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DNA Fingerprinting of Local Dried Seahorses Collected from Hong Kong Markets

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Abstract

Seahorses are under threat of endangerment because of the extensive exploitation and loss of their habitats. The major use for the seahorse is in traditional Chinese medicine, especially in Asia and China. Hong Kong is one of the major trade centers of seafood products, and dried seahorse is widely traded, but the problems of identifying dried seahorse products make the recording and census of this trade difficult. Therefore, this paper reports the use of the mitochondrial DNA genes cytochrome b and 16S ribosomal RNA and the random amplified polymorphic DNA (RAPD) technique to identify dried seahorse species purchased from local fishermen in Cheung Chau, Lau Fau Shan and Tai O in Hong Kong. Among 22 samples of seahorse individuals, three seahorse species were identified: *Hippocampus trimaculatus, H. spinosissimus* and *H. kelloggi*. Distinct banding patterns of the different seahorse species could be observed with the RAPD analysis. A strategy for seahorse conservation is also presented.

Keywords: DNA Bar-Coding; Multi-Locus Gene; Marine Conservation; RAPD

Introduction

The seahorse, genus *Hippocampus*, is a kind of special fish in the same class of bony fish (Actinopterygii) as salmon, but it is very different from others in this class. Seahorses have several unique features such as an upright posture, prehensile tail, and males having a brood pouch, as this sex gives birth to the offspring [17]. These characteristics make seahorse very sought after as live specimens and as curiosities. However, seahorses are under threat of endangerment primarily because of their speculative therapeutic effects in Traditional Chinese Medicine (TCM). They are said to be valuable in TCM for their ability to treat erectile dysfunction, nocturnal enuresis, kidney deficiency and also to promote labor, according to the Compendium of Materia Medica (*Bencao gangmu*) [1]. Even though these benefits are not scientifically proven, seahorses are heavily harvested for use in TCM and trading in China and other Southeast Asian countries. Seahorses are also threatened by the loss of habitat and by-catches (non-intended fishing of seahorses) [2,3].

In 2004, the entire genus of *Hippocampus* spp. was listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II, which means that trade of this genus is monitored and a permit is needed for export and import. Of 55 recognized species of seahorses, 11 are listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species as vulnerable or endangered [3], whereas the data are insufficient for other species according to the Ocean Park Conservation Fund Hong Kong (OPCFHK) [4]. The Protection of Endangered Species of Animals and Plants Ordinance (Cap 586) is used to enforce CITES in Hong Kong. However, the trade in seahorses is enormous for many reasons, especially in Hong Kong, which is one of the major seafood trade centers [3]. In 2011, around 7.1 tons (2.2 million individual seahorses) were imported into Hong Kong [4]. Therefore, it is important to research methods by which to identify seahorses traded in Hong Kong to protect the local population of feral seahorses.

However, challenges in the identification of dried seahorses make it hard to efficiently record the trading of seahorses [5,6], not only because the dried seahorses lose their colors and number of fin rays after drying, but also the conservative morphological features of seahorses sometimes overlap [7]. Therefore, molecular markers are vital to the identification of dried seahorses as the DNA molecule is relatively long-lived and stable during processing [8]. In this project, the study of two genes from the mitochondrial DNA (mtDNA) and random amplified polymorphic DNA (RAPD) analysis were conducted. mtDNA has been used in many different studies to identify species, and among the most frequently used mtDNA for identifying seahorses are cytochrome b and 16S rRNA) [9]. MtDNA is preferably used because of its conservative features (size, gene arrangement and content), but its rapid

mutation allows differentiation of close relatives [3,10,11]. RAPD analysis is a relatively new and rapid technique for conducting phylogenetic studies. It can be applied to an unknown genome and because it is cheaper and more efficient [12], RAPD can be a useful complement to conventional molecular techniques.

Several previous studies have identified seahorses using mitochondrial DNA as molecular markers [7,13-16]. However, only a few studies on the identification of dried seahorses have used a combination of methods. This project was aimed at collecting local dried seahorse samples in the market believed to be caught by local fishermen as by-catch and examining the use of multi-locus DNA markers (cytochrome b and 16S rRNA) with the complement of RAPD analysis to identify the dried seahorses at species level.

Materials and Methods

Collection of Samples

Local dried seahorse samples were purchased in August, September and October 2015 from local fishermen in the traditional fishing villages of Cheung Chau, Lau Fau Shan and Tai O in Hong Kong. Data on the morphology of the seahorse samples were recorded according to "A Guide to the Identification of Seahorses" [17]. Photos of the collected samples were taken and kept as a record (Figure 1). Then, the morphology record was used to compare with the checklist in "A Guide to the Identification of Seahorses" [17] and the suspected species of different samples were identified, however not all dried seahorse samples could be identified clearly by using the morphological characteristics (data not shown).

Panel A

Panel B

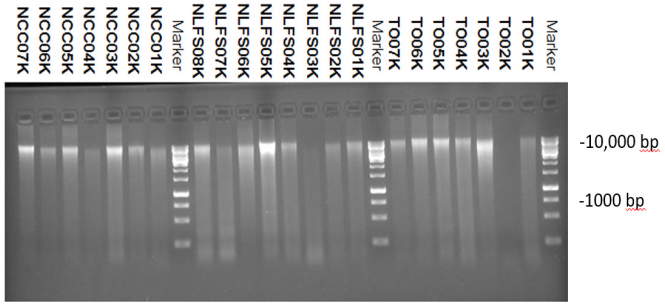
Panel C

Figure 1: Photos of dried seahorses purchased from Cheung Chau (Panel A), Lau Fau Shan (Panel B) and Tai O (Panel C). Samples are labeled with numbers that correspond to the information shown in the supplementary tables.

