of blind snakes have been discovered from time to time (Ellis et al., 2017; Giokas et al., 2011; Graboski et al., 2022; Hedges & Thomas, 1991; Wallach, 1999).

In conclusion, recent studies combining the application of molecular tools, paleontological evidence and morphological evidence have provided new insight to the evolutionary history of fossorial reptiles and proved the fossorial origin of snakes, resolving taxonomic questions and shedding light on the evolutionary mechanism of both extinct and extant species.

1.3 Ecological importance

Soil animals play an important role in maintaining a healthy soil ecosystem because they influence important ecosystem processes, including nutrient cycling, water infiltration and energy flow (Decaëns et al., 2006). The 3D structure of the soil environment and the presence of different microclimates facilitates resource partitioning among species, reducing competition (Decaëns et al., 2006). Ecosystem engineering activities performed by soil animals, which involve the construction and maintenance of the complexity of habitats, contribute to the enhancement of soil biodiversity (Decaëns et al., 2008; Wolters, 2001). Soil animals also highly interact through intra- and inter-trophic interactions. Fossorial reptiles are top predators in some soil ecosystems and can serve as bioindicators in monitoring the changes in environmental conditions (Decaëns et al., 2006). For instance, a study on habitat rehabilitation success showed that *Simoselaps bertholdi*, a fossorial snake that feeds on small lizards, reflects the recovery process of the ecosystems as high trophic level carnivores colonise rehabilitated area at a late and mature succession stage (Thompson & Thompson, 2005). Another example is *Chalcides ragazzii*, a fossorial skink that can be used as a bioindicator for monitoring pesticide contamination in Africa (Lambert, 1997). A study used the level of faecal metabolites of a worm lizard, *Trogonophis wiegmanni*, to examine soil pollution by heavy metals (Martín et al., 2021a). Furthermore, researchers observed that fossorial blind snakes exhibit commensalism and positively contribute to the growth rate and survival rate of owls because blind snakes prey upon insect larvae that are parasites of owl nestlings (Gehlbach & Baldridge, 1987). Despite the significant contribution of soil animals to terrestrial biodiversity and the general agreement on ecological importance of the soil community, including fossorial reptiles, our understanding of the ecology of soil animals is still largely lacking when compared with other groups of organisms (Copley, 2000; Wolters, 2001).

1.4 Conservation status of fossorial reptiles

Globally, 21.1% of reptile species are on the brink of extinction (Cox et al., 2022), under the threat of global climate change, habitat loss and degradation, invasive species and overexploitation (Böhm et al., 2013). A study showed that fossorial species are most impacted and sensitive to habitat degradation when compared with terrestrial species and arboreal species (Theisinger & Ratianarivo, 2015). As fossorial reptiles are often characterised by a narrow distribution range, niche specificity, sedentary habit and cryptic morphology (Lacoursière-Roussel et al., 2016), they are vulnerable to any anthropogenic pressure and environmental changes, particularly changes in soil conditions and landscape structure (Böhm et al., 2013; Hager, 1998; Henderson et al., 2016).

Although the proportion of threatened species is not high in reptiles (21.1%) compared to some other groups, such as amphibians (40.7%) (Cox et al., 2022), the actual proportion of threatened species may be higher because 55% of all described species have yet to be assessed (Bland & Böhm, 2016; Tingley et al., 2016). Among the species that have been assessed, 20% of all species are categorised as Data Deficient (Tingley et al., 2016). The situation is even more pronounced for fossorial reptiles, with 47% of species are classified as Data Deficient (Böhm et al., 2013). According to a study on the conservation status of the world's reptiles, out of 1500 randomly sampled reptile species, 49% of Typhlopidae species (blind snakes), 40% of Leptotyphlopidae species (thread snakes) and 38% of Uropeltidae species (shield-tailed snakes) were listed as "Data Deficient" under the IUCN Red List (Böhm et al., 2013). The challenges in assessing the conservation status of fossorial reptiles are attributed to the lack of ecological and population studies. Fossorial reptiles are difficult to study for three reasons (Maritz & Alexander, 2009; Measey, 2006). Firstly, they are small-sized, and have cryptic, secretive behaviour, making sampling very difficult (Measey, 2006; Measey et al., 2009). Some species are capable of burrowing deep underground and can move rapidly in the soil, making surveying or collecting these animals particularly challenging (Maritz &

Alexander, 2009). Secondly, there have been few studies evaluating survey techniques for sampling fossorial reptiles and the most commonly used methods are not cost-effective (Measey, 2006). For instance, excavation is the most common technique to be deployed, but it requires intensive effort and cause great disturbance to the habitat (Maritz & Alexander, 2009). Thirdly, currently available information is limited to data on morphology or diet, as fossorial fauna are often collected as voucher specimens (Maritz & Alexander, 2009). Altogether, the knowledge gap on fossorial reptiles requires urgent research attention from scientists and conservationists (Böhm et al., 2013). In particular, ecological studies to evaluate sampling methods for fossorial reptiles are necessary, especially for threatened species.

1.5 Fossorial reptiles in Hong Kong, their threats and conservation gap

Despite its relatively small size (0.01% of mainland China), Hong Kong is home to a high diversity of reptiles, accounting for about one-fifth of the native reptile species found in China (Agriculture, Fisheries and Conservation Department [AFCD], 2022g; Hau et al., 2005). Of the 90 species recorded by the Agriculture, Fisheries and Conservation Department (AFCD), 25 species are lizards, 53 species are snakes and 12 species are turtles (AFCD, 2022g). These reptile species thrive in a diversity of habitats, for example woodlands, streams, grasslands, and mangroves (AFCD, 2022g). In this study, we focus on the four fossorial reptile species that occur in Hong Kong: *Dibamus bogadeki*, *Indotyphlops albiceps*, *Indotyphlops lazelli* and *Indotyphlops braminus*. I detail our understanding and knowledge gaps of each target species below.

1.5.1 Dibamus bogadeki

Globally, there are 25 species in the family Dibamidae (Uetz & Hallermann, 2022). *Dibamus bogadeki* (Bogadek's Burrowing Lizard), is the only Dibamid species found in Hong Kong, which was named after Fr. Anthony Bogadek, who discovered the first specimen of the species (Lazell & Lu, 1990). The first specimen (MCZ 172041) was collected in 1987 in Hei Ling Chau and was sent to the Australian Museum for identification (Lazell & Lu, 1990). The specimen was first considered to be *Dibamus bourreti*, which is a species that occurs in Guangxi and Hunan Provinces of China (Darevsky, 1992; Lazell & Lu, 1990). Subsequently, the Hong Kong specimen was described as a new species, in view of the differences in the incompleteness of nasal sutures and the ash-white tail (Darevsky, 1992; Lazell & Lu, 1990).

The global distribution of this species is extremely small, including only three outlying islands in Hong Kong, Hei Ling Chau, Shek Kwu Chau and Sunshine Island (Chan et al., 2012). Since 2002, regular ecological surveys, using active search, pitfall traps and coverboards, have been conducted by The Herpetofauna Working Group of AFCD (Chan et al., 2012). Populations of this lizard are potentially small because there were only few individuals detected, with only seven specimens were collected between 1987 and 2011. In addition to the small number of sightings, no individual has been detected in Hei Ling Chau for 35 years and in Shek Kwu Chau for 10 years. In recent years, surveys were mainly conducted on Sunshine Island. As such, *D. bogadeki* is listed as Endangered under the IUCN Red List (Yang, 2019).

Little is known about the ecology of *D. bogadeki*. From previous sightings, this species may be nocturnal, prefer moist forest habitats and tall shrubland and is often found in soil, under dead logs or under rocks (Chan et al., 2012; Yang, 2019). Also, AFCD studied their behaviour in captivity and found that the species feeds on termites and tiny arthropods and stay below the soil surface most of the time (Chan et al., 2012; Yang, 2019).

Despite the poor understanding of ecology and population, ecological studies on *D. bogadeki* are urgently needed because it may soon be threatened by development. There has been great urbanisation pressure to provide more residential areas in Hong Kong ("Govt gives reassurances on land," 2022). A large-scale development project, Lantau Tomorrow Vision, was announced in 2018, consisting of reclamation works to build large artificial islands in Central Waters to provide more residential areas in Hong Kong (Civil Engineering and Development Department [CEDD], 2021) (Figure 1.1). The reclamation covers about 1,000 hectares around Kau Yi Chau, adjacent to Sunshine Island and Hei Ling Chau. Although the proposed formation of artificial islands will not directly encroach on the adjacent existing islands, the reclamation work is less than 500 m from the islands (CEDD, 2021; Environmental Protection Department [EPD], 2021). Several environmental groups have raised concerns about the short and long-term irreversible impacts on the marine and terrestrial ecosystems and threatened species, including sea pen, coral community, Chinese White Dolphin (*Sousa chinensis*), Indo-Pacific Finless Porpoises (*Neophocaena phocaenoides*), White-bellied Sea Eagle (*Haliaeetus leucogaster*) and Bogadek's Burrowing Lizard (*Dibamus bogadeki*) (Green Power, 2021; Hong Kong Bird Watching Society, 2019; The Conservancy Association, 2021; The Green Earth, 2020; WWF-Hong Kong, 2019).

The species living on or surrounding Sunshine Island could be impacted by the development project directly and indirectly. First, before the announcement of the project, Sunshine Island was considered to be “not under immediate development pressure in view of its remoteness” (Planning Department [PlanD], 2015); however, with the commencement of adjacent, large-scale development, development pressure is likely to increase on Sunshine Island. Although Sunshine Island has been designated as a Sites of Special Scientific Interest (SSSIs) owing to the distribution of *D. bogadeki*, it is not mapped out in the statutory zoning plan by the Planning Department (PlanD, 2022), inferring that development is neither prohibited nor regulated.

Figure 1.1 Map of the conceptual location of the proposed Kau Yi Chau artificial island, other possible reclamation area and infrastructures in Central Waters under Lantau Tomorrow Vision, and sites with *Dibamus bogadeki* recorded.

Reference: Modified from Development Bureau, 2019

Second, the proximity to highly developed areas, i.e. the artificial islands, may promote accessibility to Sunshine Island. Increased accessibility will increase human disturbance, such as minor works, excavation and removal of ruins or vegetation, which may potentially alter the microhabitats of the fossorial reptiles. Human disturbance may also threaten *D. bogadeki* through introduction of invasive species, especially Red Imported Fire Ant (*Solenopsis invicta*), one of the world's 100 worst invasive species (Lowe et al., 2000). The adverse impacts of fire ants on reptiles have been documented in previous studies (Allen et al., 1994; Allen et al., 2004; Allen et al., 2017; Tuberville et al., 2000). To my knowledge, there is no record of fire ants on Sunshine Island up to now, but the potential invasion of fire ant on Sunshine Island may devastatingly impact the abundance, distribution or even survival of soil animals, including *D. bogadeki*.

Due to the potential threats ahead, a baseline study on the biology and population status of *D. bogadeki* is urgently required. Without sufficient knowledge and understanding on *D. bogadeki*, it would be difficult to evaluate the impacts of this large-scale development project on the species, and to formulate effective conservation measures to monitor the species or mitigate the impacts of the development project during the pre-, peri- and post-construction periods (CEDD, 2021; EPD, 1997; EPD, 2021). Enhanced ecological information is a prerequisite to informing conservation decisions and actions, including designation of protected areas for translocation and to restrict human access. Specifically, translocation is based on an in-depth understanding of the target species' ontogeny, ecology and habitat preference. The endemic Romer's Tree Frog (*Liuixalus romeri*) is a successful case in Hong Kong (Banks et al., 2008), but very few rescue operations were intended for fossorial reptiles globally, one of which was the Slow Worm (*Anguis fragilis*) in the United Kingdom. Unfortunately, the recaptured individuals in this case revealed a depressed population, reproductive fitness and fecundity (Platenberg & Griffiths, 1999), reflecting that the effectiveness of translocation in conserving a species is doubtful. As translocation should be the last resort when formulating mitigation measures (EPD, 1997), it is important to obtain sufficient information beforehand that can guide us in making the most appropriate conservation decisions.

In this study, I conducted intensive surveys to reveal population status of *D. bogadeki*. Further analysis was carried out to compare the effectiveness of different sampling methods for the species. The results reveal the potential impacts of this potential large-scale development project on *D. bogadeki*. I suggest corresponding conservation actions to minimise and mitigate the negative ecological impacts.

1.5.2 *Indotyphlops albiceps*

Indotyphlops albiceps (White-headed Blind Snake), is a fossorial species native to southeast Asia, including Peninsular Malaysia, Myanmar and Thailand (Neang et al., 2017; Taylor, 1965; Wogan et al., 2018). A study in 1993 reported the occurrence of *I. albiceps* in Singapore, which was subsequently considered as an error (Wallach & Pauwel, 2004; Zhao & Adler, 1993). In Hong Kong, the first potential specimen of *I. albiceps* (BMNH 1954.1.13.4) was reported by J. D. Romer in 1952 on Caine Road, Hong Kong Island. In 1966, the second specimen (BMNH 1983.946) was collected in Caroline Hill Road, Hong Kong (Lazell & Lu, 1990; Romer, 1970; Wallach & Pauwel, 2004). Some scientists have questioned the existence of *I. albiceps* in

Hong Kong due to the morphological difference (number of mid-dorsal scales) with specimens from Southeast Asia and disjunct distribution (no records in areas between Hong Kong and Myanmar, e.g. mainland China) (Hahn, 1980; Taylor, 1965). Subsequently, Wallach (1998) examined more specimens and confirmed the species validity of *I. albiceps* specimens collected from Hong Kong. To date, this species is only found in Hong Kong but nowhere else in mainland China (Wogan et al., 2018). Given the disjunct distribution (Hong Kong population is distant from other populations in southeast Asia), the taxonomic status of the Hong Kong population needs to be re-evaluated.

With a wide global distribution in Southeast Asia, *I. albiceps* is classified as Least Concern under the IUCN Red List (Wogan et al., 2018). However, with insufficient information of the population status in Hong Kong (the only distribution site in China), the species was listed as Data Deficient under the China Biodiversity Red List (Jiang et al., 2016) and was categorised as a species with regional concern by Fellowes et al. (2002). In Hong Kong, the species is rare (Wogan et al., 2018), with most records on Hong Kong Island, including Mount High West, Chung Hom Kok, and Pok Fu Lam (Karsen et al., 1998; Lazell & Lu, 1990; Wallach & Pauwel, 2004; School of Biological Sciences, HKU, 2012). Recently, there were potential sightings of *I. albiceps* recorded in Kowloon and central New Territories (Duncan Cheung, personal communication, April 29, 2022; Ray So, personal communication, n.d.). Apart from clarifying the taxonomy of different populations, studies are needed to investigate the distribution and population status of *I. albiceps* in Hong Kong.

1.5.3 Indotyphlops lazelli

Two blind snake specimens collected in 1988 and 1992 were originally identified as *I. albiceps* and subsequently described as a new species *Indotyphlops lazelli* (Hong Kong Blind Snake) (Wallach & Pauwel, 2004). The distinctive features of *Indotyphlop lazelli* include no enlarged occipitals and a single-chambered tracheal lung (Wallach & Pauwel, 2004). *Indotyphlops lazelli* could be distinguished from *I. albiceps* by a combination of morphological differences: 1) presence of 18 midbody scale rows, 2) a different supralabial imbrication pattern and 3) only snout, chin and throat are white (Wallach, 1993; Wallach & Pauwel, 2004). Other major morphological differences among *I. albiceps*, *I. braminus* and *I. lazelli* are summarised in Table 1.1.

Indotyphlops lazelli is a species endemic to Hong Kong, with only two specimens collected from Pok Fu Lam and the campus of University of Hong Kong (Wallach & Pauwel, 2004). Of the two sites where *I. lazelli* was found, one site has been developed, and the remaining forests in the surroundings of this site are under development pressure (Lau, 2012). In view of the scarcity of records, *I. lazelli* was listed as Critically Endangered in both the IUCN Red List and China Biodiversity Red List (Jiang et al., 2016; Lau, 2012). With only two records for this species, information on its ecology and population status is lacking; therefore, intensive ecology surveys should be conducted to acquire this baseline information. Besides, genetic analysis should be conducted simultaneously to evaluate its taxonomic status and phylogenetic relationship with *I. albiceps* and *I. braminus*.

Table 1.1 Scutellation data for *Indotyphlops albiceps*, *I. braminus* and *I. lazelli*.

SIP: supralabial imbrication pattern; MSR: midbody scale rows; TMD: total middorsals scales; SC: subcaudal scales; LOA: total length (mm); L/W: total length/midbody diameter ratio and PTO: number of postoculars. References: Wallach, 2009; Wallach & Pauwel, 2004.

	Scutellation variables						
	SIP	MSR	TMD	SC	LOA	L/W	PTO
I. braminus	T-III	20	261–368	8–15	35–203	30–60	1
I. albiceps	T-III	20	301–424	8–25	117–302	39–104	2 (3–4)
I. lazelli	T-V	18	409–427	9–10	92–158	77–83	1

1.5.4 Indotyphlops braminus

Originating from southern India, *I. braminus* (Common Blind Snake) has been referred to as a "Flowerpot Snake" due to its worldwide distribution in almost 118 countries, resulting from the indirect transportation of potted plants (Bamford & Prendergast, 2017; Global Invasive Species Database, 2022; Shea et al., 2021). *Indotypholops braminus* inhabits a wide range of habitats, including disturbed areas and areas with human habitation, such as gardens, leaf litter and decaying woods (Broadley & Wallach, 2009). *Indotyphlops braminus* is primarily fossorial and it is unusual to spot them coming up to the surface during the daytime; however, they are often found foraging or moving along the surface after rains when the soil becomes waterlogged (Paolino et al., 2019; Wallach, 2020). Its diet consists mainly of ants and termites, particularly their larvae, pupae and eggs (Bamford & Prendergast, 2017; Wallach, 2009). A study recorded that *I. braminus* demonstrates a special feeding behaviour of decapitating termite prey to remove the indigestible parts (Mizuno & Kojima, 2015). Although *I. braminus* is not of conservation concern (Shea et al., 2021), I included *I. braminus* as one of the target species in this study because of their higher abundance and potentially similar ecology with other blind snakes, including *I. albiceps* and *I. lazelli*.

1.6 Survey methods for sampling fossorial reptiles

To sample fossorial reptiles, active and passive techniques can be used; active search is to search for fossorial reptiles through turning over natural or artificial objects, such as dead logs, rocks and coverboards, or through digging soil or leaf litter; while passive search involves the deployment of pitfall traps and drift fences (Henderson et al., 2016).

During the process of selecting and designing survey methods for target fossorial species, we need to consider five major factors. Firstly, compared with qualitative surveys to confirm the existence of a certain species, quantitative surveys that aims at investigating the density or population status, requires a higher level of prior knowledge and experience of the researcher to select study sites with previous records of the target species or sites with the most suitable habitats for the target species (Measey, 2006; Maritz & Alexander, 2009).

Secondly, the level of disturbance to the target species or the surrounding environment should be taken into consideration. For example, a quantitative study on fossorial herpetofauna in South Africa made use of earth-moving machinery for sampling. Although this innovative method created an opportunity for researchers to dig deeper into soils and resolve the problem of escaping individuals, it had a high ecological footprint (Maritz & Alexander, 2009). Another study on the density of legless lizards compared the effectiveness of two time-constrained searches of different impacts to the environment (low-impact: observe the surface or under objects within a specific area without causing disturbance to vegetation, moderate-impact: apply hand tools to excavate and remove surface vegetation beforehand) (Kuhnz et al., 2005). Although the detection rate of moderate-impact searches was twice that of low-impact searches, excavation is not preferred when conducting surveys in sites with sensitive and fragile habitats (Kuhnz et al., 2005). Therefore, it is crucial for researchers to strike a balance between the efficacy of detecting target species and the degree of disturbance to the environment.

Thirdly, the time frame of the survey period or availability of time could affect the choice of methods. Kuhnz et al. (2005) suggested that coverboards are not suitable for short-term study on population status. Goodman & Carter (2017) shared similar views by summarising studies using coverboards for sampling herpetofauna. They found that the encounter rate ranges from 0.04% to 38.4% with 1,122 to 51,006 coverboard checks, so increasing the number of checks may give a more reliable result. Furthermore, a local Hong Kong study on sampling terrestrial herpetofauna recommended the use of transect surveys when time is limited and the use of both transect surveys and pitfalls traps if time and manpower allow (Sung et al., 2011).

Fourthly, in relation to the biology of the target species, the choice and design of method could influence the encounter rate. For example, with advances in technology, camera traps allow researchers to collect data on rare species, including fossorial snakes (Neuharth et al., 2020; Ryberg et al., 2021); however, this technique is still unable to detect blind snakes with relatively small size (snout–vent length <50 mm) (Dundas et al., 2019). On the other hand, pitfall traps are relatively more effective in capturing small-sized and fossorial species than large to medium-sized species (Enge, 2001). The encounter rate could be further enhanced with explicit design with reference to previous studies that researchers compared the effectiveness of pitfall traps with different sizes in achieving a higher species richness (Friend et al., 1989; Maritz et al., 2007; Ribeiro-Júnior et al., 2011).

Fifthly, the financial budget, including cost of equipment, personnel and travelling, may put a constraint on the selection of methods. Garden et al. (2007) demonstrated a variation in cost effectiveness among methods, and found that pit-fall trap, which detected mostly fossorial reptiles, and direct observations were the most cost-effective methods. Given limited resources and project funds, researchers could carefully calculate both the cost and effort required by the methods in order to achieve the highest detection rate with the lowest cost.

In short, no method or technique can be applied to all fossorial species and different methods have their own strengths and weaknesses. Only an optimal survey method, or a combination of methods, specifically for sampling the target species could be chosen after considering all these aspects and possessing a fundamental knowledge on the species' biology. Still, it is critical to standardise survey methods for a group of target species or a type of habitat in order to make results comparable for proper monitoring of population status (Doan, 2003; Measey, 2006).

In this study, I conducted extensive surveys to evaluate the effectiveness of three survey methods for sampling target fossorial reptiles by examining the encounter rates, aiming to provide useful information that could guide future research on fossorial reptiles in Hong Kong, including environmental impact assessment.

