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Original Article

Authenticating the use of dried seahorses in the traditional Chinese medicine market in Taiwan using molecular forensics

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ABSTRACT

Seahorse, which has a unique appearance and exhibits male pregnancy, is a useful component of traditional Chinese medicine (TCM). With the growing demand for TCM, vast amounts of seahorses are harvested from the wild every year and traded internationally. This study investigated 58 dried seahorse samples collected from 23 Chinese herbal medicine stores across Taiwan using molecular forensics. Results showed that eight seahorse species were present in the Taiwan TCM market. Among them, Knysna seahorse (*Hippocampus capensis*) has an endangered status according to the International Union for Conservation of Nature (IUCN) Red List, while the West African seahorse (*Hippocampus algiricus*), tiger tail seahorse (*Hippocampus comes*), thorny seahorse (*Hippocampus histrix*), great seahorse (*Hippocampus kelloggi*), yellow seahorse (*Hippocampus kuda*), hedgehog seahorse (*Hippocampus spinosissimus*), and three-spot seahorse (*Hippocampus trimaculatus*) have vulnerable status.

1. Introduction

Seahorses (*Hippocampus* spp.), one of the most appealing groups of 300 known species in the family Syngnathidae, are celebrated for their distinctive appearances [1]. They are found in wide longitudinal and latitudinal areas [2] and all exhibit male pregnancy [3]. After receiving eggs from females, male seahorses fertilize eggs in specialized abdominal pouches; the eggs develop into embryos therein, under suitable conditions [4]. This one-of-a-kind biological feature makes seahorses very appealing to the public, making it even more important to conserve them [5].

Seahorses that feature in traditional medicine, aquarium display, and curiosities are internationally traded; the number of *Hippocampus* species that are traded in the global market annually has been estimated to be about 20 million [6]. Most of them are sold as dried traditional Chinese medicine (TCM) materials rather than live as aquarium pets [7,8]. A clear

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picture has emerged about the curative activities of seahorses because researchers have found out that, in addition to their traditional use in treating erectile dysfunction [6,9], seahorses also exhibit antitumor, antiaging, and antifatigue properties and are able to suppress neuroinflammatory responses and collagen release [8,10]. The two-millennium history of TCM guarantees a great demand for seahorses among Chinese people, and dried seahorses are mainly consumed in China, Hong Kong, and Taiwan [7]. The economic development of China, which has increased the demands for TCM, has been catastrophic for wild animals [11]. It is thus predicted that seahorses will face increasingly deleterious conditions in the future.

Even though fishery by-catch, especially from shrimp trawlers, is the principal method of seahorse capture [12], economic enticement has caused an increasing number of fishermen to employ fishing methods that specifically target seahorses in many developing nations, such as Brazil, India, and the Philippines [7]. Fishing has had drastic effects on many aspects of seahorses, such as harming individuals, destroying social structure, reducing reproduction, affecting population structure, and devastating habitats [13]; unfortunately, many of their life history traits, such as high mate fidelity, small brood sizes, lengthy parental care, and low dispersal ability, make them more susceptible to persistent exploitation [14,15].

To allay fishing pressures on seahorses, two measures have been taken by the Conservation on International Trade in Endangered Species (CITES): first, all seahorse species have officially been listed under an Appendix II designation since 2004, in order to prohibit international seahorse trade [16]; and second, a 10-cm minimum height limit (from the top of the head to the tip of the tail) policy for seahorses in trade, except for *Hippocampus kelloggi* [17], has been enforced to ensure that seahorses have sufficient time to grow up and reproduce before being captured and sold [18]. Some regional organizations or nations, such as the OSPAR Commission, as well as the IUCN Red List of Threatened Species have also cautioned the public against exploiting seahorses by listing several of them as being endangered or vulnerable. If these policies are carried out successfully, harvesting of seahorse resources can be supervised carefully, and utilization of these sensitive creatures will be sustainable.

According to documents obtained from the Bureau of Foreign Trade [19], Taiwan has imported an appreciable amount of desiccated seahorses, in the range of 3181–8797 kg each year, during 2008–2011. These desiccated seahorses have been used as ingredients in TCM and exported mainly from China and Thailand. The CITES trade database also showed that the majority of dried seahorses came from wild populations rather than captive-bred ones. However, the species composition of dried seahorses in the Taiwan market has never been ascertained. The purpose of this study was to investigate the number of seahorse species present in the Taiwan TCM market, and whether Taiwan is taking part in the trade of endangered seahorse species. Because of the difficulty of morphological identification of dried seahorses, especially because they are sometimes sliced longitudinally before being sold, and of the abundant seahorse cytochrome b (*cyt b*) gene sequences in GenBank (www.ncbi.nlm.nih.gov), DNA taxonomy was employed to identify the species of specimens obtained from the Taiwan market.