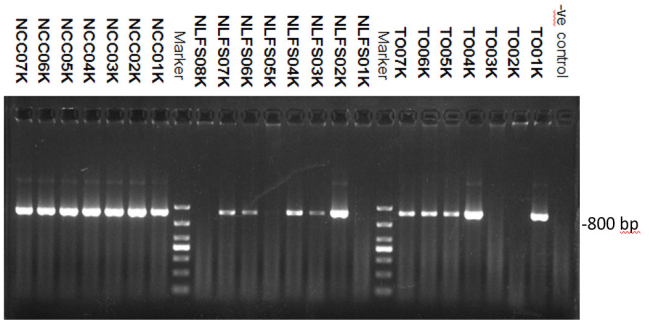
Genomic DNA Extraction

First, about 0.05 g of the tail was cut from each sample. Grinding in liquid nitrogen was performed to allow DNA extraction by cell lysis. The tissue was digested and extracted using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Version 5.0, following the kit's protocol. The extracted DNA was electrophoresed on 1.0% agarose gel to test for the presence of genomic DNA (Figure 2). A distinct band of high molecular weight (above 10,000 bp) indicated successful extraction of genomic DNA.

Panel A



Panel B



Panel C

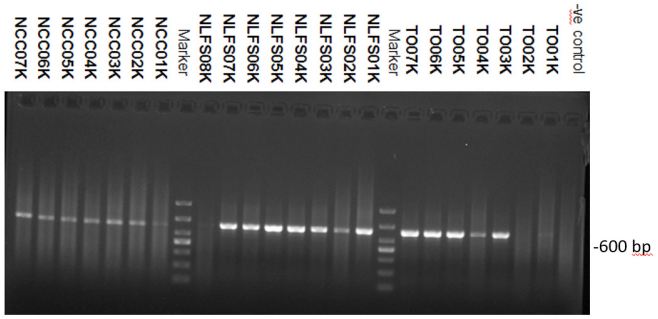


Figure 2: Agarose gel photos of genomic DNA and amplicons from genomic PCR with the two mitochondrial genes cytochrome b and 16s rRNA. **Panel A:** Gel photo of successful extraction of genomic DNA. The marker used is the Thermos Scientific Gene Ruler 1kb DNA Ladder. **Panel B:** Gel photo of PCR product using the cytochrome b gene. The marker used is the Takara DL1000 DNA Marker. A sharp band of the target product appeared around 800 bp as anticipated. **Panel C:** Gel photos of PCR product using the 16S rRNA gene. The marker used is the Takara DL1000 DNA Marker. A sharp band of the target product appeared around 600 bp as anticipated.

Study of Mitochondrial DNA (cytochrome b and 16S rRNA)

The cytochrome b and 16S rRNA genes were amplified using PCR. A pair of seahorse-specific primers were used for cytochrome b: forward primer, shf (5'-CTACCTGCACCATCAAATATTTC-3'); reverse primer: shr2 (5'-CGGAAGGTGAGTCCTCGTTG-3') (Lourie and Vincent, 2004). A pair of universal primers were used for 16S rRNA: 16sar - L (5'-CGCCTGTTTATCAAAAACAT-3') and 16sbr - H (5'-CCGGTCTGAACTCAGATCACGT-3') [18]. PCR amplifications were done in a 50-µl reaction mixture with 2 µl (around 50-100 ng) genomic DNA in the following reaction concentrations: 10X PCR buffer, 0.5 µl of EX Taq DNA Polymerase (TaKaRa) (5 U/µl), 4 µl of dNTPs (2.5 mM) and 2 µl of each forward and reverse primer (10 mM). PCR was done in TaKaRa TP600 Gradient PCR under the following conditions: 4-min initial denaturation at 94˚C, 36 cycles of the following thermal profile: 94˚C for 1 min, 55˚C for 1 min, 72˚C for 1.5 min, and a final extension for 10 min at 72˚C. After PCR, gel electrophoresis was performed on 1% agarose gel for 40 min at 120 V and visualized by a Bio-Rad Molecular Imager® Gel Doc™ XR+ Imaging System. Sharp bands of high molecular weight were extracted from the gel using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Version 4.0 following the kit protocol. The extracted amplified DNA was then sequenced by BGI Co., Ltd. After that, the sequenced DNA was visually cleaned and aligned by MEGA 6.06. The sequenced DNA was uploaded to the NCBI BLAST (The Basic Local Alignment Search Tool) for species identification. Using MEGA 6.06, phylogenetic analysis was performed by constructing a neighbor-joining tree corrected by the Kimura 2-parameter. Heuristic bootstrap analysis using 1,000 replicates was performed. The pipefish *Corythoichthys haematopterus* was used as an outgroup for comparison.

RAPD Study

The RAPD analysis was performed using 10 random 10-mer primers (Chen and Leibenguth, 1995) (Table 1). The DNA was amplified in a 10-µl RAPD-PCR reaction mixture containing 2 µl (around 50-100 ng) of genomic DNA, 0.1 µl of LA Taq DNA Polymerase (TaKaRa) (5 U/µl), 0.8 µl of dNTPs (2.5 mM) in 10X PCR buffer and 1 µl of RAPD primer (10 mM). PCR was carried out in TaKaRa TP600 Gradient PCR under the following conditions: 1.5-min initial denaturation at 94˚C, followed by 45 cycles with the following thermal profile: 94˚C for 30 s, 42˚C for 1 min, 72˚C for 2 min and a final extension for 10 min at 72˚C. The amplified DNA product was then electrophoresed on 1.5% agarose gel for 60 mins at 100 V. The products were visualized by a BIO-RAD Molecular Imager Gel DocTM XR+ Imaging System and photographed.