1.7 Research objectives

In light of the underappreciated importance of fossorial reptiles, existence of knowledge gap and conservation concern, a study on the distribution and population status in Hong Kong is required to provide useful information for formulation of conservation actions to prevent these rare and species from extinction. This study focuses on four fossorial species - *Dibamus bogadeki*, *Indotyphlops albiceps*, *Indotyphlops lazelli* and *Indotyphlops braminus*. The following research objectives are proposed:

1. To evaluate the effectiveness of three sampling methods (quadrat search, artificial refuges, opportunistic search) for target species and to investigate the environmental factors affecting the encounter rate through conducting intensive surveys regularly in four key study sites
2. To clarify the taxonomic status of *I. albiceps*, *I. lazelli* and *I. braminus* through phylogenetic analysis
3. To suggest conservations actions and further studies for *I. albiceps*, *I. lazelli* and *D. bogadeki*, and to provide recommendations on optimising ecological surveys in environmental impact assessment

CHAPTER 2 - FIELD SAMPLING TECHNIQUES AND POPULATION STATUS OF LOCAL FOSSORIAL REPTILES

2.1 Abstract

Hong Kong is home to four fossorial reptiles, *Dibamus bogadeki, Indotyphlops albiceps, Indotyphlops lazelli, and Indotyphlops braminus*. These four species are understudied and little is known about their ecology and population status. Due to their restricted distributions and potential threats from development, three of them (*D. bogadeki, I. albiceps, I. lazelli*) are of conservation concern that an urgent ecological study is needed. I conducted intensive field surveys in four key study sites with historic records of these species to examine the effectiveness of three survey methods for sampling fossorial reptiles in Hong Kong, and to evaluate the effectiveness of artificial refuges of different materials and sizes. I also investigated the correlation between encounter and environmental parameters. Only *I. albicep and I. braminus* were detected in this study. Pok Fu Lam and Sunshine Island shared the highest number of encounters. Although artificial refuges detected the highest number of individuals, active search (quadrat search and opportunistic search) was more cost-effective when sampling effort was taken into account. As the efficiency of artificial refuge increased over time, I recommend the use of active search for short-term study and a combined use of artificial refuge and active search for long-term study (>1 year). For artificial refuge of different sizes and materials, more individuals were detected under larger plywoods. Significant correlations between encounter number and environment parameters (ambient humidity, gradient and canopy cover) were revealed. The results of this study provide information which guide future ecological surveys on fossorial reptiles, for example environmental impact assessment and systematic ecological studies.

2.2 Introduction

Selection of effective sampling methods is crucial for ecological studies. To sample herpetofauna, a wide range of sampling methods have been developed, for example pitfall traps, active search, artificial refuges, drift fences and acoustic surveys (Henderson et al., 2016; McDadeh & Maguire, 2005; Mitchell et al., 1993; Ribeiro-Júnior et al., 2008; Ryan et al., 2022). A number of studies have evaluated the effectiveness of these methods based on cost (money, time and manpower), and resulting species richness and composition. The effectiveness of these sampling methods is affected by many factors, for example, target organisms, habitat types and weather variables (temperature, humidity and precipitation) (Doan, 2003; Garden et al., 2007; Greenberg et al., 1994; Hampton, 2007; Hutchens & DePerno, 2009; Kuhnz et al., 2005; Ribeiro-Junior et al., 2008; Rödel & Ernst, 2004; Ryan et al., 2002; Sung et al., 2011; Todd et al., 2007; Williams & Berkson, 2004; Vogt & Hine, 1982). In general, studies have shown that there is no single survey method suitable for sampling all herpetofauna within a particular habitat (Doan, 2013; Ryan et al., 2002; Sewell et al., 2013). As such, evaluation of sampling methods for different groups of herpetofauna in different habitats/regions is important.

To sample fossorial reptiles, active search, artificial refuges and passive below-ground trapping are commonly employed (Henderson et al., 2016). Active search through transect walk is an effective method to investigate species abundance and richness, particularly for short-term studies (Crosswhite et al., 1999; Doan, 2003; Rödel & Ernst, 2004; Sung et al., 2011). However, the effectiveness of this method may sometimes be limited by researchers' knowledge and experience to select the suitable habitats for the surveys (Crosswhite et al., 1999; Henderson et al., 2016). Passive below-ground trapping is a standardised method which is often used to capture rare species (Enge, 2001; Friend et al, 1989; Sung et al., 2011). It is particularly useful in population studies for semi-fossorial reptiles (Greenberg et al., 1994; Harikrishnan et al., 2012). However, it is time-consuming and labour-intensive that requires extra time for set up and maintenance (Sung et al., 2011; Todd et al., 2007; Welbourne et al., 2020). Among these three methods, artificial refuges are the most frequently used methods in studies on fossorial reptiles for two reasons. First, some studies showed that artificial refuges are effective in detecting fossorial reptiles (Ribeiro-Junior et al., 2008). Second, artificial refuges allow a more standardised sampling, with similar number, size and materials of artificial refuges employed, which can reduce sampling variability and is important for studies comparing diversity or abundance between sites (Grant et al., 1992). However, artificial refuges require more effort for preparation and maintenance compared to other methods (e.g. active search) (Michael et al., 2012; Michael et al., 2019).

To optimise the effectiveness of artificial refuges in sampling fossorial reptiles, the selection of material and size are crucial. Wood is commonly used because it is a good heat insulator that protects animals from excess heat. Also, wood absorbs water which can provide a moist microhabitat (Grant et al., 1992; Lamb et al., 1998). Some studies chose tin as artificial refuge material (Hesed, 2012; Hutchens & Deperno, 2009; Michael et al., 2012), as tin provides warmth/heat to animals given its high heat conductive rate (Tomasek et al., 2005). Other materials, such as clay and ceramic, represents potential artificial refuge materials because they also provide preferable (warmth and moist) microhabitats to fossorial reptiles and are more durable than wood (Basak et al., 2012; Bocanegra et al., 2019; Plagge & Teutsch, 2020).

However, only very few studies used clay as artificial refuges (Thompson, 2006), and none used ceramics. For size, Hesed (2012) showed that larger (1.2 × 0.6 m) wooden boards provide more preferable soil conditions to fossorial animals than smaller (0.1 × 0.1 m) wooden boards. However, larger boards are more challenging to install.

In Hong Kong, there are four species of fossorial reptiles, the Common Blind Snake (*I. braminus*), White-headed Blind Snake (*I. albiceps*), Hong Kong Blind Snake (*I. lazelli*) and Bogedak's Burrowing Lizard (*D. bogadeki)* (Karsen et al., 1998). Three of the four species (*D. bogadeki, I. albiceps, I. lazelli*) have restricted distribution and are rare which are of conservation concern (Lau, 2012; Wogan et al., 2018; Yang, 2019). The records of these species remain largely anecdotal. Sung et al. (2011) could not detect any fossorial reptiles using wooden coverboards. Also, three environmental impact assessment (EIA) studies could not detect the fossorial reptile species (*I. albicpes* and *D. bogadeki)* that occur in the project sites (AECOM, 2011; Capco, 2006; MTR Corporation Limited, 2010) (Table 2.1).

Given the rarity and conservation concern of the three fossorial reptile species, *I. albiceps*, *I. lazelli* and *D. bogadeki*, in this study, I aimed to optimise sampling methods for fossorial reptiles in Hong Kong. Specifically, I compared the effectiveness of sampling methods (including quadrat search and artificial refuges) for fossorial reptiles, and evaluated the effectiveness of artificial refuges of different materials and sizes. Further, I examined the correlation between environmental parameters and encounter rate of fossorial reptiles which provided insights on their habitat preference and detection.

Table 2.1 Comparison of methodology applied to designated projects under Environmental Impact Assessment Ordinance (Cap. 499) for sampling fossorial reptiles. References: AECOM (2011); Capco (2006); MTR Corporation Limited (2010)

	South Island Line (East)	**A Commercial Scale Wind Turbine Pilot Demonstration at Hei Ling Chau**	**Development of the Integrated Waste Management Facilities Phase 1**
Target species	*I. albiceps*	*D. bogadeki*	*D. bogadeki*
Survey methods	active search	active search, direct observation, coverboards	walk transect, active search, coverboards
Survey period	May–Dec 2008, Apr–Aug 2009	Aug–Oct 2005, Apr–May 2006, Nov–Mar 2006	Mar–Jul 2009, Oct 2009
Number of surveys conducted	17 × daytime, 4 × night	8 × daytime	7 × daytime, 2 × night
Result	No detection	No detection	No detection

2.3 Methods

2.3.1 Study sites

This study was conducted within Hong Kong Special Administrative Region (22°09'–22°37'N; 113°50'–114°30'E). Four field sites (Pok Fu Lam, Lung Fu Shan, Lady Clementi's Ride and Sunshine Island) were selected based on historic records of the three native fossorial reptile species (*I. albiceps*, *I. lazelli* and *D. bogadeki*) (Chan et al., 2012; Lazell & Lu, 1990; Macklin, 1988; Romer, 1970; Wallach & Pauwels, 2004) (Figure 2.1).

Figure 2.1 Historical record sites of the target fossorial species and field sites selected for this study.

All historical records of *I. albiceps* and *I. lazelli* occurred on Hong Kong Island, and three areas on Hong Kong Island were selected as study sites. In West Hong Kong Island, Lung Fu Shan covers 47 hectares of hilly terrain of elevations 150–260 m, protected within the country park area (AFCD, 2022d). Vegetation is grown with a mixture of exotic and native plants as a result of plantation and natural succession (Dudgeon & Corlett, 2004). Pok Fu Lam features a similar environment to Lung Fu Shan, with elevations from 160 to 494 m. Designated as Pok Fu Lam Country Park and one of the 67 Site of Special Scientific Interest (SSSI), the sanctuary covers 270 hectares of area (Chow, 1995). It consists of diverse habitat types, including, a sylvan reservoir, sloped woodland, a deeply elongated valley, and a few scattered barren lands with exposed rocks (AFCD, 2022f). Lady Clementi's Ride, with elevations from 80 to 120 m, is a trail meandering the foothill of Mount Nicholson (AFCD, 2022a; Kershaw & Thrash, 2005). The 8-hectare area consists of the only Fung Shui Wood on Hong Kong Island (aged 150 years) and was zoned as SSSI in 1993 because of the diverse plant diversity (Hong Kong Herbarium, 2022; PlanD, 2013).

The fourth site chosen for this study was Sunshine Island. Sunshine Island is one of the three islands where *D. bogadeki* is recorded, besides Hei Ling Chau and Shek Kwu Chau. In 1952, Sunshine Island was fractionally opened up for Chinese refugee resettlement and rehabilitation, as well as a small-scale community farming practice (Henschel, 1962). It was later transformed into a drug addiction treatment centre (PlanD, 2015). The infrastructure is to the north side of the Island, with a relatively large extent of natural terrestrial habitats remained, including woodlands and shrublands. Sunshine Island was designated as an SSSI in 2015 for the conservation of *D. bogadeki* (PlanD, 2015). From the information gathered from the only resident on the island (Mr C. N. Lam), the island used to sustain populations of introduced turtles (*Trachemys scripta elegans*), feral dogs and domestic goats (Kao, 2018). Other native reptiles, including *Python bivittatus*, *Ptyas mucosa*, *Trimeresurus albolabris*, *Naja atra* and *Plestiodon spp*., have also been encountered on the Island (C. N. Lam, personal communication, n.d.).

2.3.2 Fossorial reptile sampling

The four study sites were visited twice every month during two sampling periods, May–Dec 2021 and May–Nov 2022. The study periods encompassed the wet and warm season and the early dry and cool season (Dudgeon & Corlett, 2004). Visits at the same study sites were separated by at least seven days to minimise disturbance to the habitat. Surveys were conducted by one to four researchers. In each visit, I used three sampling techniques, including quadrat search, artificial refuges and opportunistic search, which are common methods for sampling fossorial reptiles (Henderson et al., 2016; Measey, 2006). I provide the details of the survey techniques below.

Method 1: Quadrat search

In each visit, two 100 m^2 (i.e. 10 × 10 m) quadrats were selected based on the following criteria: (1) the presence of either leaf litter forest bed or rock-type substrates, both of which offer microhabitats for fossorial reptiles; (2) the absence of dense understory vegetation and thickets and steep slopes for accessibility and ease of searching; and (3) the two selected quadrats in each visit were at least 10 m apart from each other.

Active search was done in each quadrat by searching potential microhabitats for fossorial reptiles, including under leaf litter, rocks and fallen logs. The duration of the search and number of objects turned over were recorded for each search. All overturned objects were restored to their original positions.

Method 2: Artificial refuges

I used coverboards made of three materials, plywood (Houze and Chandler, 2002; McDade & Maguire, 2005; Hutchens & Deperno, 2009), ceramic and clay (Thompson, 2006). Although previous study showed that boards as large as 1.2 × 0.6 m are more effective than smaller boards (0.1 × 0.1 m) (Hesed, 2012), it is very challenging to install due to limited man power. To optimise the effectiveness and man power, coverboards of size 0.3 × 0.3 m were used. Each coverboard is 1 cm thick. For plywood, we used untreated wood to avoid potential contamination of the environment by chemicals (Davis, 1997). To determine the effect of coverboard size on sampling efficiency, we also used plywood of size 0.45 × 0.45 m. In summary, there were four types of coverboards employed, including 0.3 × 0.3 m ceramic, 0.3 × 0.3 m clay, 0.3 × 0.3 m plywood and 0.45 × 0.45 m plywood.

In each study site, I installed 20 sets of coverboards, each set included the four types of coverboards. In each set, the coverboards were at least 0.1 m apart from each other. All sets of coverboards were separated at least 10 m from other sets (Figure 2.2). Coverboards were placed on a flat forest floor without dense herbaceous vegetation. The positions of the four types of coverboards were randomly assigned. Coverboards were allowed to acclimatise for at least a month before the sampling started (Gallegos, 2019; Grant et al., 1992; Henderson et al., 2016). In each survey, all coverboards were checked during which the soil underneath the coverboards was scanned and searched. Also, I recorded the time spent on turning over and checking coverboards to calculate sampling effort.

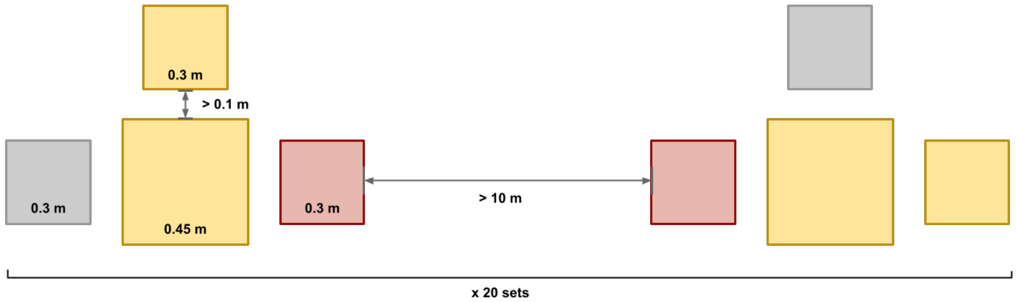

Figure 2.2 Diagram showing the arrangement of coverboards in the field. Colour indicates different materials: yellow: plywood, grey: clay and red: ceramic.

Method 3: Opportunistic search

To maximise detection of the target species, I also conduct opportunistic search in the study sites. I carried out the opportunistic search by searching suitable microhabitats for fossorial reptiles when travelling in the study sites but not restricted to sampling within a quadrat (compared to quadrat search). For each search, the duration and number of objects turned were recorded.

2.3.3 Environmental data sampling

Before sampling was done in each survey, ambient temperature and humidity were measured using a Kestrel 5000 Environmental Meter (Nielsen-Kellerman Co., Boothwyn, USA). In each quadrat, I measured the canopy cover using a spherical crown densiometer (Robert E. Lemmon, Forest Densiometers, Bartlesville, Oklahoma, USA). I counted the number of trees in two size categories, >10 cm and 5–10 cm diameter at breast height (DBH). The gradient of the quadrat area was measured by a clinometer (Suunto, Finland). Further, I randomly selected three points in each quadrat to measure soil temperature and soil moisture. I used an infrared thermometer (Model UT300C, Uni-Trend Technology, China) to measure soil temperature. For soil moisture, the soil at a depth of 0–5 cm was collected, and subsequently oven-dried in the laboratory. I calculated the soil moisture by subtracting its dry weight from its original weight. I estimated the substrate cover in the quadrat in six categories: rock, leaf litter, wood debris, plants (i.e. shallow roots and creeping stems), bare soil and other (e.g. concrete and waste). For each coverboard, I also measured ambient temperature, humidity, soil temperature, soil moisture and canopy cover using similar methods as for quadrat searching.

Besides the abiotic (environmental) factors, I also recorded biotic factors of the soil community during quadrat searching and checking of coverboards. I recorded the number of morphospecies and individual of soil animals in each quadrat and underneath each coverboard during sampling through direct observation on sites.

2.3.4 Morphological measurement

Upon detection of target species, i.e. three blind snake species (*I. braminus*, *I. albiceps*, *I. lazelli*) and one burrowing lizard (*D. bogadeki*), I measured their tail length (TL) (cm) and snout-vent length (SVL) (cm) by a ruler, and their weight (g) by a 10 g Light-Line Metric Spring Scale (Pesola 10010, Swiss). After measurement, they were released in situ.

2.3.5 Data analysis

To compare the effectiveness of different sampling methods, first, I calculated the time spent on different methods, including sampling and preparation (for both coverboards and quadrat search). The sampling time referred to the effort (man-hour) spent on checking coverboards or quadrat search. The preparation time included the effort spent on installing and maintaining coverboards and designating a quadrat. Then, the encounter rate was calculated from the number of individuals detected per unit effort and expressed as accumulation curves (individual encountered versus the effort and the number of objects turned in each method). I compared the number of encounter and mean encounter rates among study sites, and survey methods using a zero-inflated generalised linear mixed model. The tests were performed separately for each target species encountered in this study. To compare the effectiveness of different types of coverboards, I used a one-way repeated measures ANOVA to compare encounter rate.

To scrutinise the correlation between encounters of target species with environmental variables, I performed a preliminary screening of 23 abiotic and biotic variables using a test for multicollinearity as environmental variables are strongly correlated. I removed seven variables with a variance inflation factor (VIF) above 0.7 (Heikkinen et al. 2006). The VIF calculation was computed through stepwise and repeated procedures, each of which paired up the input variables with maximum linear correlation (greater than the threshold) and excluded the one with greater VIF. Given the limited number of encounters in this study, I then constructed zero-inflated generalised linear mixed models for each targeted species encountered with different combinations of factors and selected the most parsimonious models by comparing the Akaike information criterion (AIC) value. The best models were selected with the lowest AIC values (Burnham and Anderson, 2002).

All statistical analyses were conducted by IBM SPSS Statistics software (IBM Corp., Armonk, New York) or software R (RStudio Team, Boston, Massachusetts).

2.4 Results

Throughout the study, 328 surveys were conducted, with around 327.5 man-hours spent searching and 29,649 objects turned over (including coverboards and natural refuges) (Table 2.2).

Table 2.2 Overview of sampling effort of different survey methods.

	Survey method			
	Artificial Refuge	Quadrat Search	Opportunistic Search	TOTAL
Number of surveys conducted	91	163	74	328
Searching hour spent	142.4	111.9	73.2	327.5
Number of objects turned	7434	12390	9825	29649

Among the four target species, only two species, *I. albiceps* and *I. braminus*, were detected (Table 2.3). *Indotyphlops braminus* was the most common species found, accounting for 80% of all encounters. *Indotyphlops albiceps* was found in Lady Clementi's Ride and Pok Fu Lam, whereas *I. braminus* were found in Lady Clementi's Ride, Pok Fu Lam and Sunshine Island (Table 2.3). None of the target species was detected in Lung Fu Shan and this site was excluded from the subsequent analysis. Pok Fu Lam and Sunshine Island shared the same number of encounter (16). Regarding survey methods, highest number of encounters were recorded using artificial refuge (16), followed by opportunistic search (14) and quadrat search (12) (Table 2.3). In Lady Clementi's Ride and Sunshine Island, artificial refuge yielded the highest number of encounters of target species; while in Pok Fu Lam, quadrat search and opportunistic search yielded the same number of individuals.

Table 2.3 Total encounter number of target species using three survey methods in different study sites. Study sites: LCR: Lady Clementi's Ride, LFS: Lung Fu Shan, PFL: Pok Fu Lam, SI: Sunshine Island.

Target species	Survey method by site												
	Artificial Refuge				Quadrat Search				Opportunistic Search				
	LCR	LFS	PFL	SI	LCR	LFS	PFL	SI	LCR	LFS	PFL	SI	TOTAL
Indotyphlops albiceps	3	-	-	-	-	-	3	-	2	-	-	-	8
Indotyphlops braminus	3	-	4	6	-	-	3	6	2	-	6	4	34
Indotyphlops lazelli	-	-	-	-	-	-	-	-	-	-	-	-	0
Dibamus bogadeki	-	-	-	-	-	-	-	-	-	-	-	-	0
TOTAL by site	6	0	4	6	0	0	6	6	4	0	6	4	
TOTAL by method	16				12				14				

I spent the most effort (in man-hour) for artificial refuge compared to quadrat search and opportunistic search, whereas I turned over highest number of cover objects for sampling (Table 2.4). Overall, the mean encounter number and encounter rate were highest in Pok Fu Lam, followed by Sunshine Island and Lady Clementi's Ride (Table 2.5).

For the results of the statistical analysis on the two species encountered, I found that the number of *I. braminus* encounter was higher in Pok Fu Lam and Sunshine Island than Lady Clementi's Ride (Table 2.6). Although *I. braminus* was encountered at the highest rate using opportunistic search (Figure 2.3), the difference was not significantly different (Table 2.6). The encounter rate of *I. albiceps* was higher in Lady Clementi's Ride, however, the difference was not significantly different. The encounter rate of *I. albiceps* was similar using the three methods (Figure 2.3; Table 2.6).

Table 2.4 Summary of time effort (man-hour) and the number of objects sampled by three different survey methods.

Mean (± SE)	Artificial refuge	Quadrat search	Opportunistic search
Mean sampling time	3.968 ± 0.260	1.898 ± 0.064	3.039 ± 0.294
Mean preparation time	0.478 ± 0.111	0.250 ± 0.000	0
Mean of total time effort	4.446 ± 0.235	2.148 ± 0.064	3.039 ± 0.294
Mean number of objects turned over	81.380 ± 2.051	76.010 ± 6.047	132.770 ± 20.133

Table 2.5 Summary of mean encounter rate (±SE) (number of individual encountered/man-hour) in different survey sites by different sampling methods. Bold indicates the highest mean number of encounter and encounter rate.