2. Methods

2.1. Samples

In total, 58 dried seahorse samples were purchased from 23 Chinese herbal medicine stores in 2012. Because dried seahorses have exorbitant prices and a detailed morphological examination was not possible on the spot, during collection, dried seashores were classified roughly into different

Table 1 – Sampling locations and results of molecular forensics of 58 dried seahorse (*Hippocampus*) samples.

Store no.	Location	Specimen code	Haplotype code	Molecular identification
1	Taipei City	TS01MO-1	TS01MO1	*H. trimaculatus*
2	Taipei City	TS02MO-1	TS02MO1	*H. spinosissimus*
3	Taipei City	TS03MO-1	TS03MO1	NR
4	New Taipei City	TS04MO-1	TS04MO1	*H. trimaculatus*
5	New Taipei City	TS05MO-1	TS05MO1	*H. kelloggi*
		TS05MO-2	TSH4	*H. trimaculatus*
		TS05MO-3	TS05MO3	*H. spinosissimus*
		TS05MO-4	TSH1	*H. trimaculatus*
		TS05MO-5	TS05MO5	*H. trimaculatus*
6	Keelung City	TS14MO-1	TS14MO1	*H. spinosissimus*
		TS14MO-2	TSH1	*H. trimaculatus*
7	Keelung City	TS15MO-1	TS15MO1	*H. trimaculatus*
		TS15MO-2	TS15MO2	*H. trimaculatus*
		TS15MO-3	TS15MO3	*H. spinosissimus*
		TS15MO-4	TSH5	*H. trimaculatus*
		TS15MO-5	TS15MO5	*H. trimaculatus*
8	Keelung City	TS19MO-1	TS19MO1	*H. kuda*
		TS19MO-2	TS19MO2	*H. kuda*
		TS19MO-3	TSH6	*H. comes*
9	Hsinchu City	TS11MO-1	TS11MO1	*H. trimaculatus*
		TS11MO-2	TSH3	*H. trimaculatus*
		TS11MO-3	TS11MO3	*H. trimaculatus*
		TS11MO-4	TS11MO4	*H. trimaculatus*
10	Miaoli County	TS06MO-1	TSH2	*H. trimaculatus*
		TS06MO-2	TSH3	*H. trimaculatus*
11	Taichung City	TS12MO-1	TS12MO1	*H. kuda*
		TS12MO-2	TS12MO2	*H. comes*
		TS12MO-3	TS12MO3	*H. trimaculatus*
		TS12MO-4	TSH1	*H. trimaculatus*
12	Taichung City	TS13MO-1	TSH1	*H. trimaculatus*
		TS13MO-2	TSH2	*H. trimaculatus*
13	Taichung City	TS16MO-1	TS16MO1	*H. capensis*
		TS16MO-2	TSH6	*H. comes*
		TS16MO-3	TS16MO3	*H. trimaculatus*
14	Changhua County	TS09MO-1	TS09MO1	*H. comes*
		TS09MO-2	TS09MO2	*H. histrix*
		TS09MO-3	TS09MO3	*H. trimaculatus*
		TS09MO-4	TS09MO4	*H. kelloggi*
		TS09MO-5	TS09MO5	*H. spinosissimus*
		TS09MO-6	TS09MO6	*H. trimaculatus*
15	Tainan City	TS08MO-1	TS08MO1	*H. trimaculatus*
16	Kaohsiung City	TS18MO-1	TS18MO1	*H. kelloggi*

(continued on next page)

Table 1 – (continued)

Store no.	Location	Specimen code	Haplotype code	Molecular identification
17	Kaohsiung City	TS20MO-1	TS20MO1	*H. algiricus*
18	Kaohsiung City	TS21MO-1	TS21MO1	*H. spinosissimus*
19	Kaohsiung City	TS22MO-1	TSH4	*H. trimaculatus*
20	Pingtung County	TS07MO-1	TS07MO1	*H. trimaculatus*
21	Hualien County	TS17MO-1	TS17MO1	*H. trimaculatus*
		TS17MO-2	TS17MO2	*H. comes*
		TS17MO-3	TS17MO3	*H. spinosissimus*
		TS17MO-4	TS17MO4	*H. trimaculatus*
22	Kinmen County	TS10MO-1	TS10MO1	*H. comes*
		TS10MO-2	TS10MO2	*H. comes*
		TS10MO-3	TS10MO3	*H. trimaculatus*
		TS10MO-4	TS10MO4	*H. kuda*
23	Penghu County	TS23MO-1	TS23MO1	*H. spinosissimus*
		TS23MO-2	TSH5	*H. trimaculatus*
		TS23MO-3	TS23MO3	*H. trimaculatus*
		TS23MO-4	TS23MO4	*H. trimaculatus*