RAPD Primer	Sequence (5' - 3')
RAPD1	GCACCCGACG
RAPD2	CGCCCAAGCC
RAPD3	CCATGGCGCC
RAPD4	CGCCCGATCC
RAPD5	ACCCCAGCCG
RAPD6	GCACGCCGGG
RAPD7	GCACGCCGGA
RAPD8	CGCCCTCAGC
RAPD9	GCACGGTGGG
RAPD10	CGCCCTGGTC

Table 1: Sequence of random amplified polymorphic DNA (RAPD) 10-mer primers.

Results

Sample Collection and Genomic DNA Extraction

Of the 22 dried seahorse samples (Figure 1, Table 2) purchased as described in the Methods, all samples were claimed to have been caught in Hong Kong coastal waters by the local fishermen. Sai Kung was also one of the areas proposed for sample collection, but no dried local seahorses could be found. Then, the morphological data of the samples were recorded (Figure 1; other details are listed in Supplementary Tables 1-3), and species identifications were done according to their morphology following "A Guide to the Identification of Seahorses" [17]. The genomic DNA of 21 of the 22 samples was successfully extracted (Figure 2), with DNA concentrations ranging from 24.7 ng/µl to 75.4 ng/µl and the quality is good enough for PCR analyses.

Origin	Abbreviation	Number of samples	Number of successful extractions	Number of successful amplifications	
				Cytochrome b	16S rRNA
Cheung Chau	NCC##K	7	7	7	7
Lau Fau Shan	NLFS##K	8	8	7	8
Tai O	TO##K	7	6 (TO02K unsuccessful)	5	6
Total		**22**	**21**	**19**	**21**

Table 2: Samples collected and results of successful extractions and amplifications for all samples.

Sequence identity matrix-Cytochrome b			
	H. trimaculatus	*H. spinosissimus*	*H. kelloggi*
H. trimaculatus	ID	0.828	0.828
H. spinosissimus	0.828	ID	0.859
H. kelloggi	0.828	0.859	ID

Table 3: Nucleotides of the cytochrome b gene sequence.

Study of Mitochondrial DNA (cytochrome b and 16S rRNA)

For cytochrome b, 19 of the 21 successfully extracted DNA samples were amplified with an amplicon of around 800 bp. From the NCBI BLAST results (Supplementary Table 4), three species of seahorses were identified: *H. trimaculatus, H. kelloggi,* and *H. spinosissimus*. For 16S rRNA, all of the successfully extracted DNA could be amplified with an amplicon of around 600 bp, and three seahorse species were also identified using NCBI BLAST: *H. spinosissimus*, *H. kelloggi*, and *H. trimaculatus*. Both the cytochrome b and 16S rRNA results were consistent with each other. Gel photos of the PCR products of both cytochrome b and 16S rRNA are shown in Figure 2B and C.

Sequence Identity Matrix - 16S rRNA			
	H. trimaculatus	*H. spinosissimus*	*H. kelloggi*
H. trimaculatus	ID	0.900	0.862
H. spinosissimus	0.900	ID	0.907
H. kelloggi	0.862	0.907	ID

Table 4: Nucleotides of the 16S rRNA gene sequence.

The neighbor-joining trees constructed using cytochrome b and 16S rRNA are shown in (Figure 3 and Figure 4), respectively. Both trees were also consistent with each other and could be used to confirm the species of the different samples. Sequence alignments among the three species obtained in this study are also shown in Figure 5 and Figure 6.