	Number of encounter (Mean ± SE)	Encounter rate (Mean ± SE)
Sampling sites		
Lady Clementi's Ride	0.11 ± 0.039	0.042 ± 0.021
Lung Fu Shan	0	0
Pok Fu Lam	**0.22 ± 0.087**	**0.098 ± 0.038**
Sunshine Island	0.17 ± 0.065	0.045 ± 0.018
Sampling methods		
Artificial refuge	**0.23 ± 0.093**	0.071 ± 0.032
Quadrat search	0.10 ± 0.040	0.045 ± 0.018
Opportunistic search	0.21 ± 0.073	**0.073 ± 0.031**

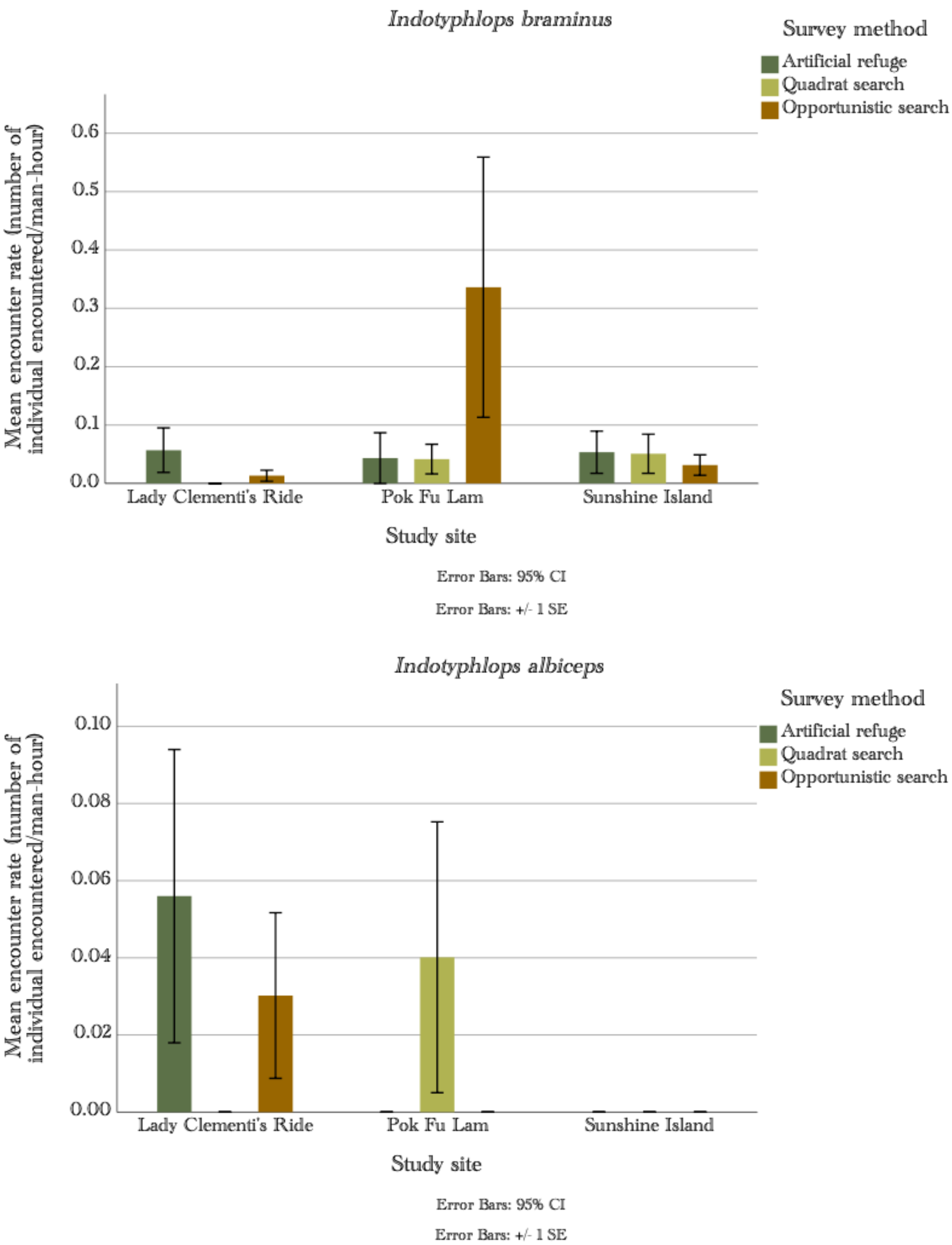

Figure 2.3 Mean encounter rate (± SE) of *I. braminus* (above) and *I. albiceps* (below) individuals per survey using three survey methods at three study sites.

Table 2.6 Estimates of study sites and study methods influencing the number of encounters and encounter rate of *I. braminus* and *I. albiceps* in the zero-inflated generalised linear mixed models. Lady Clementi's Ride and artificial refuge were set as the reference and compared with the other two sites and methods. Bold indicates significant correlations. Values are corrected to the 3 decimal places.

Species | Explanatory | Response	Estimate	Standard error	*Z*	*P*
Indotyphlops braminus | Site | Number of encounter				
Pok Fu Lam	**1.369**	**0.615**	**2.226**	**0.026**
Sunshine Island	**1.182**	**0.590**	**2.003**	**0.045**
Indotyphlops braminus | Method | Number of encounter				
Quadrat search	-0.796	0.556	-1.433	0.152
Opportunistic search	0.262	0.503	0.522	0.6
Indotyphlops braminus | Site | Encounter rate				
Pok Fu Lam	1.389	0.873	1.592	0.111
Sunshine Island	0.888	0.899	0.988	0.323
Indotyphlops braminus | Method | Encounter rate				
Quadrat search	-0.504	0.734	-0.687	0.492
Opportunistic search	0.164	0.729	0.225	0.822
Indotyphlops albiceps | Site | Number of encounter				
Pok Fu Lam	-0.162	0.814	-0.198	0.843
Sunshine Island	-20.947	15961.358	-0.001	0.999
Indotyphlops albiceps | Method | Number of encounter				
Quadrat search	-0.486	0.916	-0.530	0.596
Opportunistic search	-0.330	1.008	-0.328	0.743

Species | Explanatory | Response	Estimate	Standard error	*Z*	*P*
Indotyphlops albiceps | Site | Encounter rate				
Pok Fu Lam	-0.006	NaN	NaN	NaN
Sunshine Island	-44.481	NaN	NaN	NaN
Indotyphlops albiceps | Method | Encounter rate				
Quadrat search	-0.368	1.157	-0.318	0.751
Opportunistic search	-0.418	1.388	-0.301	0.763

From the accumulation curves, opportunistic search was more effective (steepest slope), detecting more individuals using less effort in terms of man-hour (Figure 2.4). Accounting for the number of objects turned over, the effectiveness of artificial refuge was lower at the beginning, then increased and became the most effective after around 4500 objects turned (Figure 2.5).

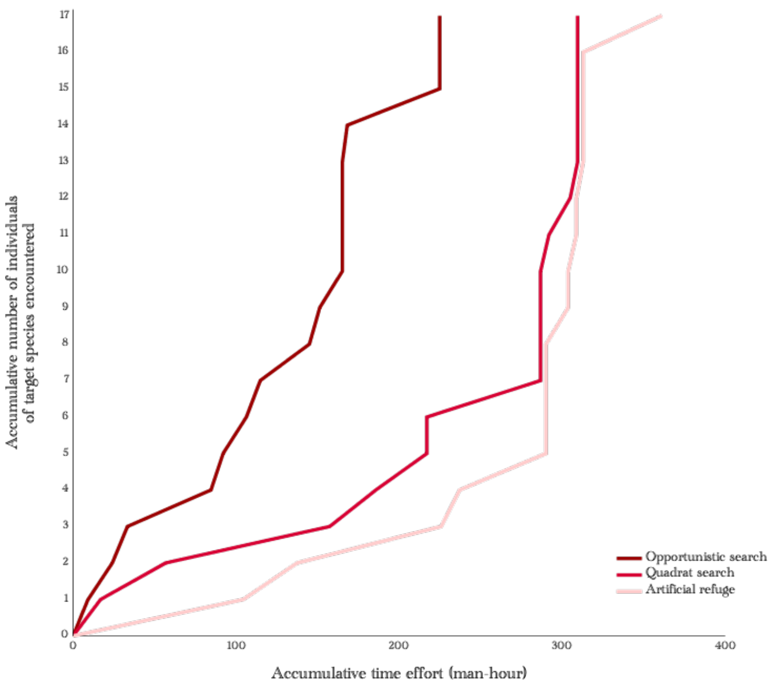

Figure 2.4 Accumulation curves related to the number of individuals of target species encountered by time effort (man-hour) by three survey methods.

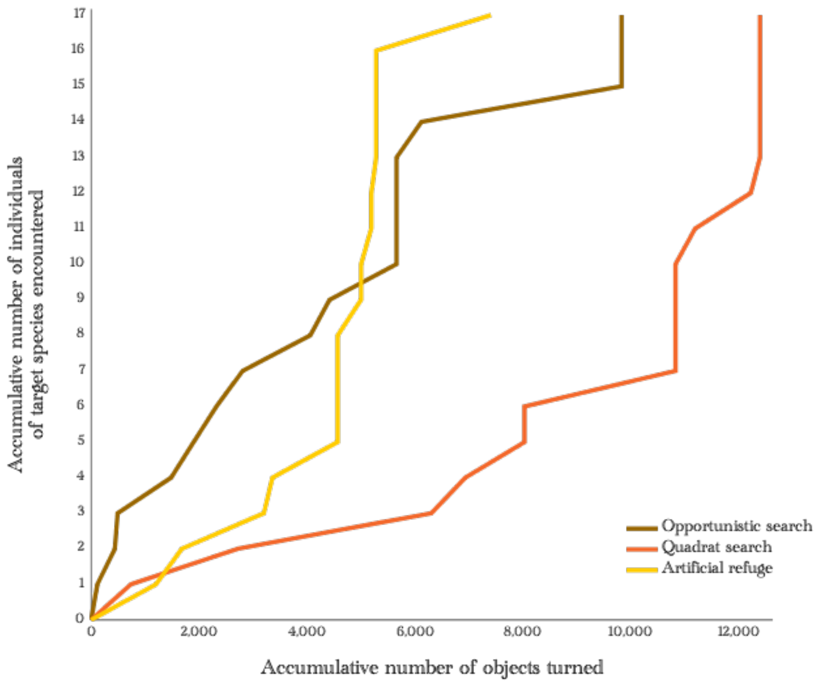

Figure 2.5 Accumulation curves showing the number of individuals of target species encountered by the number of objects turned by three survey methods.

Apart from the target fossorial species, 17 reptile species were recorded, including 10 lizard and 7 snake species (Table 2.7). *Gekko chinensis*, *Hemidactylus bowringii* and *Scincella modesta* were the most abundant species, contributing to more than 50% of all encounters of reptiles. For study sites, Sunshine Island contributed to the highest record of other reptile species. And opportunistic search yielded higher number of encounters than the other two methods.

Table 2.7 Total encounter number of other (non-fossorial) reptile species using three survey methods in different study sites. Study site: LCR: Lady Clementi's Ride, LFS: Lung Fu Shan, PFL: Pok Fu Lam, SI: Sunshine Island.

Other reptile species	Survey method												
	Artificial Refuge				Quadrat Search				Opportunistic Search				
	LCR	LFS	PFL	SI	LCR	LFS	PFL	SI	LCR	LFS	PFL	SI	TOTAL
Trimeresurus albolabris	-	-	-	-	1	-	-	2	1	3	-	2	9
Naja atra	-	-	-	-	-	-	-	-	-	-	1	-	1
Python bivittatus	-	-	-	-	-	-	-	-	-	-	-	2	2
Sibynophis chinensis	-	-	-	-	-	-	-	-	1	-	-	-	1
Ptyas mucosus	-	-	-	-	-	-	-	-	-	-	1	-	1
Achalinus rufescens	1	3	2	-	-	-	-	-	-	-	-	-	6
Pareas margartitophorus	-	-	1	-	-	-	-	-	-	-	-	-	1
Calotes versicolor	-	-	-		-	-	-	-	-	1	-	-	1
Tropidophorus sinicus	-	-	-	-	-	-	1	-	1	1	-	-	3
Plestiodon quadrilineatus	-	-	-	-	-	-	-	1	-	-	-	1	2
Sphenomorphus indicus	1	-	-	-	-	-	-	-	1	-	-	-	2
Eutropis longicaudata	-	-	1	-	-	-	-	-	-	-	-	-	1
Scincella reevesii	-	-	1	-	1	2	3	-	-	-	1	-	8
Scincella modesta	2	1	3	-	4	2	-	-	1	-	-	-	13
Hemidactylus bowringii	-	-	-	-	-	-	1	2	-	2	1	11	17
Gekko chinensis	-	-	-	17	-	-	1	1	1	1	-	5	26
Hemiphyllodactylus hongkongensis	-	-	-	-	-	-	-	-	1	-	-	-	1
TOTAL by site	4	4	8	17	6	4	6	6	7	8	4	21	
TOTAL by method	33				22				40				

Five species of amphibians were encountered, in which *Duttaphrynus melanostictus* and *Eleutherodactylus planirostris* were the most common, making up more than 90% of amphibian records (Table 2.8). They are also the only two amphibian species utilising the coverboards. I found the highest number of amphibians in Pok Fu Lam, but none were recorded on Sunshine Island. Over 50% of amphibians were sampled by quadrat search.

Table 2.8 Total encounter number of amphibian species using three survey methods in different study sites. LCR: Lady Clementi's Ride, LFS: Lung Fu Shan, PFL: Pok Fu Lam, SI: Sunshine Island.

Amphibian species	Survey method												
	Artificial Refuge				Quadrat Search				Opportunistic Search				
	LCR	LFS	PFL	SI	LCR	LFS	PFL	SI	LCR	LFS	PFL	SI	TOTAL
Duttaphrynus melanostictus	1	2	5	-	2	8	19	-	1	6	2	-	46
Kaloula pulchra pulchra	-	-	-	-	-	-	3	-	-	-	1	-	4
Polypedates megacephalus	-	-	-	-	1	-	1	-	-	-	-	-	2
Eleutherodactylus planirostris	2	4	2	-	4	6	6	-	1	7	7	-	39
Megophrys brachykolos	-	-	-	-	-	1	-	-	-	-	-	-	1
TOTAL by site	3	6	7	0	7	15	29	0	2	13	10	0	
TOTAL by method	16				51				25				

For the design of artificial refuges, although the statistical difference was not significant, the large plywood detected the highest number of target species, followed by ceramic and clay ($F_{1.961, 154.918} = 1.127$, $p = 0.326$) (Table 2.9; Figure 2.6). Small plywood yielded less encounters (1) compared to large plywood (7) (Table 2.9; Figure 2.7), although the difference was marginally significant ($F_{1, 79} = 3.723$, $p = 0.057$).

Table 2.9 Number of target species encounters by different coverboard types.

Species	Coverboard types			
	45 cm Plywood	30 cm Plywood	Ceramic	Clay
Indotyphlops albiceps	1	0	1	1
Indotyphlops braminus	6	1	3	3
TOTAL	**7**	**1**	**4**	**4**

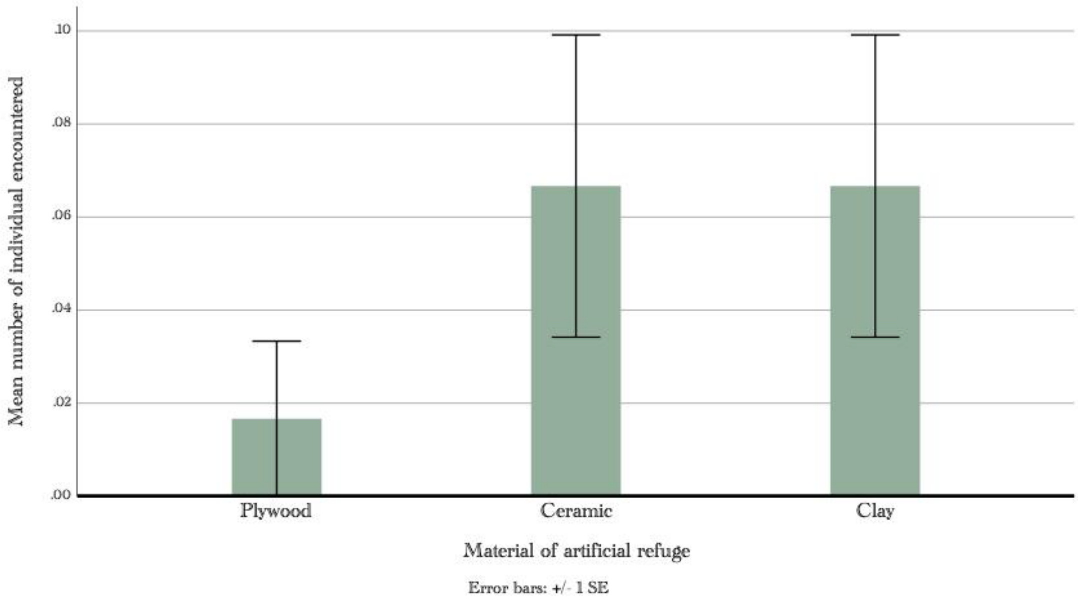

Figure 2.6 Mean number of individuals of target species encountered (± 1 SE) per coverboard with a size of 30 × 30 cm of three different materials.

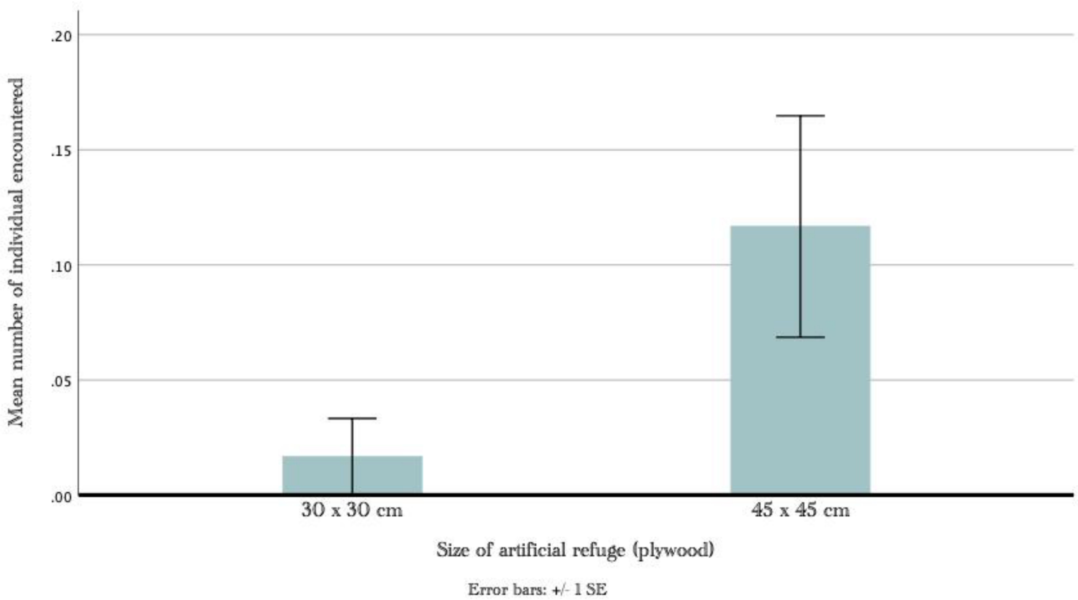

Figure 2.7 Mean number of individuals of target species encountered (± 1 SE) per plywood coverboard of two sizes (30 × 30 cm and 45 × 45 cm).

To determine the environment parameters that influence the encounter of fossorial species, 13 biotic and abiotic variables (ant colony, termite colony, solitary ant, solitary termite, gradient, elevation, leaf litter depth, ambient temperature, soil temperature, ambient humidity, soil humidity, time of day, canopy cover) were selected for the analysis to determine their correlation with the detection of the target species. Separately, I found that the best models for the *I. braminus* included ambient humidity, ant colonies, canopy cover, gradient, leaf litter depth, time of the day and termite colonies (Table 2.10). For *I. albiceps*, the best models constituted ambient humidity, ambient temperature, canopy cover, gradient, leaf litter depth, soil humidity (Table 2.11). Taking both target species into account, the overall best model is composed of ambient humidity, canopy cover, gradient, leaf litter depth, time of the day, soil humidity and termite colonies (Table 2.12).

Table 2.10 Representative zero-inflated generalised linear models explaining the correlation between different environmental parameters and the encounter number of *Indotyphlops braminus*. All values were corrected to the 2 decimal places.

Model	AICc	delta	weight
Ambient humidity + Canopy + Gradient + Time of day	183.33	0	0.33
Ambient humidity + Canopy + Gradient + Leaf litter depth + Time of day	184.15	0.82	0.22
Ambient humidity + Canopy + Gradient + Time of day + Termite colonies	184.49	1.15	0.18
Ambient humidity + Canopy + Gradient	184.86	1.53	0.15
Ambient humidity + Ant colonies + Canopy + Gradient + Time of day	185.29	1.96	0.12

Table 2.11 Representative zero-inflated generalised linear models explaining the correlation between different environmental parameters and the encounter number of *Indotyphlops albiceps*. All values were corrected to the 2 decimal places.

Model	AICc	delta	weight
Ambient humidity + Canopy + Gradient	62.66	0	0.21
Ambient humidity + Canopy + Gradient + Soil humidity	63.07	0.40	0.17
Canopy + Gradient	63.58	0.92	0.13
Ambient humidity + Ambient temperature + Canopy + Gradient	63.93	1.26	0.11
Ambient humidity + Gradient	64.29	1.63	0.09
Ambient humidity + Ambient temperature + Canopy + Gradient + Soil humidity	64.31	1.65	0.09
Ambient humidity + Canopy + Gradient + Leaf litter depth	64.33	1.67	0.09
Gradient	64.57	1.90	0.08

Table 2.12 Representative zero-inflated generalised linear models explaining the correlation between different environmental parameters and the encounter number of *Indotyphlops spp.* All values were corrected to the 2 decimal places.