NR = negative response in the polymerase chain reaction (PCR).

morphological forms and one individual of each form was purchased randomly and brought back to the laboratory. Details are given in Table 1.

2.2. DNA extraction, polymerase chain reaction, and sequencing

DNA samples were extracted from fin tissues using a QuickGene DNA tissue Kit S (Fujifilm, Tokyo, Japan). Polymerase chain reaction (PCR) amplifications of mitochondrial cytochrome b (*cyt b*) (1140 bp) were performed in a mixture with a final volume of 100 μL, containing 10 ng template DNA; 25 μmol of each specific primer; SH-F (5′-AAC YAG GAC YAA TGR CTT GA-3′) and SH-R (5′-GCA SWA GGG AGG RKT TTA AC-3′), which were designed on the basis of the mitochondrial genomes of *Hippocampus kuda* (NC_010272) and *Microphis brachyurus* (NC_010273) and located, respectively, at tRNA-Glu (14311–14330) and tRNA-Thr (15550–15569) according to the sequence (NC_010272); 50 μL of Fast-Run™ Advanced *Taq* Master Mix (ProTech, Taipei, Taiwan); and distilled water. Thermal cycling began with one cycle at 94 °C for 4 minutes; followed by 35 cycles of denaturation at 94 °C for 1 minute, 45–51 °C for 1 minute, and 72 °C for 1 minute; and finally, a single extension step at 72 °C for 10 minutes. PCR products were purified using a PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan). Approximately 50 ng of the purified PCR products were employed as the template for sequencing, which was performed by following the protocol of the ABI PRISM BigDye Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) with the SH-F and SH-R primers.

2.3. Data analysis

As suggested by Wilson and Orr [1] and Sanders et al [20], the *cyt b* DNA sequences of *Syngnathus schlegeli* (AY786429) and *Corythoichthys haematopterus* (AY787229) from GenBank served as outgroups for the phylogenetic analysis. To construct the reference database, data on 30 *Hippocampus cyt b* haplotypes of 22 *Hippocampus* species studied by Casey et al [21] and Chang et al [22] were downloaded (Table 2). The entire *cyt b* gene was 1141 bp; however, the last nucleotide of the incomplete stop codon was deleted from all *cyt b* gene sequences analyzed in this study so that they had the same length (1140 bp) and could be aligned directly. A maximum-likelihood (ML) tree was constructed utilizing RAxML 7.0.4 [23]. In the parameter setting of the software RAxML 7.0.4, data were partitioned by codon position, which was performed under the GTR + G + I model. The ML tree was obtained by performing 100 different runs using the default algorithm of the program, and the best ML tree was chosen based on the likelihood scores of suboptimal trees created in each run. Nodal support was certified by a bootstrap analysis with 1000 nonparametric bootstrap iterations. The software Mega 4 [24] was utilized to calculate the K2P genetic distance among taxa.

3. Results and discussion

DNA markers, such as 12S rRNA, cytochrome b, and the internal transcribed spacer, are applied widely in taxonomy, and are also used to identify the species compositions of TCMs

Table 2 – Set of 30 cytochrome b (*cyt b*) haplotypes from 22 *Hippocampus* species used as the reference database for the maximum-likelihood phylogenetic analysis.