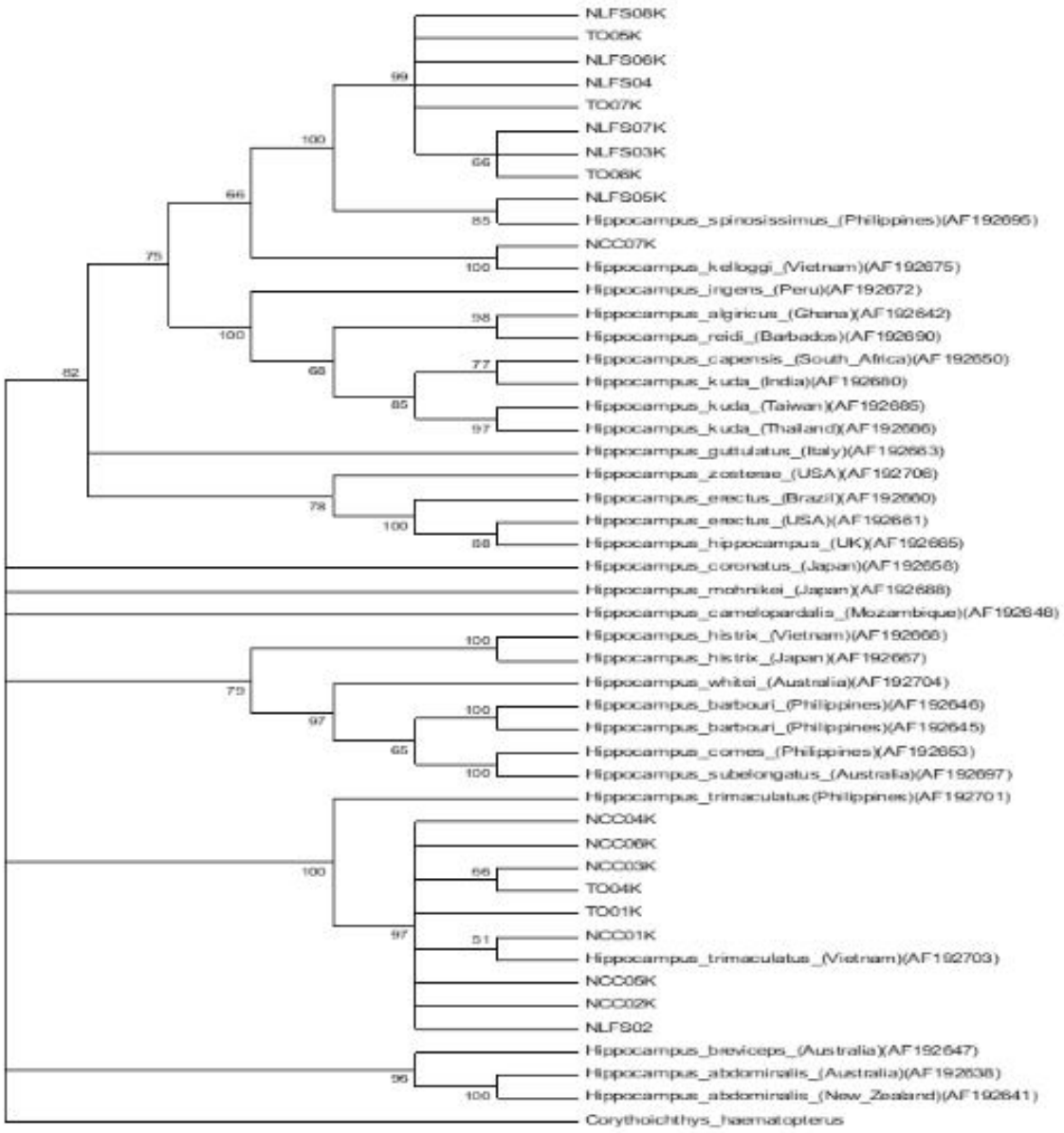


Figure 3: A neighbor-joining tree constructed using the cytochrome b gene and corrected by the Kimura 2-parameter. Heuristic bootstrap analysis using 1,000 replicates was performed. Twenty-nine reference sequences [15] were used, and the pipefish *Corythoichthys haematopterus* was used as an outgroup.

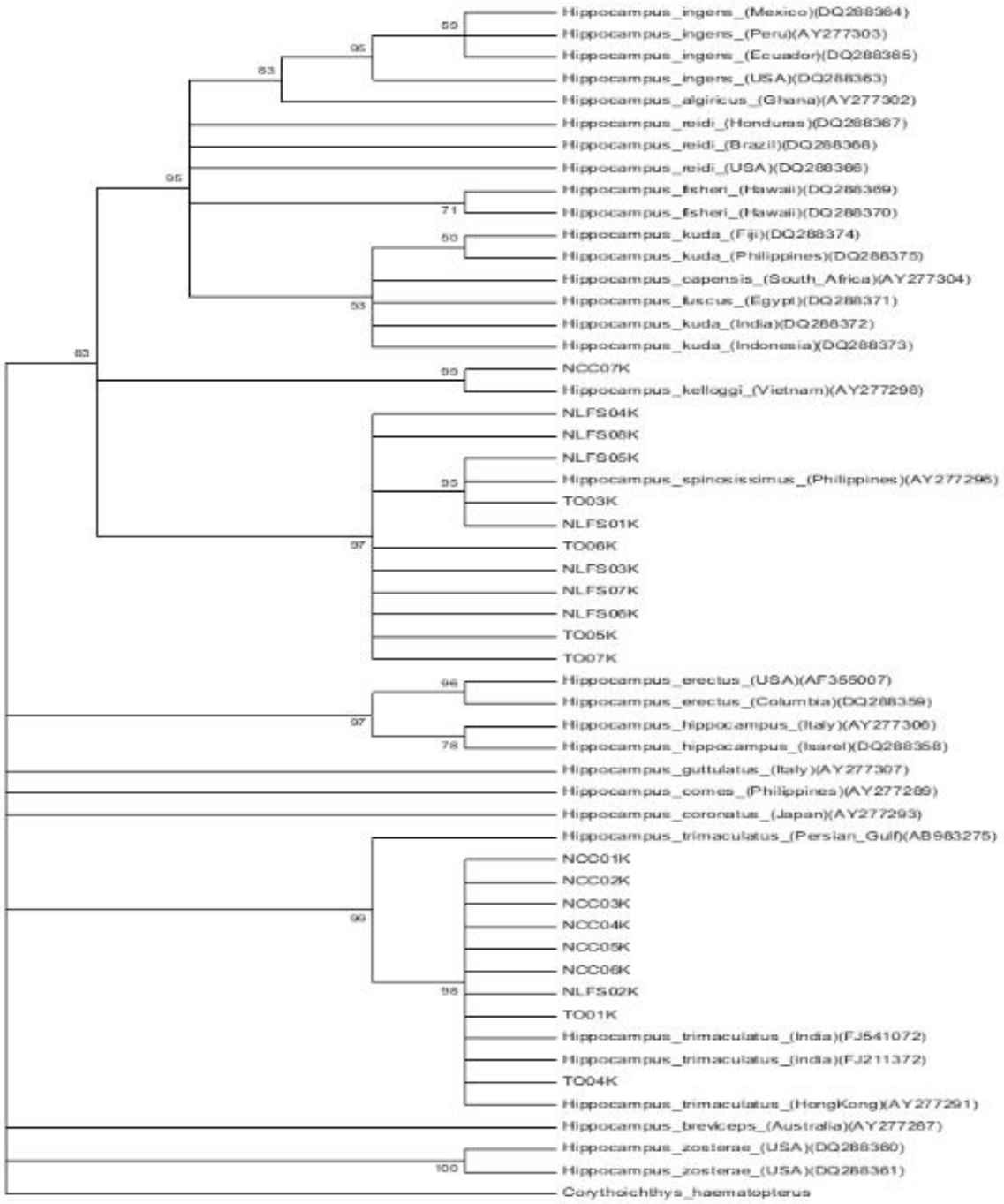


Figure 4: A neighbor-joining tree constructed using the 16S rRNA gene and corrected by the Kimura 2-parameter. Heuristic bootstrap analysis using 1,000 replicates was performed. Thirty-two reference sequences [19,20] were used, and the pipefish *Corythoichthys haematopterus* was used as an outgroup.