Model	AICc	delta	weight
Ambient humidity + Canopy + Gradient + Time of day	213.74	0	0.26
Ambient humidity + Gradient + Time of day	214.12	0.37	0.22
Ambient humidity + Canopy + Gradient + Leaf litter depth + Time of day	214.97	1.23	0.14
Ambient humidity + Canopy + Gradient	215.09	1.35	0.13
Ambient humidity + Canopy + Gradient + Time of day + Termite colonies	215.19	1.44	0.13
Ambient humidity + Gradient + Time of day + Soil humidity	215.37	1.62	0.12

Among these factors, there was a significant positive correlation between the number of encounters of *I. braminus* and ambient humidity; as well as a significant negative correlation between its encounter number to the canopy cover and the gradient (Table 2.13). For *I. albiceps*, there was no significant correlation with all variables (Table 2.14). Overall, the ambient humidity positively affected the encounter number of the target *Indotyphlops* spp. and the gradient had a negative effect on encounter (Table 2.15).

Table 2.13 Environmental parameters included in the best zero-inflated generalised linear models explaining the correlation with the encounter number of *Indotyphlops braminus*. Bold indicates significant correlations. All values were corrected to the 3 decimal places.

Parameter	Estimate	Standard error	*Z*	*P*
Ambient humidity	**0.050**	**0.024**	**2.120**	**0.034**
Canopy cover	**-0.134**	**0.055**	**2.433**	**0.015**
Gradient	**-0.084**	**0.041**	**2.016**	**0.044**
Time of the day	0.003	0.001	1.800	0.072
Leaf litter depth	0.255	0.221	1.149	0.250
Termite colony	0.067	0.069	0.963	0.336
Ant colony	0.013	0.028	0.452	0.652

Table 2.14 Environmental parameters included in the best zero-inflated generalised linear models explaining the correlation with the encounter number of *Indotyphlops albiceps*. All values were corrected to the 3 decimal places.

Parameter	Estimate	Standard error	*Z*	*P*
Ambient humidity	0.125	0.099	1.252	0.210
Canopy cover	0.315	0.201	1.562	0.118
Gradient	-0.310	0.163	1.894	0.058
Soil humidity	-0.149	0.117	1.265	0.206
Ambient temperature	-0.181	0.189	0.953	0.341
Leaf litter depth	-0.194	0.384	0.501	0.617

Table 2.15 Environmental parameters included in the best zero-inflated generalised linear models explaining the correlation with the encounter number of *Indotyphlops spp*. Bold indicates significant correlations. All values were corrected to the 3 decimal places.

Parameter	Estimate	Standard error	*Z*	*P*
Ambient humidity	**0.045**	**0.022**	**2.009**	**0.045**
Canopy cover	-0.075	0.046	1.641	0.101
Gradient	**-0.096**	**0.039**	**2.414**	**0.016**
Time of day	0.002	0.001	1.776	0.076
Leaf litter depth	0.188	0.198	0.946	0.344
Termite colonies	0.040	0.047	0.842	0.400
Soil humidity	0.036	0.039	0.924	0.355

Although ambient temperature and soil surface temperature did not significantly correlate the number of encounters in the zero-inflated generalised linear models, I further explored their potential correlation using scatter plots (Figure 2.8 & 2.9). Instead of generating a linear correlation, the plot reflected a dome-shaped relationship between the number of individuals of target species encountered per survey and temperature. The encounter number peaked at ambient and soil temperatures of 28–30°C.

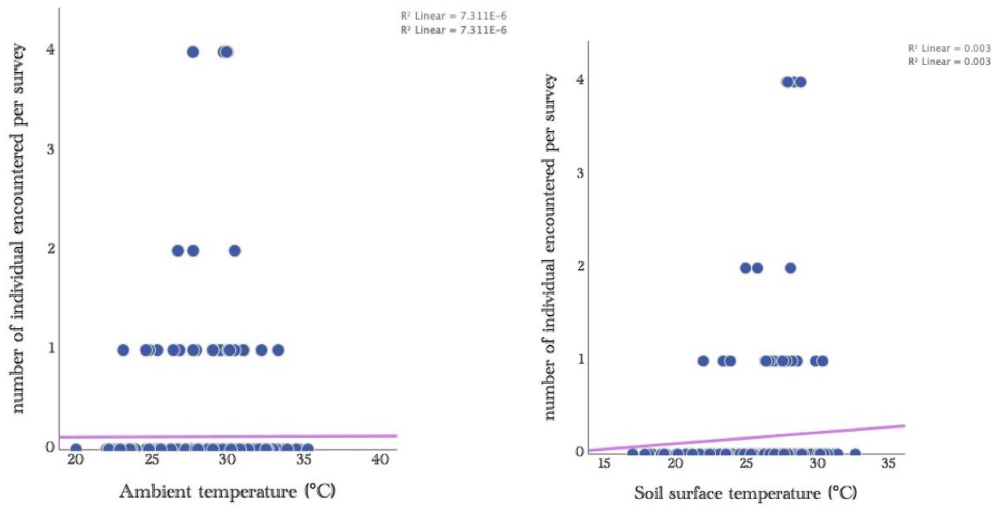

Figure 2.8 (left) & 2.9 (right) Number of individual of target species encountered per survey versus ambient temperature (°C) (left) and soil surface temperature (°C) (right).

Overall, the highest mean encounter rate occurred in July (Figure 2.10). The period from April to July had a higher encounter rate than the period from August to November.

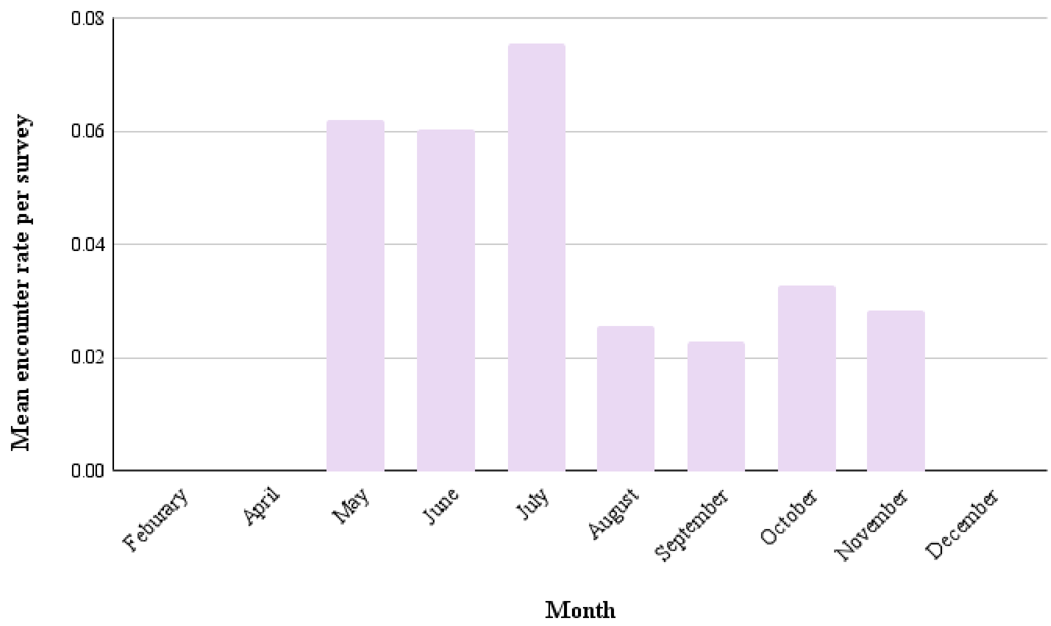

Figure 2.10 Mean encounter rate of target species encountered per survey in different months throughout the study in 2021-2022.

2.5 Discussion

2.5.1 Absence of Indotyphlops lazelli and Dibamus bogadeki in the study

Neither *I. lazelli* nor *D. bogadeki* were detected in this study, even within their known distribution areas. Their absence may be because of unsuitable survey methods and/or small populations. The first reason may be less likely because we were able to capture many *I. albiceps* and *I. braminus* at the sites. Also, several *D. bogadeki* individuals were detected using coverboards in surveys conducted by Agriculture, Fisheries and Conservation Department (Chan et al., 2012). The survey methods I used in this study should be useful in detecting *D. bogadeki* and *I lazelli*. The survey effort spent in this study is significantly greater than previous surveys. It is of conservation concern that we could not detect *D. bogadeki* and *I. lazelli* because the result of this study may indicate their populations are very small and declining. Urgent actions are needed to understand more about their population status and distribution. First, surveys should be carried out on the other two islands where *D. bogadeki* occurs, Hei Ling Chau and Shek Kwu Chau, which was not possible because the pandemic limited access to the islands. Second, more studies should be done to evaluate other sampling methods for fossorial reptiles, such as environmental DNA (eDNA) (Kyle et al., 2022; Matthias et al., 2021) and below-ground trapping (Enge, 2001; Friend et al., 1989; Greenberg et al., 1994; Ribeiro-Júnior et al., 2011). Third, for *I. lazelli*, there were only two records prior to this study (Wallach & Pauwel, 2004). In 2021, potential specimen of *I. lazelli*, was collected by naturalists in urban areas in Tsuen Wan, New Territories (Pan Lau, personal communication, n.d.). The species identity was later validated by morphological analysis. This discovery in Tsuen Wan indicates the distribution of *I. lazelli* is wider than previously known. Given the low detection rate of fossorial reptiles yielded in this study, besides employing other sampling methods, citizen science projects may be useful in revealing their distribution and population status, by encouraging amateur naturalists to gather sightings of blind stakes across Hong Kong.

2.5.2 Efficiency of sampling methods

Fossorial reptiles were detected using all three methods, although there was no statistically significant difference in the encounter rate among the methods. Our results suggest that active search (quadrat search and opportunistic search) is more cost-effective in detecting fossorial reptiles. Of the two active search methods, opportunistic search is more cost-effective than quadrat search because no preparation (setting up quadrat) is required (Garden et al., 2007; Hutchens & DePerno, 2009). Opportunistic search covers a larger area which allows sampling of more habitat types (Gillespie et al., 2005; Oldenburg, 2017), thus it is applicable to detect fossorial species of relatively higher abundance, like *I. braminus*; as well as those with smaller population size, like *I. albiceps*. In contrast, quadrat search is confined to smaller areas, and based on my experience, there is a higher chance that disturbance caused by surveyors scare away target species so they cannot be detected (Noon et al., 2006). Sampling of environmental parameters is difficult for opportunistic search, and so it is not suitable for detailed ecological studies. Opportunistic search (or transect search) is preferable for studies that aim to detect fossorial species, but generally not for detailed ecological studies (Harikrishnan et al., 2012), for example studies investigating habitat preference of fossorial species.

Despite the lower encounter rate, a substantial proportion (38%) of encounters were made by artificial refuge. One important finding is that the efficiency of artificial refuge increased over time, which supports previous studies showing acclimation, allowing the soil faunal community to get used to and colonise the newly added refuges, is crucial for the use of coverboards (Gallegos, 2019; Grant et al., 1992; Henderson et al., 2016; Pittman & Dorcas, 2006). Compared to active search, artificial refuge is a more standardised sampling method with lower sampling variability (for example observer bias), but requires more effort for installation and maintenance (Michael et al., 2018; Ribeiro-Junior et al., 2008). In particular, in this study, around 10% of coverboards were disturbed or damaged by mammals, probably Wild Boars. Given the high density of Wild Boars in some areas in Hong Kong (AFCD, 2022h), the application of coverboards requires more maintenance efforts. As such, I would suggest employing artificial refuge for longer term (>1 year) studies on fossorial reptiles, but not for short term studies or studies with limited manpower.

2.5.3 Correlation with environmental parameters

Despite the relatively low encounter rate of target species in this study, several significant correlations between the encounter number of *I. braminus* and the environmental parameters were revealed in this study. First, the detection rate of *I. braminus* increases with the ambient humidity. Higher ambient humidity lowers their risk of desiccation when they are exposed in air. This increases their foraging time above the soil environment and the probability of being detected on the surface. Previous study found that two blind snakes had a higher detection rate following rains (Spence-Bailey et al., 2010). Although measurement of rainfall was not conducted in this survey, it is likely that the target species could be detected more often after rainfall events, which increase ambient humidity.

Second, higher canopy cover may be beneficial to the occurrence of blind snakes, but detrimental to their detectability. Generally, forest with higher canopy cover associates with more thriving and mature vegetation. The understory is usually supplied with higher quality organic matters, such as fallen leaves. The abundant leaf litter and prosperous plant materials support a healthy soil community, including the prey items of blind snakes; and provide sufficient refuges for the blind snakes when they are out from the soil. As such, the complex environment on the ground may increase the difficulty for surveyors to detect the hiding individuals. Furthermore, canopy coverage may affect the light intensity on the forest floor. For instance, moon phase conditions may influence the detection of some reptiles, particularly nocturnal species (Spence-Bailey et al., 2010). This is also associated with the size of eyes of reptiles which affect their foraging behaviours, that species with smaller eyes tend to forage under brighter conditions (Werner & Seifan, 2006). However, the impact of light on the daily activity cycle of blind snakes is not well-studied.

Third, it is easier to sample fossorial reptiles in flat areas. First, the superficial soil layer is thinning in sloped areas because of surface runoff, and thus providing less suitable microhabitats for fossorial reptiles. Also, it is more challenging for surveyors to conduct surveys on steep slopes which may reduce detection rate.

Fourth, my results showed that the detection of fossorial reptiles was the highest at temperature of 28–30°C, which may infer the optimal temperature for sampling fossorial reptiles. As reptiles are ectotherms, temperature can directly affect their metabolic rate (Lilywhite, 1987; Shine, 2005). At lower temperature, they become inactive with decelerated metabolism and stay underground whereas higher temperature beyond optimum may render them vulnerable to desiccation with increased evaporation rate. An optimal temperature favours the activity of fossorial reptiles, increasing their frequency to emerge on surface for food resources, and thus enhancing the chance for them to be detected (Henderson et al., 2016). A previous study further suggested that the minimum temperature is the most influential factor impacting the activity pattern of blind snakes (Parpinelli & Marques, 2008).

Lastly, in this study, most (70%) individuals of target species were detected in the early wet season (May to July) and the mean encountered rate was higher. First, this may be related to the increased humidity during rainy seasons as discussed previously. Second, another possible explanation is the reproductive behaviour of fossorial reptiles. In Japan, ovulation of *I. braminus* starts in May and hatching takes place in August (Kamosawa & Ota, 1996). Third, the rise in the availability of food items, particularly ants, in wet season also contributed to the increasing activities of blind snakes (Parpinelli & Marques, 2008). Ants at all life stages (adult, pupae and larvae) are primary food items to blind snakes (Bamford & Prendergast, 2017; Wallach, 2009). The strong correlation with and dependence of ant determine the food availability, explaining the occurrence of the blind snakes. The blind snakes probably forage with the colony emigration of their targeted ant species. The interactions between fossorial reptile conspecifics and other members in the soil community represents a vast knowledge gap, more studies on their ecology are needed and worthwhile (Amo et al., 2004; Cooper et al., 1999; Downes and Shine, 1998; Langkilde and Shine, 2004). Recording the presence of ants and even identifying the species during sampling would be a step further in enhancing the detectability of blind snakes. All in all, activity on the soil surface may increase during the early wet season and enhance the chance of detection.

2.5.4 Encounter rate among study sites

In this study, the highest number of encounters was recorded in Pok Fu Lam, highlighting the importance of this site for fossorial reptiles in Hong Kong. Compared with other study sites, Pok Fu Lam offers a wider diversity of microhabitats for the subsistence of different fossorial reptiles, particularly, several encounters were made in the same patch of forest, surrounded by human settlements and slopes. Despite lower encounter rates of target species in Lady Clementi's Ride and Sunshine Island, the two sites hold one of the very few populations of *I. albiceps* and *D. bogadeki*, respectively. Although there were previous records of *I. albiceps*, *I. braminus* and *I. lazelli* in the region around Lung Fu Shan, and there were occasional encounters of *I. braminus* in Lung Fu Shan within the study period but not during the surveys, none is detected in this study. It is likely that the population of fossorial reptiles is lower in Lung Fu Shan compared to other study sites.

2.5.5 Application of artificial refuges to sample other species

Besides fossorial reptiles, I was able to detect a number of other reptile and amphibian species. In particular, I recorded a relatively high number of *Scincella modesta*, *Achalinus rufescens* and *Gekko chinensis* under artificial refuges than the targeted species. These three species are forest inhabitants. *S. modesta* is ground-dweller, while *A. rufescen* is a cave-dweller; and the *G. chinensis*, despite being arboreal-dwellers, was found clinging to the underside of the coverboards. Also, in this study, *Eleutherodactylus planirostris* was detected in three study sites using all three survey methods. *E. planirostris* is an exotic species first discovered in 2000 in Hong Kong and it has become a widespread species, distributed in more than 18 locations, including The University of Hong Kong (HKU) near Lung Fu Shan (Lee at al., 2016). Although Sung et al. (2011) also conducted surveys in Lung Fu Shan, no *E. planirostris* was recorded from 2008 to 2009. The recent spread of *E. planirostris* may explain the presence of *E. planirostris* in this study, which is concordant with Lee et al. (2016) that the species was first recorded at HKU in 2010. As *E. planirostri* could potentially threaten the endemic *Liuixalus romeri* (Lee et al., 2016), and could often be detected under coverboards (Rizkalla, 2009), I suggest that artificial refuges could be deployed with quadrat/active search for future studies on investigating the impact of *E. planirostris* on local ecosystems. Besides herpetofauna, *Nanhaipotamon hongkongense*, a tropical freshwater crab endemic to Hong Kong, was also detected under boards on several occasions. These encounter results can provide preliminary data for future research about relevant taxa with the application of similar sampling methods.

2.6 Conclusion

In conclusion, two of the target species (*Indotyphlops albiceps and Indotyphlops braminus*) were detected using three survey methods (quadrat search, artificial refuges and opportunistic search) in this field study. Active search (quadrat search and opportunistic search) is a more desirable and cost-effective method for short-term study, while artificial refuges could be deployed with active search for long-term study. The encounter correlates significantly with a set of environmental factors, including ambient humidity, gradient and canopy cover. Extra survey effort or novel sampling methods are required to detect *Dibamus bogadeki* and *Indotyphlops lazelli*. These findings will be useful to guide future ecological surveys on fossorial reptiles.

CHAPTER 3 - CLARIFICATION OF TAXONOMIC STATUS OF BLIND SNAKES IN HONG KONG

3.1 Abstract

The diversity of fossorial herpetofauna, such as, blind snakes, is underestimated because of the challenges in distinguishing different species and collecting specimens. The advancement of molecular analysis has led to the discovery of many new species in the last decade. In Hong Kong, there are three blind snake species described and recorded, namely *Indotyphlops braminus*, *I. albiceps* and *I. lazelli*. Of these three species, the taxonomic status of two species, *I. albiceps* and *I. lazelli*, is questionable, given the rarity of specimens and lack of genetic studies. In this study, we conducted a phylogenetic analysis to determine if *I. albiceps* is a new species and *I. lazelli* is a valid species. I found that there is a significant genetic difference (5.96% for *AMEL*; 3.84% for *BDNF*; 26.6% for *Cytb*) between the populations of *I. albiceps* in Hong Kong and Southeast Asia. This result suggests that the Hong Kong population represent a new, cryptic species. *Indotyphlops lazelli* also showed a significant disparity (5.36% for *AMEL*; 3.49% for *BDNF*; 26.7% for *Cytb*) between other congeners, revealing *I. lazelli* as a valid species. Also, I found that the *I. braminus* population in Hong Kong is potentially hybrids of *I. braminus* and *I. pammeces*, suggesting the species was introduced into Hong Kong. Further work, including morphological analysis on *I. albiceps*, collection of additional blind snake samples from across the species range and more systematic surveys for fossorial reptiles in nearby regions (for example, South China), are needed to resolve the understudied taxonomy and inform the conservation of blind snakes.

3.2 Introduction

Traditionally, the species delimitation determines the species boundaries and its diversity based on various levels of classifications, for example, morphological distinction, different ecological niche occupation and reproductive isolation (De Queiroz, 2007). However, sole dependence on the above traits might not be capable of achieving the norm of the taxonomical diagnosability (Vences et al., 2013).

With the popularisation of molecular systematics in species classification, the extent of genetic diversity has been resolved in different taxa (Elejalde et al., 2008; Guo et al., 2014; Hellborg et al., 2005; Metzger et al., 2010; Thum & Harrison, 2009). Phylogenetic taxonomy of Serpentes has been revised across different regions of different geographical scales (Guicking et al., 2009; Guo et al., 2014; Metzger et al., 2010). For example, in Hong Kong, an integrative approach using both genetic and morphological data has helped elucidate the taxonomic status of several species, for instance, *Bungarus wanghaotingi* (Yuan et al., 2022), *Trimerodytes balteatus* (Francis, 2021), *Pareas margaritophorus* (Vogel et al., 2020), *Fowlea flavipunctatus* (Purkayastha et al., 2018) and *Ovophis tonkinensis* (Fong et al., 2017). These studies evidenced that snake taxonomy in Hong Kong and nearby regions deserve more research attention.

Species delimitation faces exceptional challenges in species lineages that share similar life history, adaptation and morphology. Given similar fossorial lifestyle, many blind snake species have similar ecomorphotype and are difficult to distinguish from each other. Taxonomically, blind snake (Infraorder Scolecophidia) species were differentiated by their anatomical (for example visceral and osteological) and morphological traits (for example scalation) (Miralles et al., 2018; Wallach & Pauwels, 2004). Differentiation using anatomical traits require high level of skills, for example, one of the distinctive features of *I. lazelli* is having a unicameral tracheal lung (Wallach & Pauwel, 2004), which is very challenging to examine given the small size of the species.

Molecular phylogenetic analyses have been applied to resolving taxonomy status of blind snake species (Figueroa et al., 2016; Ren et al., 2019; Vogel et al., 2020; Yuan et al., 2022). Many new species of blind snake were “rediscovered” and described across different continents (Ellis et al., 2017; Giokas et al., 2011; Graboski et al., 2022; Hedge & Thomas, 1991; Torki, 2017; Wallach 1999). Molecular data has also helped to clarify the evolutionary relationships of blind snakes (Nagy et al., 2015), and supported that the blind snake diversity is underestimated (Ellis, 2016; Hedges et al., 2014; Marin et al., 2013b). Many species complexes have yet to be resolved (Ellis et al., 2017; Kornillos et al., 2020).