Scientific name	Haplotype code	Accession no.	Origin
H. abdominalis	NZ.98	AF192641	New Zealand
	AUS.183.1	AF192638	Australia
H. algiricus	GHA.X1	AF192642	Ghana
H. barbouri	PH.225	AF192646	Philippines
	PH.132	AF192645	Philippines
H. breviceps	BIR.361	AF192647	Australia
H. camelopardalis	MOZ.515	AF192648	Mozambique
H. capensis	K.59	AF192650	South Africa
H. comes	PB.53	AF192653	Philippines
H. coronatus	JAP.344.1	AF192658	Japan
H. erectus	CHS.170.1	AF192661	USA
	BRZ.182.4	AF192660	Brazil
H. guttulatus	EX.221	AF192663	Italy
H. hippocampus	EX.230	AF192665	England
H. histrix	JAP.345	AF192667	Japan
	VIG.40	AF192668	Vietnam
H. ingens	PER.184.1	AF192672	Peru
H. kelloggi	VIE.32	AF192675	Vietnam
H. kuda	TH.174.9	AF192686	Thailand
	TA.207	AF192685	Taiwan
	IND.350.1	AF192680	India
H. mohnikei	JAP.346.7	AF192688	Japan
H. reidi	BAR.94	AF192690	Barbados
H. spinosissimus	PF.152	AF192695	Philippines
H. subelongatus	AUS.228	AF192697	Australia
H. trimaculatus	PA.128	AF192701	Philippines
	VIC.22	AF192703	Vietnam
	TW	JX682713	Taiwan
H. whitei	AUS.217	AF192704	Australia
H. zosterae	FK.141	AF192706	USA

and food [25–28]. Dried seahorse samples, although having undergone a manufacturing process and mostly been stored at room temperature, were demonstrated to contain qualified DNA materials that were sufficient for amplification of *cyt b* gene sequences. In this study, only one (TS03MO-1) of 58 specimens had a negative PCR response, and 49 *cyt b* haplotypes were sequenced successfully (Table 1). The DNA barcoding technologies have been applied to prevent the smuggling of endangered species, and many seahorse species have an endangered or a vulnerable status on the IUCN Red List; consequently, this study suggests that customs authorities can utilize a molecular forensic method to verify and oversee imported dried seahorses.

Fig. 1 shows the results of the phylogenetic analysis of seahorse haplotypes from the investigated samples and the GenBank database. The genus *Hippocampus* is an apparently monophyletic group with high statistical support; however, as noted by Casey et al [21], some seahorse species, such as *H. kuda*