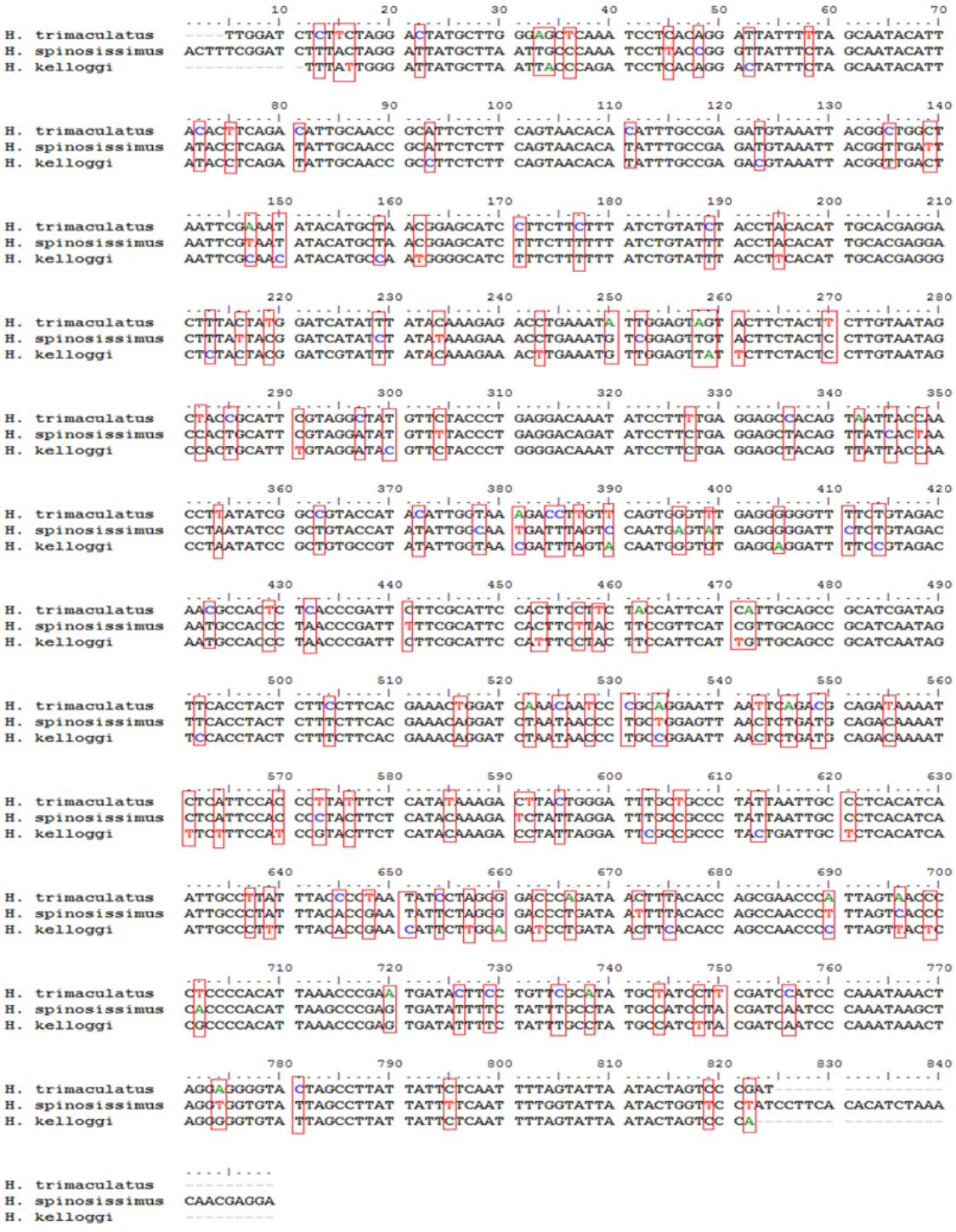


Figure 5: Cytochrome b gene sequence alignment of *H. trimaculatus, H. spinosissimus,* and *H. kelloggi.* Red rectangles indicate dissimilar nucleotide sites.