In Hong Kong, the identification of the three local blind snake species (*Indotyphlops albiceps*, *I. lazelli* and *I. braminus*) has been based on morphological and anatomical characteristics (Lazell & Lu, 1990; Wallach & Pauwels, 2004), but their genetics have not been examined. The taxonomic status of *I. albiceps* in Hong Kong requires examination because of its intriguing global distribution. It can be found in Myanmar, Thailand, Malaysia and Hong Kong (Uetz et al., 2022). Its distribution is disjunct as it has not been recorded in China, the geographic regions between Hong Kong and other countries of its range. It is suspected that the population of *I. albiceps* in Hong Kong represents a cryptic species, i.e. two or more genetically distinct species classified as single species with high superficial morphological similarities (Bickford et al., 2007). As such, a genetic study is needed to assess the taxonomic status of the *I. albiceps* population in Hong Kong which will be vital to determine if this species deserves more conservation attention (e.g. Funk et al., 2011).

Further, the genetic data of *I. lazelli* is useful to evaluating its species validity. In consideration of its “Critically Endangered” status under both the IUCN Red List and China Biodiversity Red List (Jiang et al., 2016; Lau, 2012), plus its endemism to Hong Kong, this species is of global and regional conservation concern. Consolidating its taxonomic status is crucial in acknowledging and prioritising this species in future conservation actions.

Indotypholops braminus has a wide global distribution. Although *I. braminus* has been considered as a native species in Hong Kong (AFCD, 2022b), according to the Global Invasive Species Database (2022) and a study by Shea et al., (2021), it is most likely originated from India and Bhutan, but spread across continents by decades of natural dispersal and/or accidental introduction in potted plants to the adjacent Asian regions, including Hong Kong (Wallach, 2009, Hedges et al., 2014, Pyron & Wallach, 2014). Further, Sidharthan et al., (2022) proposed that populations outside the native range of the *I. braminus* are actually hybrids of *I. pammeces* and *I. braminus* in India. As such, examination of genetic data of *I. braminus* in Hong Kong can help shed light on the origin and species identity of *I. braminus*.

Overall, in this study, by applying phylogenetic analysis, I aimed to clarify (1) the taxonomic status of *I. albiceps*, specifically the relationship between the Hong Kong and Southeast Asia populations, (2) the species validity of *I. lazelli* and (3) the phylogenetic relationship between *I. braminus* populations in Hong Kong and elsewhere.

3.3 Methods

3.3.1 Sample Collection

All specimens and tissue samples were collected within Hong Kong Special Administrative Region, China (22°09′–22°37′N; 113°50′–114°30′E). Intact specimens of six *I. albiceps*, three *I. braminus* and one *I. lazelli* were obtained from fieldwork or donation across Hong Kong from 2019 to 2021 (Figure 3.1). After basic morphological measurement of their snout-vent length (cm), tail length (cm) and body weight (g), the specimens were dissected to extract their liver tissues. The specimens were preserved in 95% ethanol and liver tissues and stored in a -20°C refrigerator at the Natural History Collection of Lingnan University (Specimen voucher numbers of *I. albicep*s are Lingu:Herp: 15, 16, 17, 18, 882, 883; *I. braminus*: Lingu:Herp: 827, 828, 844; and I. *lazelli*: Lingu:Herp: 590) (Table 3.1).

Figure 3.1 Collection localities of specimens of *Indotyphlops albiceps, Indotyphlops braminus and Indotyphlops lazelli* in Hong Kong between 2019 and 2021. Numbers indicate number of specimens collected from each location.

Table 3.1 Information of genetic sequences of blind snakes (*Indotyphlops albiceps, I. braminus and I. lazelli*) specimens obtained in this study.

Species	Locality	Date of collection (year/month)	Specimen voucher number
Indotyphlops albiceps	South District	2019/06 2021/09	LINGU:Herp:15 LINGU:Herp:882
	Aberdeen Country Park	2019/06	LINGU:Herp:16 LINGU:Herp:18
	Kwai Chung	2019/06	LINGU:Herp:17
	Pok Fu Lam	2021/11	LINGU:Herp:883
Indotyphlops braminus	Pok Fu Lam	2021/05	LINGU:Herp:827
	Sunshine Island	2021/06	LINGU:Herp:828
	South District	2021/07	LINGU:Herp:844
Indotyphlops lazelli	Tsuen Wan	2021/02	LINGU:Herp:590

3.3.2 DNA Extraction & Polymerase Chain Reaction (PCR)

DNA was extracted by digesting the preserved liver tissues with proteinase k and following the manufacturer's procedures of the DNeasy® Blood & Tissue Kit (Qiagen®, Hilden, Germany). DNA yields were quantified by Qubit™ Fluorometer (Invitrogen™, Waltham, Massachusetts, USA). One mitochondrial protein-coding gene, cytochrome b (*Cytb*) and two nuclear protein-coding genes, brain-derived neurotrophic factor (*BDNF*) and amelogenin genes (*AMEL*), were selected for amplification (Burbrink et al., 2000; Noonan & Chippindale 2006; Vidal et al., 2010). The targeted loci were amplified by the designated primer pairs under respective thermal cycling conditions (Table 3.2).

PCR amplification was performed in 20 µl reactions, containing 1µl extracted DNA and 19 µl Master Mix prescribed by the Accupower® PCR PreMix (Bioneer Corporation) for each primer pair. After that, 3 µl of the PCR products were run on a 1.5% agarose gel for gel electrophoresis to visually estimate the base pair size of the PCR amplicons. The remaining volume of the PCR products were purified using Bioneer AccuPrep® PCR/Gel Purification Kit (Ref. K-3037, Bioneer Corporation) before submission to BGI Genomics (Hong Kong) for Sanger sequencing. DNA sequencing was performed in both forward and reverse directions using the PCR primer pairs.

Table 3.2 Details of primers and corresponding programmed PCR conditions.

Loci	Primer pairs	Reference	Sequence (5'–3')	Thermal cycle condition
Cytb	L14910	Burbrink et al., 2000	GAC CTG TGA TMT GAA AAA CCA YCG TTG T	1. Pre-denaturation: 94°C for 7 minutes 2. 40 cycles: 1. Denaturation: 94°C for 30 seconds 2. Annealing: 46°C for 30 seconds 3. Extension: 72°C for 1 minute 3. Post extension: 72°C for 7 minutes
	H16064		CTT TGG TTT ACA AGA ACA ATG CTT TA	
BDNF	BDNF-F	Noonan & Chippindale, 2006	GAC CAT CCT TTT CCT KAC TAT GGT TAT TTC ATA CTT	1. Pre-denaturation: 94°C for 3 minutes 2. 40 cycles: 1. Denaturation: 94°C for 3 minutes 2. Annealing: 50°C for 40 seconds 3. Extension: 72°C for 1 minute 3. Post extension: 72°C for 10 minutes
	BDNF-R		CTA TCT TCC CCT TTT AAT GGT CAG TGT ACA AAC	
AMEL	LAMSQ	Vidal et al., 2010	ATG GGA GGA TGG ATG CAC CA	1. Pre-denaturation: 94°C for 3 minutes 2. 40 cycles: 1. Denaturation: 94°C for 3 minutes 2. Annealing: 50°C for 40 seconds 3. Extension: 72°C for 1 minute 3. Post extension: 72°C for 10 minutes
	HAMSQ		GGC CAT GRT TCA AGA GGY GTA T	

3.3.3 Phylogenetic analysis

I obtained 29 new sequences from the samples, with one of the *BDNF* sequences of *I. albiceps* failing in the PCR process and omitted from latter analyses. Additional sequences of blind snakes were downloaded from the GenBank, comprising 38 *Cytb*, 43 *BDNF* and 36 *AMEL* sequences (Table 3.3). I also included another seven unpublished sequences, four *BDNF* and three *AMEL* for analysis; they were obtained from four donated specimens of *I. albiceps* that were collected in Hong Kong outside of this study. For each locus, all sequences were assembled and aligned using the MUSCLE algorithm (Edgar, 2004) in the software Geneious R11 (Kearse et al., 2012). The aligned sequences were trimmed and corrected manually to generate a consensus sequence matrix in Geneious. New sequences were first screened out if they have low pairwise sequence identity with those of the targeted genus *Indotyphlops.* All sequences of low-quality (e.g. existence of double peaks) or with missing data were removed from the analysis.

Table 3.3 Localities and accession number of sequences of blind snakes downloaded from GenBank.

Species	Locality	Accession number in GenBank		
		Cytb	*BDNF*	*AMEL*
Acutotyphlops kunuaensis	Mt. Balbi, North Solomons, Papua New Guinea	KT316466.1	GU902419.1	GU902339.1
Afrotyphlops elegans	Ribeira Macoia, Principe Island, São Tome and Principe	KT316472.1 KT316473.1 KT316474.1 KT316475.1	GU902391.1 KF992882.1	GU902314.1
Amerotyphlops brongersmianus	Guyana	KF993239.1	GU902390.1 MH925778.1	GU902313.1
Amerotyphlops reticulatus	Paramakatoi. Guyana	KT316483.1 NC_010971.1	GU902396.1	GU902319.1
Anilios australis	Perth Airport, WA, Australia	AM236346.1	JQ910311.1 JQ910312.1	GU902331.1
Anilios bicolor	Ora Banda, WA, Australia	KT316485.1	JQ910391.1 GU902410.1	GU902332.1
Anilios bituberculatus	Ora Banda, WA, Australia	KT316486.1	JQ910314.1 GU902403.1	GU902325.1
Anilios longissimus	Bandicoot Bay, Barrow Island, WA, Australia	JQ910525.2	JQ910301.1	GU902330.1
Antillotyphlops catapontus	Neptune's Treasure, Anegada, British Virgin Islands	KF993243.1	GU902426.1	GU902346.1
Antillotyphlops dominicanus	near town of Soufriere, Dominica	KF993249.1	GU902428.1	GU902348.1

Antillotyphlops platycephalus	WNW Sabana Grande, Puerto Rico	AY099992.1 KF993269.1	GU902437.1	GU902357.1
Argyrophis diardii	Myanmar	KT316507.1	KF992877.1	KF992856.1
Argyrophis muelleri	Nat Ma Taung National Park, Min Dat Township, Min Dat District, Chin State, Myanmar	KT316508.1	KF992878.1	KF992857.1
Cubatyphlops anchaurus	Maisí, Guantánamo Province, Cuba	KF993235.1	GU902423.1 KF993231.1	GU902343.1
Cubatyphlops anousius	Tortuguilla, Guantánamo Province, Cuba	KF993236.1	GU902445.1	GU902365.1
Cubatyphlops arator	El Narigon, near Puerto Escondido, La Habana Province, Cuba	KF993237.1	GU902424.1	GU902344.1
Gerrhopilus mirus	Colombo or Uda Walawe National Park, Uda Walawe, Sri Lanka	KT316555.1	GU902394.1	GU902317.1
Indotyphlops albiceps	Tha Baik Kyin Township, Shwe U Daung Wildlife Sanctuary, Nyaung Gome Elephant Camp, Mandalay, Myanmar	KT316509.1	GU902382.1	GU902305.1
Indotyphlops braminus	Florida, USA		GU902383.1	GU902306.1
	India		MW442111.1 MW442112.1 MW442108.1 MW442109.1 MW442110.1	MW442100.1 MW442097.1 MW442098.1 MW442096.1 MW442099.1

	Madagascar	KT316548.1 KT316546.1 KT316545.1 KT316547.1		
	Phang-Nga City, MuangDistrict, Phang-Nga Province, Thailand		FJ433959.1	FJ434038.1
	Yunnan, China	DQ343649.1 NC010196.1		
	Unknown	NC_010196.1		
Indotyphlops pammeces	Colombo or Uda Walawe National Park, Uda Walawe, Sri Lanka		GU902458.1	GU902378.1
Ramphotyphlops acuticaudus	Ngermid village, Palau	JQ910543.2	GU902381.1	GU902304.1
Ramphotyphlops lineatus	Malakoni, Bengkulu, Sumatra	KT316550.1	GU902384.1	GU902307.1
Sundatyphlops polygrammicus	Brang Kua, Moyo Island, Indonesia	KT316551.1	JQ910381.1 GU902421.1	GU902341.1
Typhlops agoralionis	Marché Léon, Grand Anse, Haiti	KF993234.1	GU902422.1	GU902342.1
Typhlops rostellatus	Arecibo, Puerto Rico	KF993278.1	GU902439.1	GU902359.1
Typhlops schwartzi	Nisibon, El Seibo, Dominican Republic	KF993279.1	GU902440.1	GU902360.1
Xerotyphlops socotranus	Sokotra Island, between Eerk and Jelhiiyo, Yemen	KY660141.1	KC848452.1	KC848443.1

Xerotyphlops vermicularis	Geolazar, Armenia	KY660140.1	KC848451.1	KF992864.1
	Gilan Province, Iran	KT316553.1	KF992885.1	KC848442.1
	Zai Park, Jordan	JQ910544.2	GU902397.1	GU902320.1

Combining the sequences obtained from this study and Genbank, I constructed a phylogenetic tree for each of the three loci using Maximum Likelihood analysis in software MEGA 11 (Tamura et al., 2021). The analysis was performed with the algorithm of RAxML v.8 (Stamatakis, 2014), including 1000 bootstrap replicates to infer the phylogenetic relationships. *Gerrhopilus mirus* (Jan, 1860) was included as an outgroup. Trees were visualised and assessed with bootstrap values by software Figtree (Rambaut, 2009). Based on the generated phylogeny trees, I estimated the average evolutionary distance within and between clades of each tree using the Maximum Composite Likelihood Model. All ambiguous positions were removed for each sequence pair through the pairwise deletion option. Standard errors were calculated by the bootstrap method.

To elucidate on the taxonomic relationship *I. braminus*, I prepared another set of congeneric sequences from the Genbank, consisting of *I. braminus* and *I. pammeces* from different localities (Table 3.4). With reference to the definition of *I. braminus* distribution in India by Sidharthan et al., (2022), I selected *I. braminus* sequences from the west coast of India (the range of *Indotyphlops* hybrid of *I. braminus* and *I. pammeces*, previously also known as *I. braminus*), the rest of India (the native range of *I. braminus*) and population outside of India. Together with the sequences of *I. braminus* population in Hong Kong collected from this study, phylogenetic trees for each chosen loci were constructed. *Indotyphlops albiceps* was chosen as the outgroup. The analysis was also performed with the same algorithm and software as above.

Table 3.4 Localities and accession number of sequences of *Indotyphlops* species downloaded from GenBank.

Species	Locality	Accession number in GenBank		
		Cytb	*BDNF*	*AMEL*
Indotyphlops albiceps	Tha Baik Kyin Township, Shwe U Daung Wildlife Sanctuary, Nyaung Gome Elephant Camp, Mandalay, Myanmar	KT316509.1	GU902382.1	GU902305.1
Indotyphlops braminus	Florida, USA	AY099990.1	GU902383.1	GU902306.1
	India	OP056476.1 OP056477.1 OP056480.1 OP056481.1 OP056483.1 OP056488.1 OP056492.1 OP056495.1 OP056496.1 OP056500.1 OP056502.1	MW442108.1 MW442109.1 MW442110.1 MW442111.1 MW442112.1 ON806731.1 ON806732.1 ON806733.1 ON806735.1 ON806736.1 ON806737.1 ON806739.1 ON806741.1 ON806743.1 ON806744.1 ON806745.1 ON806746.1 ON806747.1	MW442096.1 MW442097.1 MW442098.1 MW442099.1 MW442100.1 ON806706.1 ON806707.1 ON806708.1 ON806710.1 ON806711.1 ON806712.1 ON806714.1 ON806716.1 ON806718.1 ON806719.1 ON806720.1 ON806721.1 ON806722.1
	Madagascar	KT316545.1 KT316546.1		

		KT316547.1 KT316548.1		
	Phang-Nga City, MuangDistrict, Phang-Nga Province, Thailand		FJ433959.1	FJ434038.1
	Yunnan, China	DQ343649.1 NC010196.1		
Indotyphlops pammeces	India	OP056504.1 OP056506.1 OP056510.1 OP056511.1 OP056512.1	ON806749.1 ON806750.1 ON806752.1 ON806753.1 ON806754.1	ON806724.1 ON806725.1 ON806727.1 ON806728.1 ON806729.1
	Colombo or Uda Walawe National Park, Uda Walawe, Sri Lanka		GU902458.1	GU902378.1

<u>*3.4 Results*</u>

A total of 29 new sequences of three designated loci were obtained from 10 blind snake specimens. For the loci *AMEL*, *BDNF* and *Cytb*, 450, 700 and 400 base pairs were yielded, respectively. In total, 46, 52 and 48 consensus sequences were selected for constructing the phylogenetic trees of *AMEL*, *BDNF* and *Cytb,* respectively. In overview, the three generated phylogenetic trees shared a similar topology (Figure 3.2–3.4). *Indotyphlops albiceps*, *I. lazelli* and *I. braminus* from Hong Kong generally clustered separately in three clades with small differences in pairwise distances within each clade (<1.4%). All *I. braminus* and *I. albiceps* specimens from Hong Kong were a sister lineage, which was genetically distant from the clades containing *I. lazelli* and the only *I. albiceps* (GU902305.1) collected outside of Hong

Kong obtained from GenBank. Most of the clades were supported by strong bootstrap values of (>70%). Based on the tree lineage similarity, all consensus sequences were categorised into 10 clades (A_1–A_6, A_A, $A_{A \times B}$, $A_{B \times P}$, A_L for *AMEL* and B_1–B_6, B_A, $B_{A \times B}$, $B_{B \times P}$, B_L for *BDNF*) and 11 clades (C_1–C_7, C_{A1}, C_{A2}, C_B, C_L for *Cytb*). Overall, the standard errors of all the pairwise distance values above were between 0.75% to 4.20% (Table 3.5–3.7), showing little variation in the estimates of the average evolutionary divergence over sequence pairs between and within clades.

The phylogenetic tree for locus *AMEL* is in Figure 3.2. The majority of *I. albiceps* samples (from Hong Kong) formed a strongly supported clade (clade A_A, bootstrap = 99). The sister group to clade A_A was one comprised of *I. braminus* and *I. pammeces*, forming a strongly supported clade (clade $A_{B \times P}$, bootstrap = 95). In clade $A_{B \times P}$, *I. braminus* samples were collected in Hong Kong and elsewhere, including India, Thailand and the United States. Clades A_A and $A_{B \times P}$ were the sister group to clade $A_{A \times B}$, which was comprised of one *I. braminus* (from India) and *I. albiceps* (from Myanmar). The within-clade pairwise distances of clades $A_{A \times B}$, A_A and $A_{B \times P}$ (1.1–1.5%) were lower than between-clade distances (5.5–6.4%) (Table 3.5). The *I. lazelli* sample (clade A_L) was distantly related to other *Indotyphlops* species. The pairwise distances of *I. lazelli* and the other *Indotyphlops* clades (clades $A_{A \times B}$, A_A, and $A_{B \times P}$) were 5.4–7.9% (Table 3.5).

Figure 3.3 represents the phylogenetic tree for locus *BDNF*. Similar to *AMEL,* all *I. albiceps* samples from Hong Kong formed a robust clade (clade B_A, bootstrap = 100). Clade B_A was sister to clade $B_{B \times P}$, which comprised *I. braminus* samples from Hong Kong, India, Thailand

and the United States and a *I. pammeces* (bootstrap = 100). Together, clade B_A and $B_{B \times P}$ were sister groups to clade $B_{A \times B}$. Clade $B_{A \times B}$ was formed by the *I. albiceps* from Myanmar and an *I. braminus* specimen from India (bootstrap = 91). These three clades were more distant between each other (3.8–4.4%) than within clades (0.6–1.8%) (Table 3.6). Besides, clade B_L contained the only *I. lazelli* sample, which had a pairwise distance of 3.5–4.6% with other *Indotyphlops* clades (Table 3.6).

For the phylogenetic tree of locus *Cytb* (Figure 3.4), clade C_{A1} included *I. albiceps* from Hong Kong (bootstrap = 100). Clade C_{A1} formed a sister lineage to a large group, which was constituted of clade C_{A2}, C_B and C_3 (with a single *Ramphotyphlops lineatus*). Within this large group, clade C_{A2} referred to the *I. albiceps* specimen from Myanmar. Sister to clade C_{A2}, clade C_B included all *I. braminus* sequences (bootstrap = 90), which further diverged into two distinct sister lineages, one contained samples only from Hong Kong (bootstrap = 100); another consisted of individuals from China, India, Madagascar and the United States (bootstrap = 100). The pairwise distance within clade C_{A1}, C_{A2} and C_B maintains were 9.8%, which was lower than their between-clades distance (26.6–29.3%) (Table 3.7). Exceptionally, *I. lazelli* clusters with *R. acuticaudus* from Palau (clade $C_{L \times R}$, bootstrap = 26). The within-clade pairwise distance of clade $C_{L \times R}$ was 25%, which was similar to the distance between other *Indotyphlops* clades (clade C_{A1}, C_{A2} and C_B), 26.7–30.3% (Table 3.7).

For the phylogenetic trees of *I. braminus* and *I. pammeces*, 29, 30 and 27 consensus sequences were resulted for the loci *AMEL*, *BDNF* and *Cytb* respectively. The topology of the three phylogenetic trees was different. In the *AMEL* tree, *I. braminus* from different ranges and *I.*

pammeces did not have an obvious segregation (Figure 3.5). In the *BDNF* tree, the sequences clustered into three major clades (Figure 3.6), one comprising of *I. pammeces* in India, second consisting of *I. braminus* in native range of India and one *I. pammeces* (GU902458.1) from Sri Lanka; and third consisting of *I. braminus* in other parts of the world (including Hong Kong), the hybrid range of India and, one sequence (ON806741.1) from native range in India. And for the *Cytb* tree, there were two major clades formed (Figure 3.7), one of which was composed of *I. pammeces*, *I. braminus* outside India (including Hong Kong) and from their hybrid range in India, whereas sequences of the native *I. braminus* in India were nested in another clade.

Table 3.5 The numbers of base substitutions per site from averaging over all sequence pairs between clades of the *AMEL* tree are shown. The pairwise differences within clades are shown on the diagonal (in grey). Standard error estimates (blue) are shown above the diagonal. All values are corrected to 3 decimal places.

Clade	A_1	$A_{A \times B}$	A_A	$A_{B \times P}$	A_2	A_L	A_3	A_4	A_5	A_6
A_1	0.018	0.015	0.018	0.017	0.014	0.015	0.016	0.018	0.021	0.031
$A_{A \times B}$	0.067	0.015	0.014	0.014	0.012	0.014	0.017	0.017	0.021	0.030
A_A	0.084	0.060	0.014	0.015	0.014	0.018	0.019	0.020	0.022	0.032
$A_{B \times P}$	0.079	0.055	0.064	0.011	0.013	0.017	0.018	0.020	0.023	0.034
A_2	0.076	0.058	0.077	0.066	0.045	0.013	0.017	0.017	0.021	0.030
A_L	0.061	0.054	0.079	0.075	0.063	n/c	0.018	0.017	0.020	0.029
A_3	0.072	0.078	0.101	0.094	0.092	0.074	0.004	0.017	0.020	0.032
A_4	0.091	0.086	0.108	0.106	0.096	0.078	0.087	0.022	0.018	0.028
A_5	0.105	0.100	0.113	0.119	0.114	0.090	0.103	0.088	n/c	0.030
A_6	0.173	0.177	0.191	0.206	0.185	0.149	0.184	0.158	0.175	n/c

Table 3.6 The numbers of base substitutions per site from averaging over all sequence pairs between clades of the *BDNF* tree are shown. The pairwise differences within clades are shown on the diagonal (in grey). Standard error estimates (blue) are shown above the diagonal. All values are corrected to 3 decimal places.

Clade	B_1	B_2	B_L	B_3	$B_{A \times B}$	$B_{B \times P}$	B_A	B_4	B_5	B_6
B_1	0.015	0.008	0.007	0.007	0.007	0.009	0.009	0.008	0.007	0.011
B_2	0.041	0.012	0.009	0.008	0.009	0.010	0.010	0.009	0.008	0.011
B_L	0.036	0.049	n/c	0.007	0.008	0.009	0.009	0.009	0.008	0.013
B_3	0.039	0.046	0.036	0.003	0.005	0.007	0.008	0.008	0.008	0.012
$B_{A \times B}$	0.039	0.053	0.035	0.029	0.006	0.008	0.008	0.009	0.009	0.013
$B_{B \times P}$	0.049	0.060	0.046	0.039	0.039	0.002	0.009	0.010	0.008	0.013
B_A	0.047	0.058	0.043	0.041	0.038	0.044	0	0.010	0.009	0.014
B_4	0.041	0.047	0.046	0.044	0.048	0.056	0.053	0.002	0.008	0.012
B_5	0.031	0.040	0.036	0.040	0.041	0.043	0.036	0.036	0	0.012
B_6	0.065	0.064	0.078	0.077	0.083	0.087	0.090	0.067	0.0696	n/c

Table 3.7 The numbers of base substitutions per site from averaging over all sequence pairs between clades of the *Cytb* tree are shown. The pairwise differences within clades are shown on the diagonal (in grey). Standard error estimates (blue) are shown above the diagonal. All values are corrected to 3 decimal places.

Clade	C_1	$C_{L\,x\,R}$	C_2	C_{A1}	C_{A2}	C_B	C_3	C_4	C_5	C_6	C_7
C_1	0.014	0.034	0.035	0.039	0.044	0.039	0.038	0.033	0.031	0.032	0.036
$C_{L\,x\,R}$	0.272	0.250	0.034	0.033	0.035	0.035	0.038	0.032	0.030	0.032	0.036
C_2	0.297	0.300	0.222	0.038	0.035	0.034	0.038	0.034	0.033	0.033	0.039
C_{A1}	0.291	0.267	0.316	0	0.036	0.036	0.036	0.032	0.033	0.032	0.045
C_{A2}	0.327	0.278	0.306	0.266	n/c	0.036	0.041	0.033	0.037	0.037	0.045
C_B	0.307	0.303	0.306	0.270	0.293	0.014	0.039	0.033	0.030	0.036	0.042
C_3	0.274	0.311	0.313	0.299	0.307	0.307	n/c	0.037	0.039	0.034	0.042
C_4	0.292	0.296	0.315	0.288	0.289	0.306	0.318	0.022	0.030	0.033	0.041
C_5	0.253	0.271	0.302	0.270	0.297	0.267	0.296	0.279	0.018	0.030	0.037
C_6	0.267	0.289	0.311	0.266	0.312	0.323	0.286	0.316	0.274	0.017	0.036
C_7	0.262	0.290	0.320	0.331	0.331	0.322	0.304	0.347	0.299	0.298	n/c

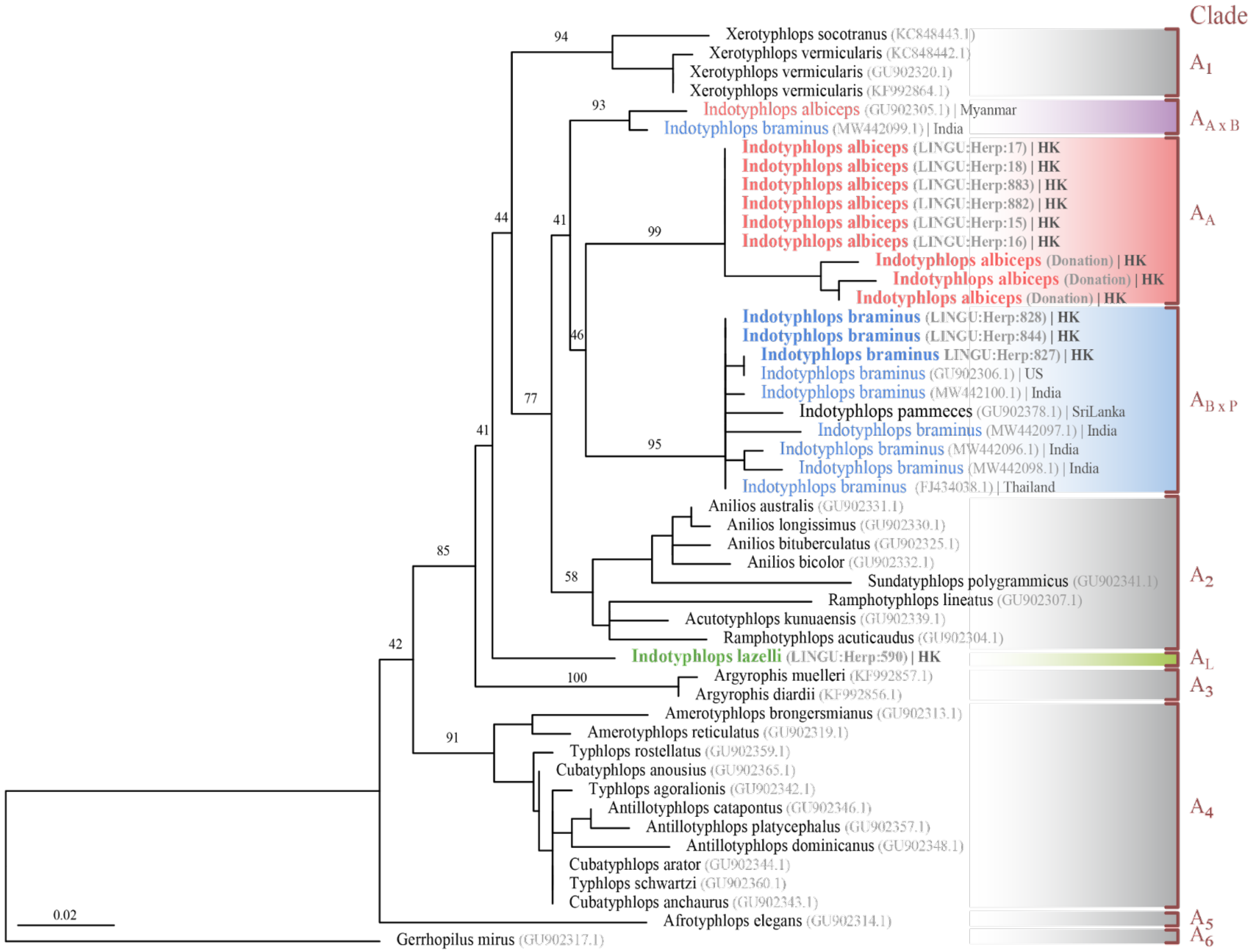

Figure 3.2 Phylogenetic tree generated with Maximum Likelihood analysis for *AMEL* loci. Numbers at nodes are bootstrap values, while the scale represents branch length. Bold indicates samples from Hong Kong. Word colour: Red: *Indotyphlops albiceps*; Blue: *I. braminus*; Green: *I. lazelli*.

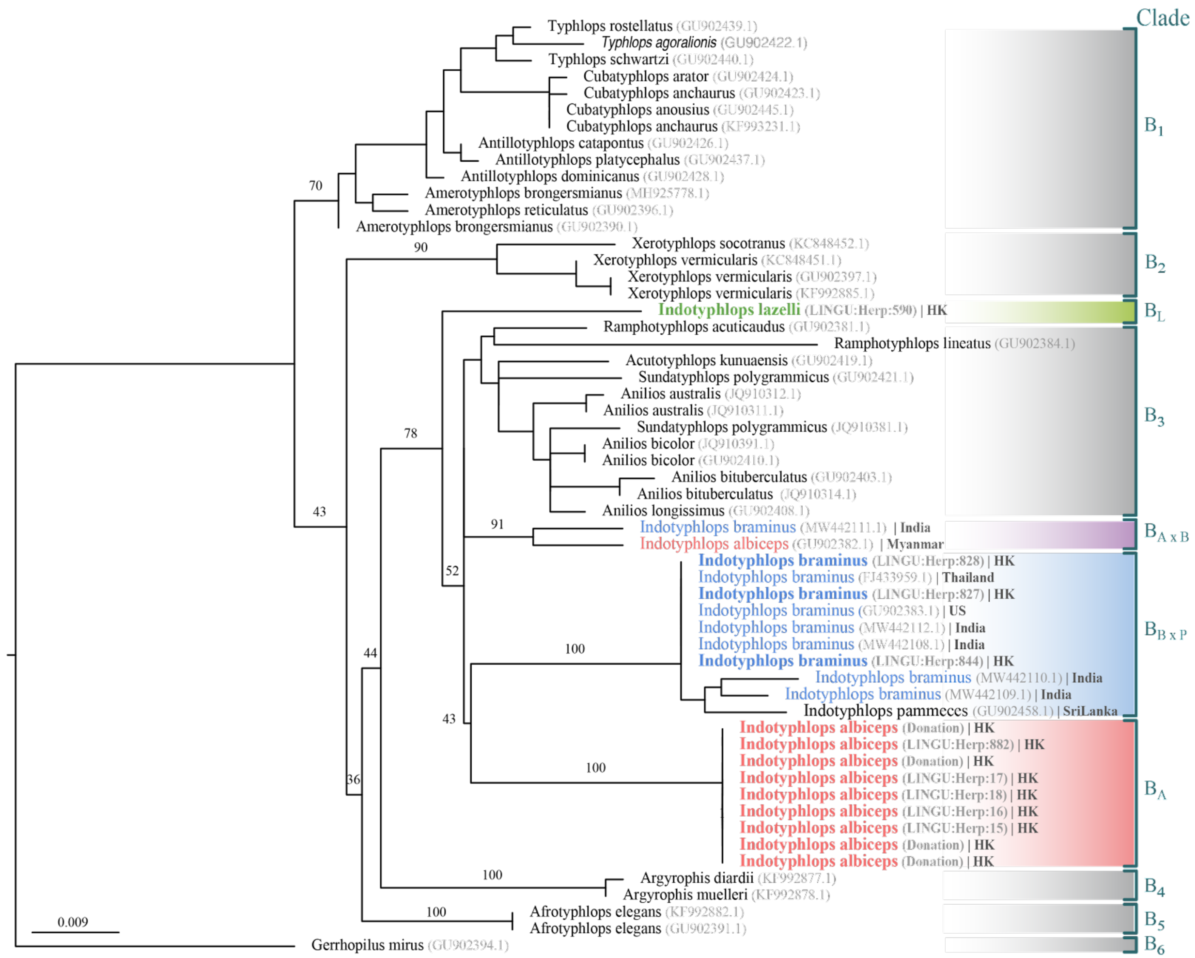

Figure 3.3 Phylogenetic tree generated with Maximum Likelihood analysis for *BDNF* loci. Numbers at nodes are bootstrap values, while the scale represents branch length. Bold indicates samples from Hong Kong. Word colour: Red: *Indotyphlops albiceps*; Blue: *I. braminus*; Green: *I. lazelli.*

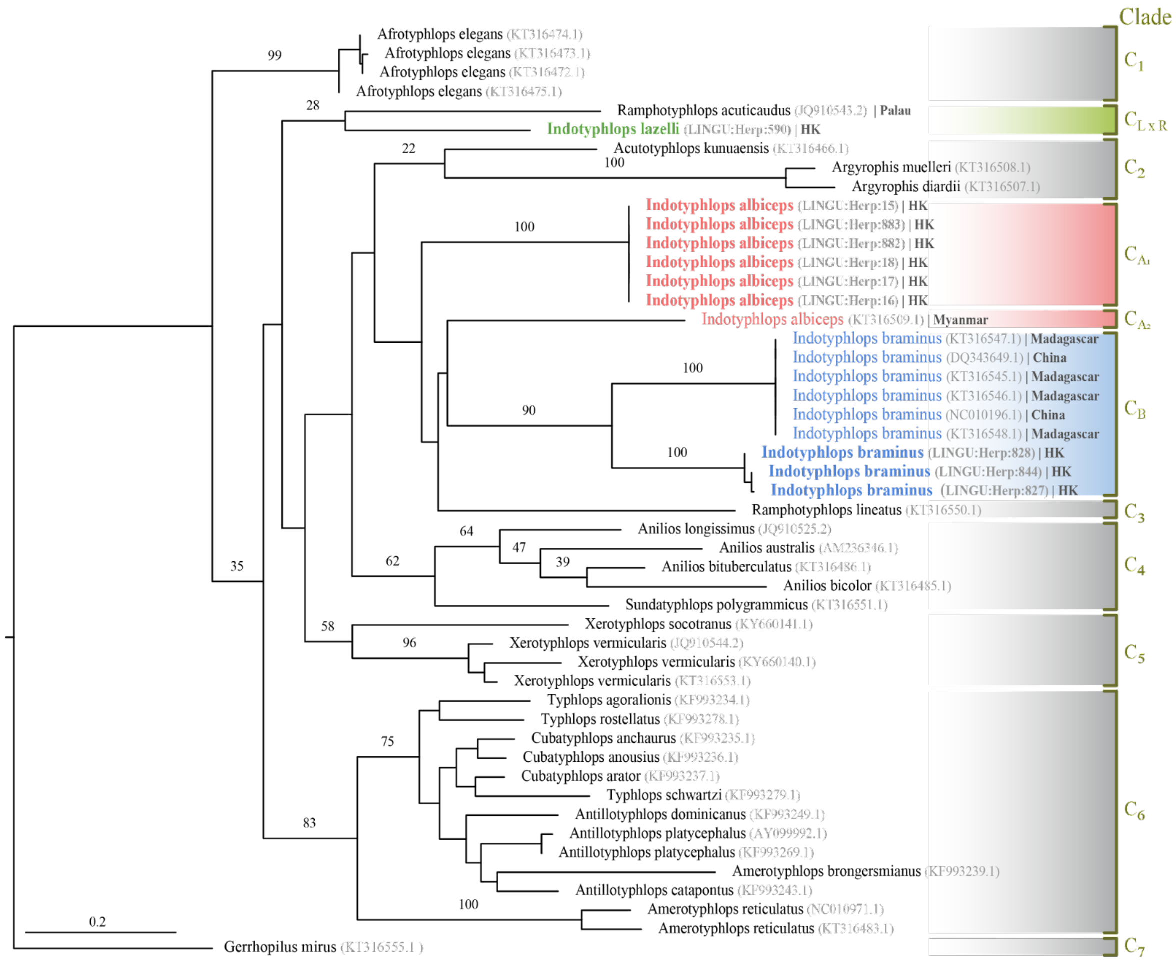

Figure 3.4 Phylogenetic tree generated with Maximum Likelihood analysis for *Cytb* loci. Numbers at nodes are bootstrap values, while the scale represents branch length. Bold indicates samples from Hong Kong. Word colour: Red: *Indotyphlops albiceps*; Blue: *I. braminus*; Green: *I. lazelli.*

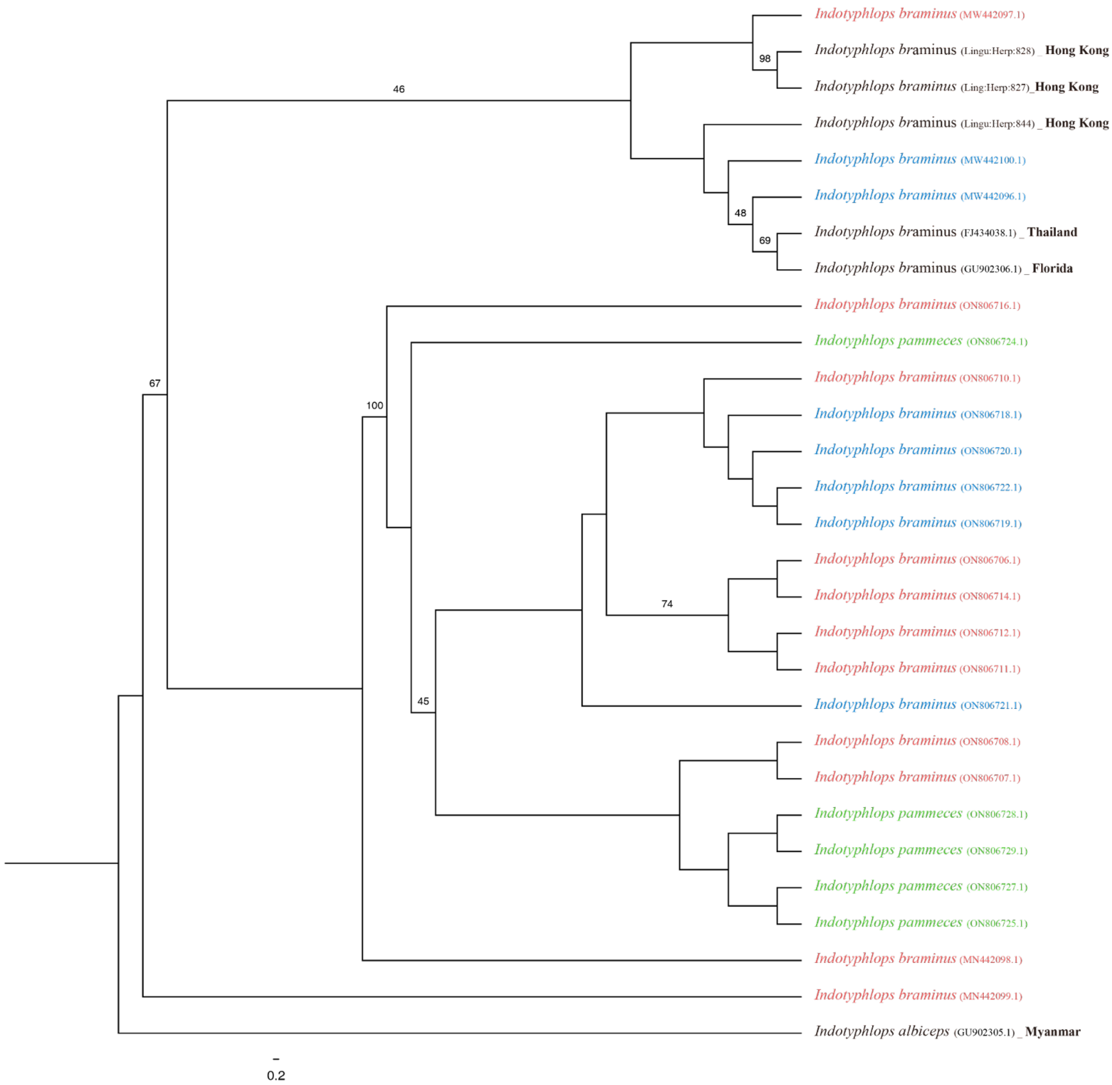

Figure 3.5 Phylogenetic tree of *I. braminus* and *I. pammeces* generated with Maximum Likelihood analysis for *AMEL* loci. Numbers at nodes are bootstrap values, while the scale proportionally represents branch length. Red: *I. braminus* from native range in India; blue: *I. braminus* from hybrid range in India; green: *I. pammeces* from its native range; black: *I. braminus* outside India.

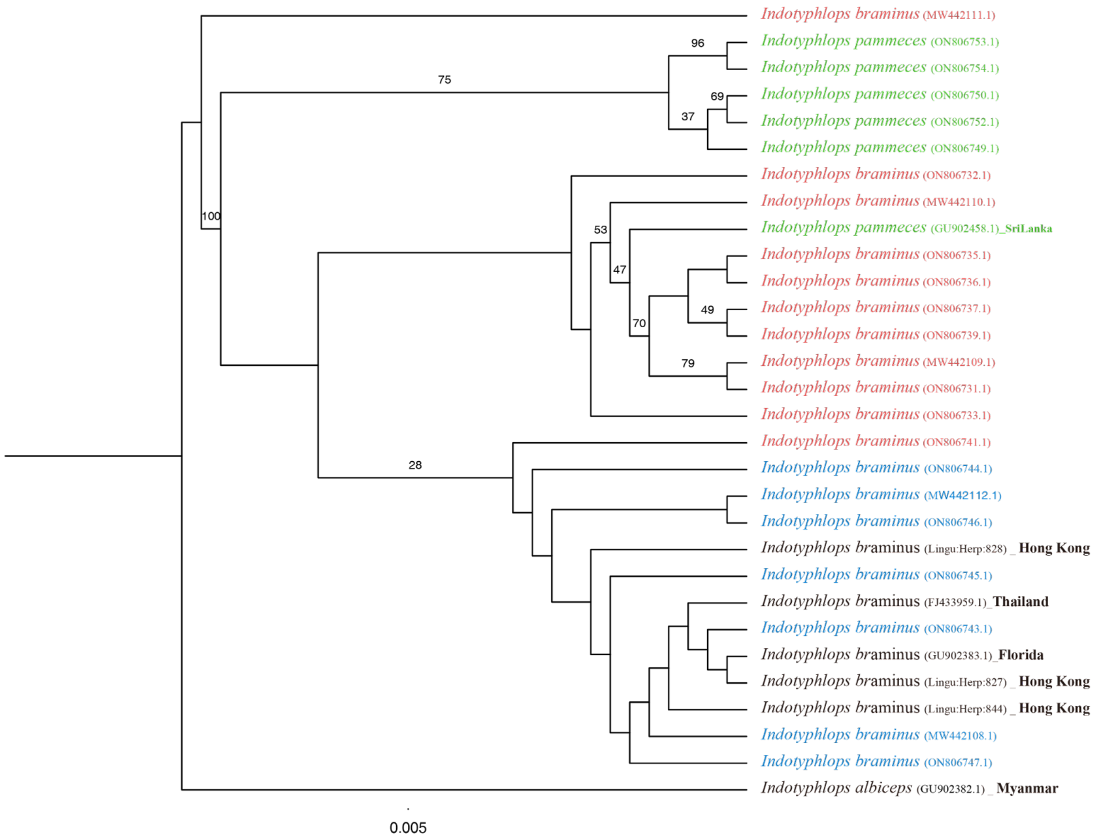

Figure 3.6 Phylogenetic tree of *I. braminus* and *I. pammeces* generated with Maximum Likelihood analysis for *BDNF* loci. Numbers at nodes are bootstrap values, while the scale proportionally represents branch length. Red: *I. braminus* from native range in India; blue: *I. braminus* from hybrid range in India; green: *I. pammeces* from its native range; black: *I. braminus* outside India.