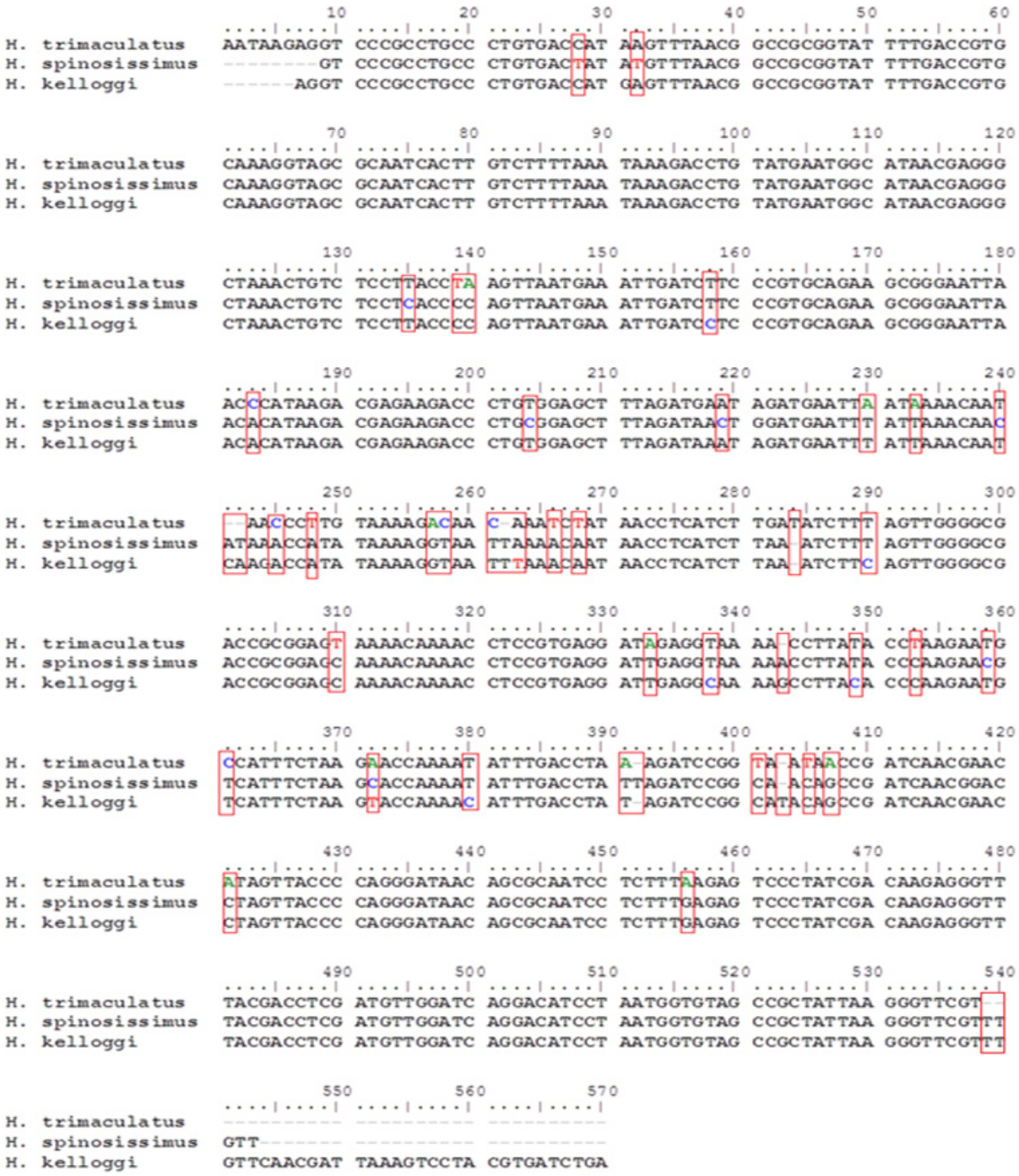
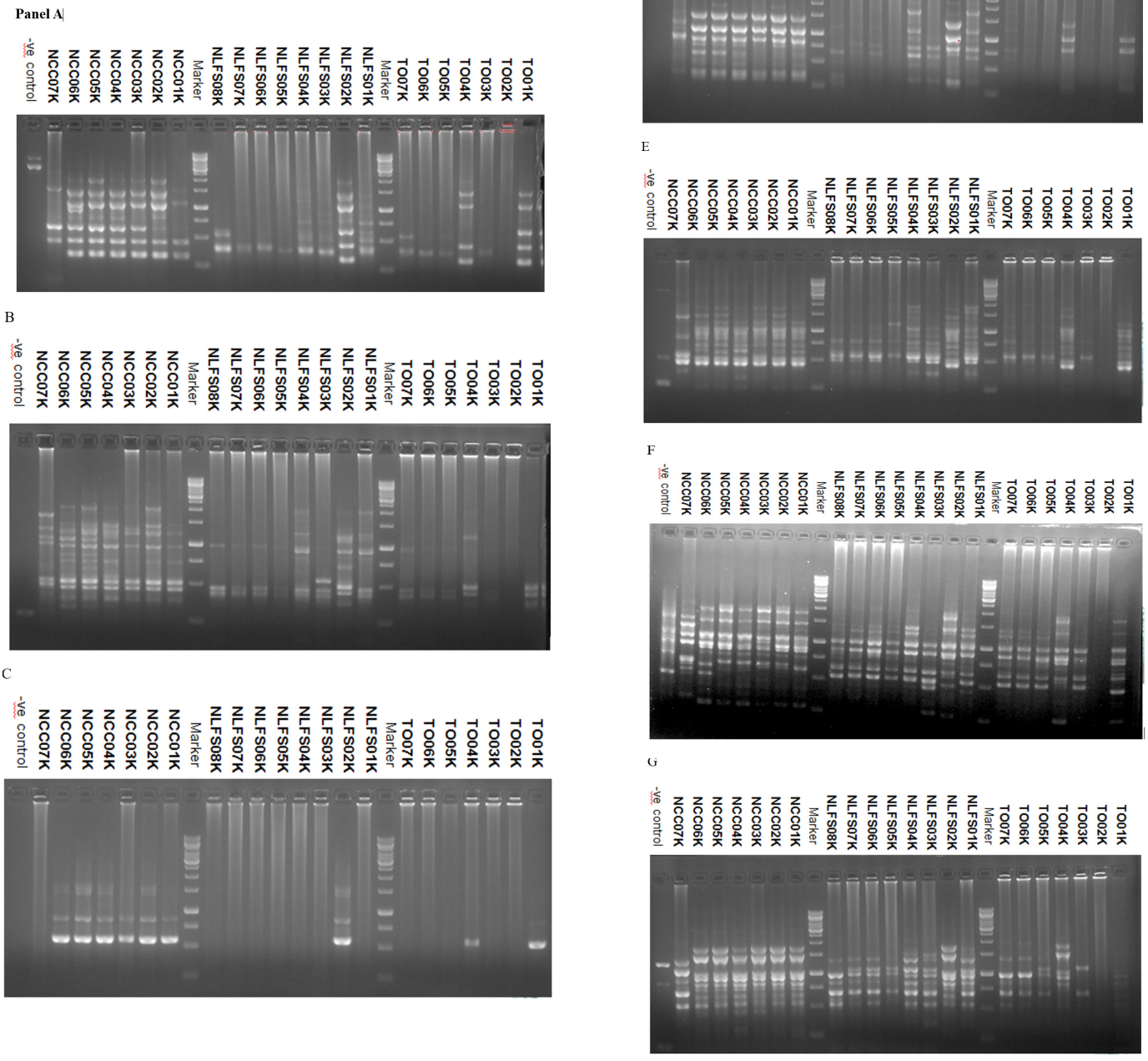