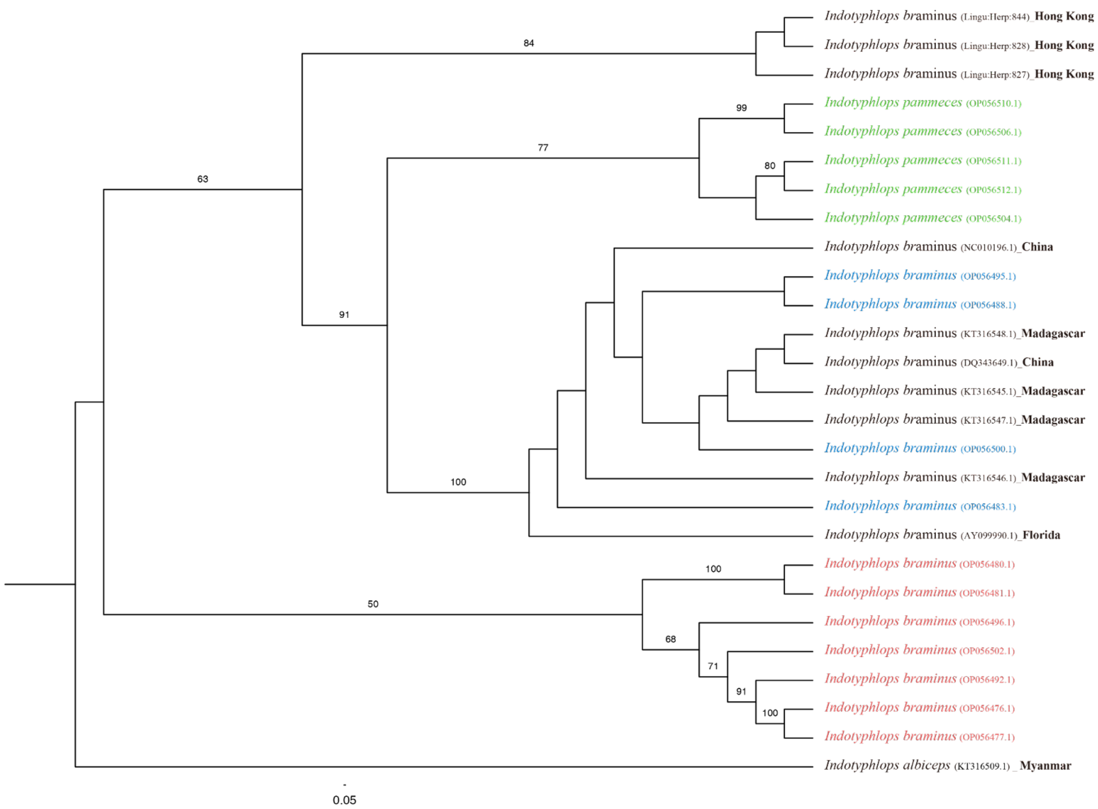

Figure 3.7 Phylogenetic tree of *I. braminus* and *I. pammeces* generated with Maximum Likelihood analysis for *Cytb* loci. Numbers at nodes are bootstrap values, while the scale proportionally represents branch length. Red: *I. braminus* from native range in India; blue: *I. braminus* from hybrid range in India; green: *I. pammeces* from its native range; black: *I. braminus* outside India.

3.5 Discussion

In this study, I used phylogenetic approach to resolve the taxonomy of blind snakes in Hong Kong. In particular, I found that the *I. albiceps* from Hong Kong are genetically distinct from *I. albiceps* from Southeast Asia, and is potentially a cryptic species. Furthermore, I provided the first genetic sequence of the *I. lazelli* and further confirmed that it is a valid species, or even another genus, given its genetic difference with other *Indotyphlops* species. I discuss the findings of this study in more detail below.

3.5.1 Establishment of phylogenetic status of blind snake species in Hong Kong

The genetic analysis validated the phylogenetic status of the three targeted blind snakes in Hong Kong. In the phylogenetic trees of the three chosen loci *AMEL*, *BDNF* and *Cytb*, *I. albiceps*, *I. braminus* and *I. lazelli* separated into three monophyly clades. The three clades were supported by strong bootstrap value (> 95%) with relative small discrepancies within their clades (< 1.4% for *AMEL*; < 0.6% for *BDNF*; and < 9.81% for *Cytb*). In general, as a reference, the genetic distance between clades of described species were about 16–24% for *Cytb* (John & Avise, 1998), and were >0.27% for nuclear DNA sequences (for example *AMEL* and *BDNF*) (Miralles et al., 2016; Petzold & Hassanin, 2020; Sidharthan et al., 2022). In practical, the minimum pairwise distances among the three clades of the three target species were 26.7% for *Cytb*; 6.4% for *AMEL*; and 4.3% for *BDNF*, which are greater than those among described species. This implied that the three blind snake species from Hong Kong were phylogenetically separated and distinct from each other, reinforcing their distinct species identity.

3.5.2 Indotyphlops albiceps from Hong Kong represents a potential new species

The genetic results suggested that the *I. albiceps* population in Hong Kong is a single, new species. First, the Hong Kong populations and Myanmar population were positioned in a monophyletic way with other blind snake sequences. The minimum pairwise distance between the *I. albiceps* populations and other clades was 3.58%, which was higher than the distance between described species for the same loci *BDNF*, e.g. 3.17% between *I. braminus* and *I. albiceps* in Sidharthan et al. (2022). Specifically, the *I. albiceps* sample from Myanmar formed a clade with another *I. braminus* sample in India in both *AMEL* (clade $A_{A \times B}$) and *BDNF* (clade $B_{A \times B}$) trees. These may represent a possible species misidentification, which is further discussed below. Second, the *I. albiceps* specimens from Hong Kong are genetically distinctive (pairwise distance 5.96% for *AMEL*; 3.84% for *BDNF;* 26.6% for *Cytb*) from that from Myanmar (GenBank accession number: GU9023053.1). The latter sample is the only *I. albiceps* specimen available in GenBank. This sample was collected from the Shwe U Daung Wildlife Sanctuary in Mandalay, Myanmar, which is close (approximately 120 km) to the type locality of *I. albiceps*, Chanthaburi in southeast Thailand. Further, *I. albiceps* collected from Hong Kong exhibited low genetic variation (pairwise distances: *AMEL = 1.4%, BDNF* = 0%, *Cytb* = 0%) among each other and phylogenetically grouped into a clade with strong support (bootstrap support > 99%). In other words, the *I. albiceps* in Hong Kong population are highly identical in phylogeny, further supporting that the *I. albiceps* in Hong Kong represents a single species. Altogether, *I. albiceps* from Hong Kong represent a potential cryptic, new species. To verify this, more genetic samples from across its native range, also a detailed morphological and osteological examination of the *I. albiceps* and the holotype of specimen from Hong Kong and the type locality is needed (Hawlitschek et al., 2021).

3.5.3 Indotyphlops lazelli is a genetically distinct species

For *I. lazelli*, I obtained the first genetic sequence of this species and compared it with other blind snake species. The genetic results showed that the *I. lazelli* was segregated from other congeners. The clade of *I. lazelli* has larger minimum pairwise distances (5.36% for *AMEL*; 3.49% for *BDNF*; 26.7% for *Cytb*) with other clades than that between described species (e.g. 4.43 for *AMEL* and 3.17 % for *BDNF* between *I. braminus* and *I. albiceps*) (Sidharthan et al., 2022). Although *Ramphotyphlops acuticaudus* clustered with *I. lazelli* in the *Cytb* tree, however, the pairwise distance between these two species was high (25.0%), suggesting that they are distinctive species.

Indotyphlops lazelli appears to be more closely related to other genera. Coincidentally, *I. lazelli* was distant from the congeneric group, including *I. braminus*, *I. albiceps* and *I. pammeces*, in the *AMEL*, *BDNF* and *Cytb* trees whereas it was sister to the clades containing species of other genera, including *Acutotyphlops*, *Anilios*, *Ramphotylops* and *Sundatyphlops*. Such results imply that *I. lazelli* may potentially belong to another genus which require further phylogenetic studies upon collection of more specimens.

3.5.4 Phylogenetic relationship between Indotyphlops braminus populations in Hong Kong and elsewhere

I found that the Hong Kong specimens of *I. braminus* were generally clustered with overseas sequences (including China, Florida and Madagascar) in a clade, but segregated from populations in India in *AMEL* and *BDNF* trees whereas the topology was different for *Cytb*. The discordance among the phylogenetic trees topologies of mitochondrial and nuclear markers may be a consequence of hybridization (Funk & Omland, 2003) as the genetic materials from the parents of different species may be partially inherited to their hybrid descendant. Specifically, the Hong Kong populations were nested with the *Indotyphlops* hybrids in the *BDNF* and *Cytb* trees. This is coherent to the results in Sidharthan et al. (2022), which proposed that populations of *I. braminu*s outside of native range in India were *Indotyphlops* hybrids of *I. braminus* and *I. pammeces*. As such, the *I. braminus* population in Hong Kong possibly belongs to the hybrid descendant. Further taxonomic studies including additional samples of *I. braminus* and *I. panmmeces* across Southeast and South Asia will be useful in verifying the native range of *I. braminus* and the origin of Hong Kong population. Direct chromosomal analysis on the local specimens to examine the presence of triploid females derived from the obligate parthenogenesis, which often associated with interspecific hybridization, will also be helpful (Ota et al., 1991; Patawang et al., 2016; Wynn et al., 1987).

3.5.5 Possible misidentification of specimens from database

Our results show that there may be misidentification of species identity deposited in GenBank. Misidentified specimens and sequences is problematic for taxonomic research but is a common problem (e.g. Maia et al., 2016; Vasconcelos et al., 2016). Usually, taxonomic groups with unresolved taxonomy and/or insufficient sampling effort are prone to misidentification. In this study, I found that the sequences of *I. albiceps* from Myanmar clustered with that of an *I. braminus* in the *AMEL* (MW442099.1) and *BDNF* (MW442111.1) trees. The *I. braminus* was collected from Tamenglong, Manipur of India and stated as *Indotyphlops sp.* in Sidharthan & Karanth (2021). This *I. braminus* specimen was separated from all other *I. braminus* sequences and have a small genetic difference (<1.5% for *AMEL*; <1.8% for *BDNF*) with the *I. albiceps* from Myanmar. The result suggested that the *I. braminus* specimen may belong to *I. albiceps*. If possible, further morphological examination of this *I. braminus* specimen is desirable, which will help future taxonomic studies of the genus *Indotypholops* and also help assess the distribution of *I. albiceps* which have not been recorded in India.

Incidentally, there is an inclusion of *I. pammeces* (GU902378.1 in *AMEL* and GU902458.1 in *BDNF*) in the monophyletic clade of *I. braminus*. Previous study by Sidharthan et al. (2022) discussed the taxonomic relatedness of *I. pammeces* and *I. braminus*. Unlike the *albiceps-braminus* clade, they are phylogenetically similar to each other by only <0.277 % (*AMEL*) and <0.794 % (*BDNF*). Morphologically, they share similar characters with a subtle distinction in their scale arrangement. Therefore, the *I. pammeces* in the *I. braminus* clade may not represent a misidentification of sequence but a close phylogenetic hierarchy between two *Indotyphlops* species. The *braminus-pammeces* composition in this study recalled the nested phylogeny in the clade of Indian *Indotyphlops* in Sidharthan et al. (2022) and also the significance of incorporating morphological features in species differentiation.

3.5.6 Phylogenetic tool for blind snake species delimitation in Hong Kong

The multi-locus information incorporated in this study can be a reliable species delimitation tool for blind snake species in Hong Kong. In this study, both nuclear and mitochondrial markers were taken into consideration. The difference in the distance values of various DNA segments can be explained by the rate of molecular evolution at different sequences of genome varying across taxa, populations and individuals (Will & Rubinoff, 2004). Therefore, involvement of multiple DNA segments could widen the coverage over the diversity within lineages. To delimit species based on phylogenetic structure and a distance-based approach, the pairwise distance calculated from both nuclear and mitochondrial genetic materials may result differently by taxa. But the result demonstrated a high level of concordance between the nuclear and mitochondrial DNA markers used for inter-species phylogenetic reconstruction (Metzger et al., 2010). Supported by the pairwise distance results between groups, the three lineages of local blind snake species were phylogenetically distinguishable. This underlined the applicability of phylogenetic analysis in blind snake species delimitation in Hong Kong.

3.6 Conclusion

In short, *I. albiceps* is a potential cryptic species isolated from the Southeast Asian population which require further examination of morphology and anatomy to describe the species. The species validity of *I. lazelli* is consolidated and the taxonomy status of species needs further study because my data showed this species is genetically closer to other genus than *Indotyphlops.* Lastly, the *I. braminus* populations in Hong Kong is potentially hybrid of *I. braminus* and *I. pammeces*, revealing their non-native origin.

CHAPTER 4 - CONSERVATION IMPLICATIONS

In this study, I was able to detect two target species of fossorial reptiles (*I. albiceps* and *I. braminus*) in three study sites (Pok Fu Lam, Sunshine Island, Lady Clementi's Ride) through intensive field surveys using three sampling methods (quadrat search, artificial refuges and opportunistic search). My results showed that artificial refuge can be applied to long-term study with sufficient manpower, while active search (quadrat search and opportunistic search) is a more effective tool for short-term study. I also found correlations between encounter rate and some of the environmental parameters (gradient, canopy cover and ambient humidity), which can help optimise future ecological surveys. Since *D. bogadeki* and *I. lazelli* could not be detected in this study, it is important to explore novel sampling methods and carry further surveys to determine their distribution and population status. On the other hand, I carried out a phylogenetic study to clarify the taxonomic status and phylogenetic relationship of *I. lazelli*, *I. albiceps* and *I. braminus*. I found that *I. albiceps* is a potential new species and *I. lazelli* is a genetically valid species. In the following sections, I will provide specific recommendations for conservation of fossorial reptiles in Hong Kong based on the results of this study and suggest further studies to fill the vast knowledge gaps in ecology of fossorial reptiles.

4.1 Optimisation of ecological surveys methods in ecological impact assessment (EIA)

The environmental impact assessment (EIA) process is a gatekeeping mechanism to assess and mitigate the environmental impacts of development projects. In Hong Kong, the implementation of EIA complies with the Environmental Impact Assessment Ordinance (Cap. 499). The implementation of EIA follows the instructions and recommendations listed in the EIA technical memorandum (EPD, 2011a). To comprehensively assess the ecological impacts of a development project, the project proponent should carry out a robust assessment of the ecological community, particularly species diversity, and abundance of species of conservation concern (EPD, 1997). However, neither the technical memorandum nor guidance notes provide guidelines on survey methods specified for fossorial reptiles, which may be overlooked using conventional survey methods for reptiles. Given the critical conservation status of fossorial reptiles (including *I. albiceps*, *I. lazelli* and *D. bogadeki*) in Hong Kong, specific guidelines should be given for sampling threatened species of fossorial reptiles in EIA. This study complies with Action 9d to "enhance the practices in addressing ecological impacts of projects through EIA process" under Hong Kong Biodiversity Strategy Action Plan 2016-2021 (Environment Bureau, 2016) by providing recommendations to enhance current assessment methods. Based on the results of this study, I provide three specific suggestions to optimise ecological surveys in EIA below.

4.1.1 Survey methods

In this study, I was able to sample the target species using both active search (quadrat search and opportunistic search) and coverboards (artificial refuges). Many studies have shown that using multiple survey methods are crucial for comprehensively assessing reptile diversity (Doan, 2003; Hutchens & DePerno, 2009; Measey, 2006; Sung et al., 2011). Given that active search is a common survey method to sample reptiles in EIA studies in Hong Kong, I recommend adding the application of coverboards for study sites where threatened fossorial reptiles are recorded in the past. In a previous project on Integrated Waste Management Facilities at Shek Kwu Chau, where *D. bogadeki* occurs, the EIA study applied coverboards to sample the fossorial reptiles (AECOM, 2014). Similar approach should be adopted, particularly in other areas with records of threatened fossorial reptiles, for example other outlying islands and Hong Kong Island, which will help increase the detection rate of the target species. A previous study found that Red Imported Fire Ant (*Solenopsis invicta*) can colonise coverboards, turning the coverboard from a refuge to a trap for herpetofauna (Todd et al., 2008). Also, in this study, coverboards were frequently unearthed by Wild Boars (*Sus scrofa*). Therefore, special attention should be paid when applying coverboards to sites with the presence or record of Fire Ants and Wild Boars.

In many cases, surveys are limited in terms of efforts and duration for EIA studies. The application of coverboards is labour-intensive and requires at least three months for acclimatisation before fossorial reptiles utilise the coverboards. It is recommended that if manpower is limited and/or the survey period is less than a year, active search is employed over coverboards. To increase the chances of detecting fossorial reptiles, during active search, surveyors are highly recommended to turn over objects, such as rocks and fallen logs, and to search through leaf litter. Additionally, although time-constrained opportunistic search could not provide detailed ecological information, it is useful for rapid detection of the presence or absence of the species at sites during the survey.

4.1.2 Survey period and number of surveys to be conducted

In most cases, efforts to assess biodiversity are constrained by availability of the time for comprehensive sampling, and thus rapid biodiversity assessments are often used in unexplored or poorly known areas (Sung et al., 2011). Presumably, blind snakes have a seasonal activity pattern that they mate in spring, oviposit in mid-summer, and hatch in autumn (Shine & Webb, 1990). In the study, I detected a seasonality that higher encounter rates were recorded during the wet season (April to October), with one-third of all encounters occurring in July. Therefore, for surveys to study or detect blind snakes, sampling should be conducted mainly in the wet season particularly July, during which there was a higher chance to detect blind snakes in this study.

Besides, my results also advise the number of surveys required to detect fossorial reptiles using different methods. On average, to detect one individual of *I. albiceps* or *I. braminus*, it took 14.1 man-hours for artificial refuges, 22.4 man-hours for quadrat search and 13.6 man-hours for opportunistic search respectively. Therefore, for example, for a daytime or night survey that normally lasts 3–5 hours, at least 4 surveys using artificial refuges are needed to yield one encounter. It is reminded that the detection rates could sometimes be site-specific, and certain methods may be more effective at certain sites. Since no *I. lazelli* and *D. bogadeki* was detected in the field study, more survey effort and/or employing novel sampling methods may be required to detect these two species.

4.1.3 Regular review of guidance notes

Many studies have shown the shortcomings of ecological surveys conducted for EIA projects (Jefferson et al., 2009; Tam, 2007; Teng, 2010). In particular, the guidance notes relevant to ecological assessment of EIA (GN 6/2010, GN 7/2010, GN 10/2010, GN 11/2010), which were issued in 2010 (EPD, 2011b), should be regularly reviewed and updated based on previous EIA studies and scientific studies. I suggest that the guidance notes should provide additional information on sampling fossorial reptiles to the project proponents with reference to the suggestions made in this study, such that the impacts on threatened fossorial reptiles can be comprehensively assessed and mitigated. The consistency of the survey methods used for different projects could yield comparable results that give a more comprehensive evaluation on the vulnerability of the target species to the proposed development project.

4.2 Conservation actions for native fossorial reptiles

The results from the phylogenetic analysis suggest that the *Indotyphlops albiceps* population in Hong Kong is a new species. To validate this, further morphological studies to differentiate the Hong Kong and other populations are needed. If the Hong Kong populations represent a new species, the new species is likely an endemic species to Hong Kong because there has been no record of *I. albiceps* in China yet. With such a restricted distribution and low density, the new species would be a globally threatened species, which warrants immediate conservation actions.

For *Indotyphlops lazelli*, this study provides genetic evidence supporting that it is a valid species, reinforcing the need for more conservation efforts in conserving this extremely rare species, which is now listed as Critically Endangered under the IUCN Red List (Lau, 2012) and China Biodiversity Red List (Jiang et al., 2016).

Although intensive survey efforts were spent in field study, no *I. lazelli* and *D. bogadeki* was detected in the field study, even within their known distribution areas. The survey effort spent in this study is significantly greater than previous studies which targeted on the same species. *D. bogadeki* is another species of threatened endemic fossorial reptile, which is now listed as

Endangered under the IUCN Red List (Yang, 2019). Therefore, it is of conservation concern that *D. bogadeki* and *I. lazelli* could not be detected in this study.

I suggest conservation recommendations for these three native fossorial species below.

4.2.1 Conservation actions for Indotyphlops albiceps, I. lazelli and Dibamus bogadeki

4.2.1.1 Territory-wide surveys with the aid of citizen science

Intensive territory-wide surveys should be carried out to determine the distribution of *I. albiceps* (or the new species) and *I. lazelli*. All historical records (>10 years ago) of these two species are from Hong Kong Island (Wallach & Pauwel, 2004), with several sporadic records outside their known distribution in Hong Kong, one from the north-western part of the Kowloon Peninsula, and two from the central New Territories of Hong Kong (Duncan Cheung, personal communication, April 29, 2022; Ray So, personal communication, n.d.; Pan Lau, personal communication, n.d.), revealing that their populations may not be as isolated and scattered as we previously believed. Although previous studies described that these three species prefer woodland habitat, two of the historic sightings were in an urbanised site (Wallach & Pauwel, 2004), which are consistent with the three new sightings occurring in peri-urban or urban areas. They were found in fragmented green areas with a matrix of plantations and artificial facilities, hiding under the stones or on concrete roads near planters. These new sighting records suggested that these species may persist in developed areas with suitable substrate for example soil in planters. Therefore, conducting systematic and comprehensive territory-wide surveys in a diversity of habitats, particularly urban areas and peri-urban areas (e.g. edge of country parks) will yield useful important to assess and update the conservation status *I. albiceps* and *I. lazelli*.

Surveys for *D. bogadeki* should be extended to other known distributed sites or even potential distributions. Due to the restricted access of Hei Ling Chau and Shek Kwu Chau, where correctional institutes and rehabilitation centres are located (Correctional Services Department, 2021; The Society for the Aid and Rehabilitation of Drug Abusers, 2019), recent surveys of *D. bogadeki* were mostly conducted on Sunshine Island. At the beginning of this study, I explored the possibility of conducting surveys in Hei Ling Chau and Shek Kwu Chau, but because of security reasons and the outbreak of COVID-19, I was unable to gain access to these two islands. With the launch of large-scale development project, including the Integrated Waste Management Facilities (i.e. incinerator) at Shek Kwu Chau and Lantau Tomorrow Vision, it is suggested that future surveys could be carried out at these two sites to deepen our understanding on the population status of *D. bogadeki* across its distribution.

I suggest that the territory-wide surveys can also be conducted through collaboration with scientists working on soil or other fossorial species. To further increase detection rate, surveys could be conducted with the aid of citizen science. Citizen science, which involves volunteers to collect data and/or to resolve questions collaboratively with scientists, has proven to be a powerful tool in monitoring environmental changes and biodiversity (MacPhail & Colla, 2020; Pocock et al., 2018; Tulloch et al., 2013). Overseas studies have demonstrated that coverboards, which is one of the traditional techniques on sampling fossorial reptiles (Henderson et al., 2016), could be used in combination with a citizen science approach for data collection in studies on herpetofauna diversity (Pittman & Dorcas, 2006; Wittmann et al., 2019). In recent years, nature studies (i.e. observation of wildlife) has become the most popular recreational activity associated with nature in Hong Kong, exceeding the number of people participating in hiking, camping and picnicking (Cheung, 2013). Additionally, owing to the outbreak of COVID-19 in 2020, there has been a growing number of people visiting protected areas to relieve stress emerging from the pandemic (Ma et al., 2021). It is expected that more people will be interested in wildlife watching, or even willing to take part in citizen science programs. Universities and NGOs have been initiating citizen science programs targeting different taxa, including some understudied groups, such as spiders (Outdoor Wildlife Learning Hong Kong, 2021), sea slugs (Chow et al., 2022), millipedes (So et al., 2022). These studies yielded new species and distribution records in Hong Kong. With reference to these successful experiences from overseas and local programs, organising a citizen science program on collecting sighting records of fossorial reptiles would be helpful in gathering distribution and ecological data, as well as arousing public awareness on the conservation of fossorial reptile in Hong Kong.

Based on findings from the territory-wide surveys, relevant government departments should formulate specific conservation measures for habitats according to the most updated distribution to protect the two species. The surveys would clarify whether these newly discovered populations are situated within or outside protected areas (i.e. country parks and special areas). If they are located inside protected areas, the government is primarily responsible for conserving the species through habitat management and monitoring. If not, with reference to the mechanism of Public-Private Partnership (PPP) or Management Agreement (MA) under New Nature Conservation Policy, similar conservation measures on private lands could be adopted (AFCD, 2022e).

4.2.1.2 Provision of legal protection of the species

The Hong Kong government should review and revise the list of protected species and include the three species, *I. albiceps*, *I. lazelli* and *D. bogadeki* in the list. To protect rare, endemic and threatened species, it is expected that the extent of legal protection should be consistent according to the conservation status of species. Whitfort et al. (2013) reviewed the laws related to protection of wild animals and plants in Hong Kong and found that since 1996, there have been no amendments on the list of protected wild animals, Schedule 2 under Wild Animals Protection Ordinance (Cap.170). This list (Schedule 2) is an official document that is used as a reference for species of conservation priority. If the list is outdated, threatened species may be overlooked and do not receive appropriate protection, for example being susceptible to hunting and overlooked in the EIA process.

The importance of providing legal protection to these three species could be further explained by the case of High West Site Development of The University of Hong Kong (HKU). There used to be a co-occurrence of the three local blind snake species in one of the campuses of HKU (Wallach & Pauwel, 2004). Unfortunately, in 2018, HKU initiated a development for two student residences, together with the redevelopment of staff quarters at the site (Andrew Lee King Fun & Associates Architects, 2021; Great Harvest Group, 2021; HKU Estates Office, 2022). Given its relatively small scale, the development project is not a designated project under Environmental Impact Assessment Ordinance (Cap. 499) (EPD, 2011a) and an EIA study was not required. Still, it was required to apply for permission under the Town Planning Ordinance (Cap. 131) (Town Planning Board [TPB], 2022a). However, ecological impact assessment (EcoIA) is not a document that must be included in the application under Cap. 131

(TPB, 2022b). As a result, whether the conservation concern of a threatened species distributed in the application site could be adequately addressed to Committee members of TPB during the process depends largely on several aspects: 1) the "initiative" of the applicant to submit EcoIA, 2) comments from relevant government departments, particularly AFCD, and 3) comments from public consultation. As a result, HKU, the applicant, did not conduct and submit any EcoIA in support of its application (TPB, 2019a), and AFCD had no comment on the application (PlanD, 2019). The application was eventually approved in 2019 (TPB, 2019b), and extensive construction with excavation, piling and foundation work has already begun, which probably harms the fossorial reptile community. All in all, the threatened fossorial reptile species should be included in the protected species scheduled under Cap.170, which will raise the conservation attention on the species, which hopefully will incentivise AFCD and project proponents to comprehensively evaluate and mitigate ecological impacts on fossorial reptile from development projects.

4.2.2 Conservation actions for Dibamus bogadeki

In this study, despite intensive survey efforts, I was not able to detect *D. bogadeki*. This result may be attributed to two reasons. First, the survey methods employed (quadrat search and coverboards) are ineffective despite most, if not all, previous records being revealed by these methods. Second, the population of *D. bogadeki* is low and even declining on Sunshine Island. Recent surveys assessing the impacts of Kau Yi Chau Artificial Islands have been conducted on Sunshine Island, and the preliminary findings echoed with the results of this study that no *D. bogadeki* has been recorded (Development Bureau et al., 2022). Based on the results and these two potential causes, I suggest three actions for the conservation of *D. bogadeki*.

4.2.2.1 Investigation on new sampling methods

Apart from applying other conventional methods (e.g. pitfall traps), further studies should be carried out to explore new sampling methods (e.g. environmental DNA and scent detection dogs) for fossorial reptiles in Hong Kong, increasing the chances of getting more ecological information on the species. I provide more details in the section on further studies below.

4.2.2.2 Protection of habitats through legislations

Since most recent records of *D. bogadeki* occurred on Sunshine Island (Chan et al., 2012), which is the smallest and most undisturbed among distributed islands (PlanD, 2015), enhanced protection of the natural habitats on Sunshine Island is vital. Although Sunshine Island is listed as a Site of Special Scientific Interest (SSSI) (PlanD, 2015), the degree of protection is far from enough to protect *D. bogadeki* from the large-scale development project. Due to the increasing development pressure of Sunshine Island with the surrounding areas and the absence of Outline Zoning Plan (OZP) for the site (PlanD, 2022), the species could be affected directly by habitat degradation or loss from unauthorised development. It is worrying that the situation of "destroy first, build later" may happen to lower the ecological value to facilitate development of Sunshine Island in the future.

Additionally, the island is freely accessible to the public and during my work, I observed that there has been an increasing level of human disturbance. I noticed some minor work, such as removal of rubbles and creation of new hiking trails, as well as human activities, such as raising goats, barbecuing on the beach, and organising boat parties in the surrounding waters. Mr C. N. Lam (personal communication, n.d.), the only resident on the island, also mentioned that there have been groups occasionally landing on Sunshine Island to renovate the ruins of the past drug rehabilitation centre. It is suspected that some of these activities may change the natural habitats which may affect the population of *D. bogadeki* on the island. Further, the degree of human disturbance is expected to increase after the construction of the Kau Yi Chau Artificial Islands because the water between the Artificial Islands and Sunshine Island will be used for water recreation (Figure 4.1) (Development Bureau et al., 2022).

To address the potential threat on the habitats of Sunshine Island, first, I suggest that the government includes Sunshine Island in the statutory Development Permission Areas (DPA) Plan, followed by an OZP, with a general presumption against development. Second, to restrict access and to minimise human disturbance, the government should designate the core habitats of *D. bogadeki* in Sunshine Island as Restricted Area under Cap. 170 with reference to the case of Sham Wan Restricted Area for conservation of Green Turtle (*Chelonia mydas*) (AFCD, 2022c).

4.2.2.3 Investigation on potential impacts of invasive species

Apart from disturbance to the habitats, accidental or intentional introduction of invasive species, in particular Red Imported Fire Ant (*Solenopsis invicta*), may pose a threat to *D. bogadeki*. In Hong Kong, Fire Ants were first recorded in 2005 (Wong & Yuen, 2005) and it is expected that Fire Ants will expand their range because current management methods are ineffective (Chan & Guénard, 2020). A previous study documented that there is a negative correlation between relative abundance of herpetofauna and Fire Ant (Allen et al., 2017). Due to their egg-laying behaviour, reptiles are particularly vulnerable to the invasion of the Fire

Ants (Allen et al., 1994). Besides preying upon their eggs and hatchlings (Diffie et al., 2010; Landers et al., 1980; Newman et al., 2014), Fire Ants may indirectly impact reptiles by reducing the availability of food and decreasing the diversity of soil invertebrates (Donaldson et al., 1994; Wang et al., 2019; Xi et al., 2010). In China, as many as 18 reptile species are known to be threatened by Fire Ants (Lin et al., 2006). Studies have shown that fossorial species may be particularly vulnerable to the invasion of Fire Ants (Allen et al., 2004). For example, the population decline of a highly fossorial reptile, Southern Hognose Snake (*Heterodon simus*), coincided with the spread of Fire Ants in the southeastern United States (Tuberville et al., 2000). As human disturbance is a major driving force for the invasion (King & Tschinkel, 2008; MacDougall & Turkington, 2005), and abundance of Fire Ants (Todd et al., 2008), it is of great concern that the commencement of Lantau Tomorrow Vision may further promote the invasion of Fire Ants to Sunshine Island. As such, studies to evaluate the ecological impacts and monitor the invasion of invasive ants are needed for the conservation of *D. bogadeki* in the long run.

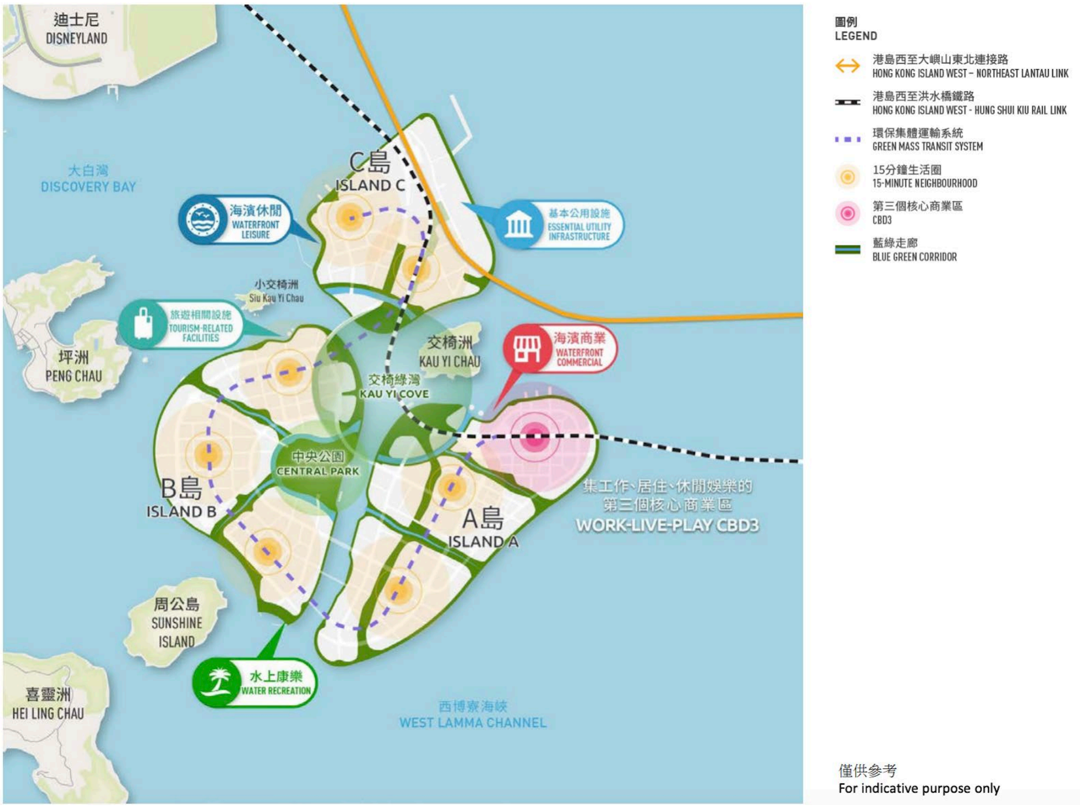

Figure 4.1 Broad Land Use Concept Plan of Kau Yi Chau Artificial Islands.

Reference: Development Bureau et al., 2022

4.2.3 Conservation actions for Indotyphlops albiceps

4.2.3.1 Morphological and osteological examination

To validate if *I. albiceps* is a new species, a detailed morphological and osteological examination of the specimens from Hong Kong and the type locality is needed.

4.2.3.2 Surveys in mainland China

To our knowledge, there have been no systematic studies on fossorial reptiles in mainland China. As *I. albiceps* and other congeners are widespread in regions adjacent to mainland

China (including Southeast Asia and Hong Kong), intensive surveys targeting fossorial reptiles should be carried out in mainland China to fill the knowledge gap.

4.3 Further research on fossorial reptiles

This study sheds light on the effectiveness of conventional survey methods for sampling fossorial reptiles in Hong Kong and the taxonomic status of threatened species. To enhance detection rate and to further understand the ecology and ecological importance of fossorial reptiles in Hong Kong, I suggest further studies on four aspects: novel sampling methods, behavioural ecology, relationship with other members of the community and monitoring population status.

4.3.1 Enhancement of detection rate through novel sampling methods

Environmental DNA (eDNA) has been widely applied and tested to detect and sample a variety of animal groups. In principle, organisms can be detected by sampling eDNA because they may leave their DNA (e.g. excretion, shed skin, or other body parts) in the environment (e.g. soil sediment or water) (Taberlet et al., 2018). In some cases, this method is more effective than field survey methods in detecting rare and cryptic species, for example, Big-headed Turtle (*Platysternon megacephalum*) (Lam et al., 2022), Alabama Sturgeon (*Scaphirhynchus suttkusi*) (Pfleger et al., 2016) and Hellbender (*Cryptobranchus alleganiensis*) (Spear et al., 2015).

Although soil environments are more prone to confine the eDNA detection sensitivity because of the presence of humic substances, enzyme inhibitors, and DNA photodegradation inducers (Matthias et al., 2021; Thomsen & Willerslev, 2015), some studies revealed the longevity of eDNA materials in the soil, persisting from days to decades (Foucher et al., 2020; Kyle et al., 2022; Taberlet et al., 2018). Previous studies have proven that eDNA is an efficient and feasible method to elevate the detection rate of semi-fossorial reptiles (Kyle et al., 2022; Matthias et al., 2021), and it could be used in combination with conventional field methods to detect target fossorial species through collecting eDNA materials from soil underneath coverboards (Adams et al., 2019; Kyle et al., 2022; Matthias et al., 2021). Therefore, by developing an assay of primers specific for local species, it is possible to apply eDNA techniques to enhance detection rate and conservation strategies of fossorial reptiles in Hong Kong (Katz et al., 2021; Lacoursière-Roussel et al., 2016).

Another novel survey method that is worth trying is the utilisation of scent detection dogs in field surveys. These well-trained conservation dogs, led by experienced handlers, could detect animals by scent in the field and are capable of locating rare and cryptic species (Bennett et al., 2020; DeMatteo et al., 2019). When the target species is located, the dogs alert handlers through performing a response (e.g. sitting), which involves no touching or barking to avoid disturbance (DeMatteo et al., 2019). This method was found to be useful for finding reptiles, such as Tuatara (*Sphenodon punctatus*) (Browne et al, 2015), Eastern Box Turtle (*Terrapene carolina carolina*) (Kapfer et al., 2012), Brown Tree Snake (*Boiga irregularis*) (Savidge et al., 2010) and Carolina Anole (*Anolis carolinensis*) (Fukuzawa & Sasahara, 2018). Several studies revealed that detection dogs possess high ability and potential in detecting fossorial reptiles and amphibians, for example, Pygmy Bluetongue Lizard (*Tiliqua adelaidensis*) (Nielsen et al., 2016), California Tiger Salamander (*Ambystoma californiense*) (Powers, 2018) and African Bullfrog (*Pyxicephalus adspersus*) (Matthew et al., 2021). Therefore, to enhance detection rate of fossorial reptiles in Hong Kong, particularly threatened species, it may be feasible to use scent detection dogs in addition to conventional survey methods.

4.3.2 Behaviour study in the field and in captivity

Concerning the secretive life history and behaviour of fossorial reptiles, it is challenging to study their behavioural ecology by direct non-invasive observations. Equipment, such as endoscope or borescope, can be deployed because scolecophidians usually either cohabit existing underground tunnels or trench their passageways in soil (Herrel et al., 2021). These devices have been used to study different taxa of burrowing organisms, such as Indian Pangolin (*Manis crassicaudata*), Naked Mole-rats (*Heterocephalus glaber*), Eel Gobies *(Odontamblyopus lacepedii)* and Eastern Brown Snake (*Pseudonaja textilis*) (Gonzales et al., 2006; Herrel et al., 2021; Holtze et al., 2018; Karawita et al., 2018; Kopcznski et al., 2017; Whitaker & Shine, 2002). As I observed that *I. albiceps* and *I. braminus* made tunnels in captivity, it is possible to apply endoscope or borescope to examine the internal structure of their tunnels and burrows, underground locomotion and commensal organisms of blind snakes.

Furthermore, behavioural studies in captivity can be carried out given the rarity of the fossorial species in the wild and their secretive lifestyles. In this study, live *I. albiceps* and *I. braminus* were collected and a trial was done to record their behaviour using a top-view surveillance camera with a motion-triggered function. I observed that both species foraged on the surface mostly at night, and they made their tunnels under the soil, which echoed the descriptions of the behaviour of their family Typhlopidea (Herrel et al., 2021). A systematic behaviour study in field or in captivity with advanced experimental design and equipment will be useful to gather data of their natural history, for example activity pattern and foraging behaviour (Kamosawa & Ota, 1996; Shine, 1980; Shine & Webb, 1990; Thierry et al., 2009; Webb & Shine, 1992).

4.3.3 Relationship with other members of the community

Wild Boars (*Sus scrofa*) are abundant in three of my study sites on Hong Kong Island—Pok Fu Lam, Lung Fu Shan and Lady Clementi's Ride. They display a rooting behaviour for foraging, during which they utilise their sturdy snout to shovel the earth rummaging for food underneath (Shek, 2006). Their rooting depth ranges from 10 cm to more than 30 cm (Pitta-Osses et al., 2022), and this behaviour may cause disturbance to the forest floor and the soil environment (Barrios-Garcia & Ballari, 2012), or even damage underground burrows created by the fossorial organisms. As Wild Boars are opportunistic consumers, which feed on any animals (including fossorial reptiles) and plant matters available (Howe et al., 1981; Pavlov & Edwards, 1995; Singer et al., 1984; Taylor & Hellgren, 1997). Although Wild Boars are native to Hong Kong, it is of conservation concern that their populations have increased rapidly in recent years (Leung, 2022) that may adversely affect the sympatric fossorial reptile populations through disturbance of habitats or increasing predation pressure.

Biotic threats can also come from underground. *I. braminus*, one of the target species in this study, is widespread worldwide by natural dispersal and inadvertent anthropogenic introduction (Bamford & Prendergast, 2017; Global Invasive Species Database, 2022; Shea et al., 2021). It has an overlapping niche with other congeners (i.e. *I. albiceps*, *I. lazelli* and *D. bogadeki*) in the native ecosystem, and such coincidence has not been explicitly studied. Taking advantage of their parthenogenetic reproduction strategy, *I. braminus* is able to procreate at a higher successful rate (Wallach, 2020). Concerns have been raised in regions with *I. braminus* introduction that the comparable feeding habits and vigorous reproductive potential of this alien species could outcompete the native blind snake species (Fisher, 2011; Ineich et al., 2017; Snyder et al., 2019; van den Burg et al., 2021).

A detailed examination of the diet of the Wild Boars and local fossorial reptiles by stable isotopic analysis will be useful to understand the potential predation pressure and competition (Rush et al., 2014; Wurster et al., 2012).

4.3.4 Population study

In this study, several individuals of *I. albiceps* and *I. braminus* were encountered in separate surveys but in the same area of the study sites, suggesting the occurrence of confined and spatially restricted populations. This raises the possibility of capture-recapture studies which can yield more accurate estimation of demographic parameters, such as survival rate and population size (Muñoz et al., 2016). Capture-recapture studies have been conducted on several fossorial reptiles, such as Worm Snake (*Carphophis amoenus*) (Russell & Hanlin, 1999), Slow Worm (*Anguis fragilis*) (Schmidt et al., 2017) and Checkerboard Worm Lizard (*Trogonophis wiegmanni*) (Martín et al., 2021b). The visible implant fluorescent elastomer (VIE) is a feasible method for marking of fossorial reptiles in capture-recapture studies (Major et al., 2020; Penney et al., 2001; Troast et al., 2022). If encounter rate of fossorial species can be enhanced with innovative sampling methods, it is desirable to carry out mark-recapture studies on fossorial reptiles in Hong Kong which will provide useful parameters for population monitoring and conservation.

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