Figure 6: 16S rRNA gene sequence alignment of *H. trimaculatus, H. spinosissimus,* and *H. kelloggi.* Red rectangles indicate dissimilar nucleotide sites.

RAPD Study

The gel photos of the amplified products using the 10 random primers are shown in Figure 7. However, not all of the primers showed good-quality banding patterns: for example, the RAPD 3, 4 and 10 primers. Among the 10 primers, RAPD 6 and 9 appeared to be better than the others in generating distinct banding patterns for different seahorse species.



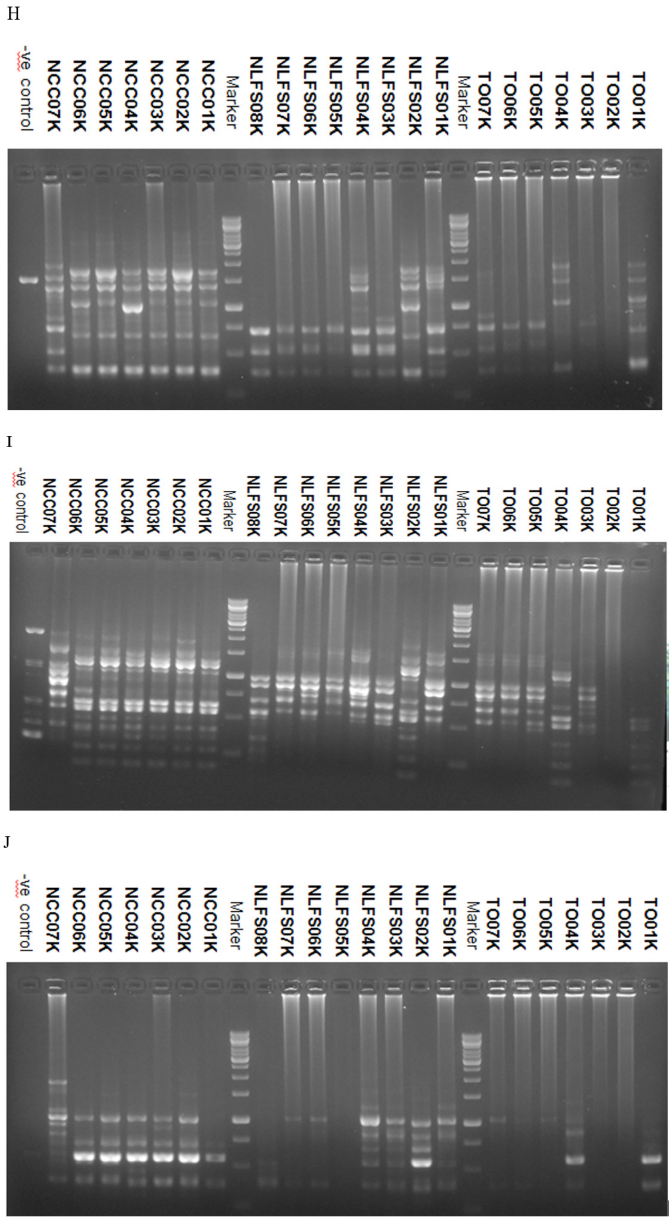


Figure 7: Gel photos of RAPD products. Panels A to J respectively refer to the RAPD primers 1-10 used in this project. The marker used is the Thermo Scientific Gene Ruler 1kb DNA Ladder. NCC01K-NCC06K, NLFS02K, TO01K and TO04K are *H. trimaculatus*. NCC07K is *H. kelloggi*. NLFS01K, NLFS03K-08K, TO03K and TO04K-07K are *H. spinosissimus*.

Discussion

Genomic DNA Extraction

The genomic DNA of one of the seahorse samples (TO02) was not successfully extracted. As the color of TO02K (Figure 1C) was comparatively whiter than that of the other samples and some dark patches were found along its body, it is suspected that this seahorse may have been dried with an unknown process such as bleaching or that chemicals were added that somehow might have degraded the genomic DNA or hindered the PCR reactions. The time at which the seahorse was caught and dried was also unknown. It is also possible that the dried seahorses were stored for a very long time, which caused the DNA to be degraded by mold.

Study of Mitochondrial DNA (cytochrome b and 16S rRNA)

Both cytochrome b and 16S rRNA were effective in identifying the different seahorse species, and the results were consistent. The nucleotide sequence of cytochrome b and 16S rRNA of *H. trimaculatus*, *H. spinosissimus* and *H. kelloggi* can be compared in Figs 6 and 7, respectively. Tables 3 and 4 show the percentage differences of the nucleotide sequence of each species. Although the sequences of the three species are highly similar, there are differences that allow the differentiation of the three species. mtDNA is useful and popular as a molecular marker to identify the species of an organism because mtDNA has a relatively higher mutation rate, which allows differences between species to be observed. Woodall et al. [21] used cytochrome b to identify a specimen of *Hippocampus erectus* from the eastern Atlantic Ocean.

RAPD Study

Problems with primer-derived nonspecific amplification products have been reported previously [22]. Bands of varying lengths were observed in the negative control of the RAPD-PCR products although no DNA template was added, probably due to the high number of PCR cycles used. The whole set-up was replicated several times using different solutions and apparatus in the hope of minimizing the problems of contamination, but the same problem recurred. This problem was as described by [22], but the reason behind it could not be determined. The sharp bands of the RAPD-PCR products were extracted for sequencing in this project to find the sequence of each particular band. However, the quality was not good for sequencing because multiple sequence peaks were reported. Nevertheless, the use of RAPD was still effective in identifying different seahorse species as it generated distinct banding patterns for different seahorse species without doing DNA sequencing, thus saving time and money. Among the 10 random primers, RAPD primers 6 and 9 were better than the others because they generated different banding patterns that could be seen in all samples of the seahorses. Also, this RAPD method is quicker and less labor-intensive than DNA sequencing. Still, it is important to note that the same PCR conditions and DNA quality should be used to obtain reproducible results [12]. The RAPD method could become a quick and easy screening method.

Current Survey of Local Seahorses in Hong Kong

H. spinosissimus was found unexpectedly and has not been recorded in the wild in Hong Kong. According to OPCFHK, there

are three native seahorse species in Hong Kong: *H. kelloggi, H. trimaculatus,* and *H. kuda*. The presence of *H. spinosissimus* in the samples bought from local fishermen in Hong Kong may indicate that the claimed origin of the samples is not accurate, which means that fisherman may catch this seahorse nearer to China and not exactly in Hong Kong. Another possible reason is that the current existing survey and recording method, which mainly involves skin divers, may not be comprehensive enough. Because *H. spinosissimus* is usually found in seas of more than 8 m in depth [23], this species is commonly caught in fishermen's nets by trawling. Therefore, it is possible that the current recording method cannot cover seahorses that inhabit a deeper habitat. As similarly reported in a study by OPCFHK in 2011-2012, only six *H. kuda* were found in Sai Kung [4]. A later study by the Eco-Education and Resources Centre (ERC) and Green Power recorded 25 *H. kuda* on the east side of Hong Kong [24]. It is surprising to note that only *H. kuda* was found in both studies. However, because *H. kuda* is found in shallow regions (2-7 m) such as river run-offs and mangrove areas [24], it is easier for divers to observe their existence. Therefore, there is a need to examine the current method of surveying seahorses to better maintain a complete record of the seahorses living in the different habitats around Hong Kong.

Recommendations for Seahorse Conservation in Hong Kong

This project only examined local dried seahorses that were purchased from three areas on the west side of Hong Kong. Samples should also be collected from areas on the eastern side of Hong Kong, such as Tap Mun, Tai Po and Sai Kung, because according to the studies by OPCFHK and ERC and Green Power, the east side of Hong Kong is a nursery ground for *H. kuda.*

Furthermore, the authors recommend the collection of samples from local TCM retailers who usually sell exotic seahorses. Considering that the *H. trimaculatus* and *H. spinosissimus* found in this project are the top two most-traded seahorses globally [25], population studies should be done to compare the samples bought from local fishermen and from TCM retailers to trace the origin of different seahorses bought from different sources.

Presently, Hong Kong does not have specific laws to protect local seahorses. The Protection of Endangered Species of Animals and Plants Ordinance (Cap 586) was only enacted to enforce CITES in Hong Kong to control the trading of seahorses. Nonetheless, the trading of seahorse has not seemed to stop. Unfortunately, this ordinance does not control the sale of seahorses that are caught by local fishermen through trawling. Even though a trawling ban has been enforced since 2012 and the by-catch of seahorses may have decreased, the sale of dried seahorses continues. Thus, the authors highly recommend that the Hong Kong government enact more stringent laws and regulations regarding the sale of local seahorses by local fishermen to comply with the CITES II guidelines and recommendations.

By the same token, education is of paramount importance to raise public awareness of this genus. It is important to teach people not to consume dried seahorse as TCM, but to use substitutes like psoralea and fenugreek to decrease the demand for dried seahorse in the market. As the presence of seahorses in a particular area indicates a healthy habitat and rich biodiversity, it is critical to protect these habitats and conserve this genus.

Conclusion

In light of the widespread trading of dried seahorse products around the world, especially in Asian regions, it is crucial to keep better track of the number of individual seahorses at a species level. However, identifying dried seahorses using morphology alone is not adequate. This project examined the use of DNA fingerprinting by studying mtDNA with the sequencing of cytochrome b and 16S rRNA and used RAPD with primers 6 and 9 to identify different seahorse species purchased from local fishermen in Hong Kong. Using mtDNA as the genetic marker could be a more reliable and standard method, and RAPD could become a more rapid approach for the identification and screening of unknown dried seahorse species.

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