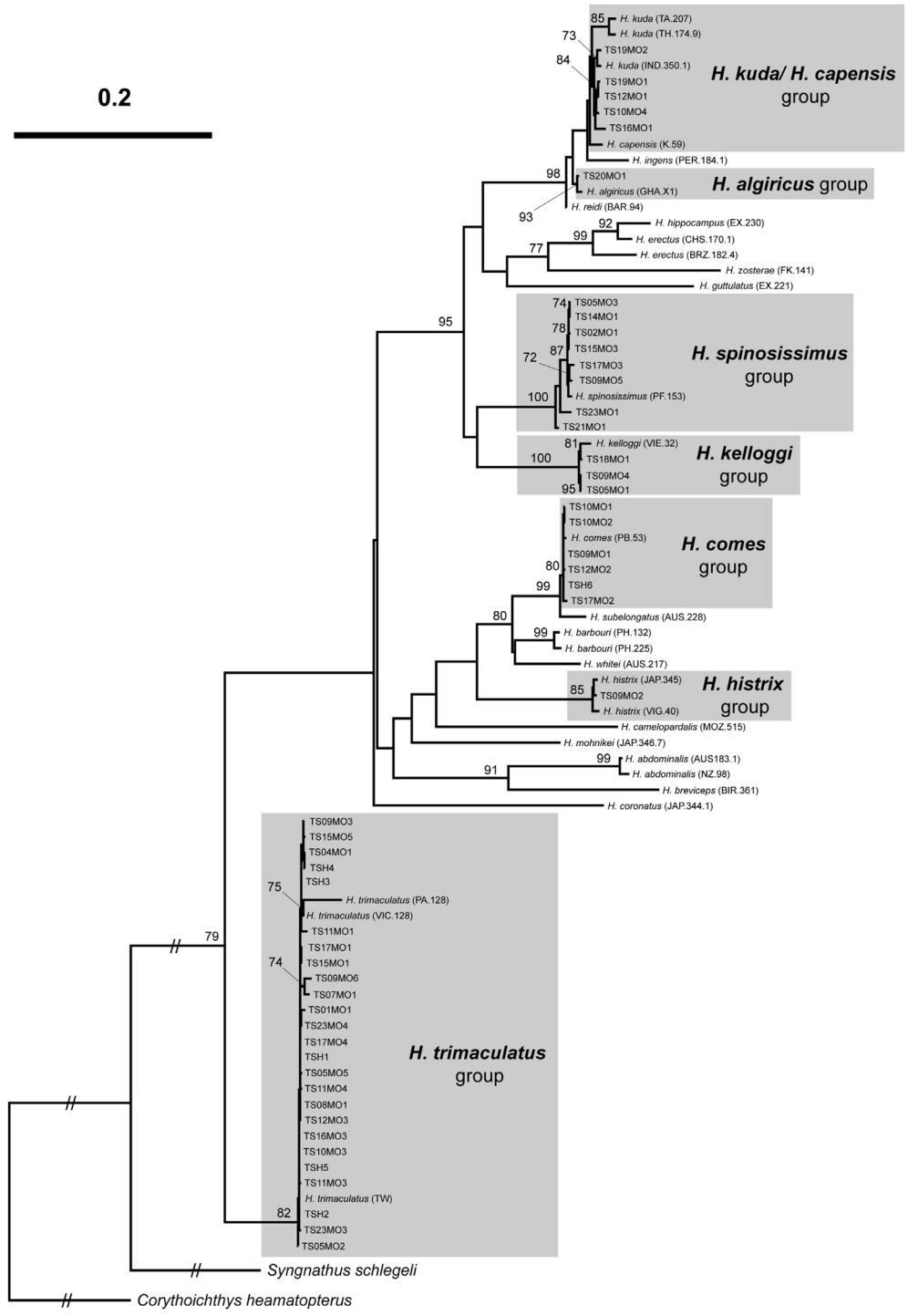

Fig. 1 – ML tree of *Hippocampus* inferred from mitochondrial cytochrome b sequences with 1000 bootstrap replicates. Bootstrap values of >70% are indicated. Seven groups were defined. ML = maximum likelihood.

and *Hippocampus capensis*, have close genetic relationship owing to recent differentiation (<1 million years ago), which results in an incomplete lineage sorting and blurs resolution of the phylogenetic analysis. The ML tree revealed that haplotypes of investigated samples were distributed in seven groups: *Hippocampus trimaculatus* group (K2P genetic distance ranging from 0.1% to 3.8% within group), *Hippocampus histrix* group (K2P genetic distance ranging from 0.6% to 1.1% within group), *Hippocampus comes* group (K2P genetic distance ranging from 0.1% to 11.9% within group), *H. kelloggi* group (K2P genetic distance ranging from 0.2% to 1.3% within group), *Hippocampus spinosissimus* group (K2P genetic distance ranging from 0.2% to 2.1% within group), *Hippocampus algiricus* group (K2P genetic distance being 0.6% within group), and *H. kuda/H. capensis* group (K2P genetic distance ranging from 0.2% to 3.3% within group). All groups, except for the *H. kuda/H. capensis* group, represented one *Hippocampus* species. The close lineage between *H. kuda* and *H. capensis* circumscribes genetic authentication. Instead, the morphological character, with or without a coronet, can serve as a good marker to distinguish one from the other [29]. The K2P genetic distances among the 79 *Hippocampus* haplotypes ranges from 0.1% to 23.2%. The average interspecific K2P genetic distances range from 1.2% (*Hippocampus algiricus* vs. *Hippocampus reidi*) to 23.2% (*Hippocampus guttulatus* vs. *Hippocampus coronatus*), and the average of intraspecific K2P genetic distances range from 0.3% (*H. comes*) to 5.9% (*H. erectus*). In general, the K2P values calculated in this study are tantamount to those of Casey et al [21], as long as there is only 1 bp difference in length between the two studies.

Eight *Hippocampus* species were found in the Taiwan TCM market: the West African seahorse (*H. algiricus*) (Fig. 2A), Knysna seahorse (*H. capensis*) (Fig. 2B), tiger tail seahorse (*H. comes*) (Fig. 2C), thorny seahorse (*H. histrix*) (Fig. 2D), great seahorse (*H. kelloggi*) (Fig. 2E), yellow seahorse (*H. kuda*) (Fig. 2F), hedgehog seahorse (*H. spinosissimus*) (Fig. 2G), and three-spot seahorse (*H. trimaculatus*) (Fig. 2H). Among them, the three-spot seahorse was the most common species in the Taiwan TCM market and was present in 73.9% of investigated stores, in contrast to the rarest species, West African seahorse, Knysna seahorse, and thorny seahorse, for each of which only one specimen was found. Compared to the US Californian shops selling seahorses native to Asia, Eastern Africa, American, Oceania, and Europe [20], the Taiwan TCM stores sold seahorses that were mainly from Asia, in addition to the Knysna and West African seahorses. Fig. 2 also shows that the 10-cm minimum height limit was violated, and young juveniles were found in the market. After the manufacturing process, samples from a single species may have a diversity of appearances, making morphological identification difficult. The Knysna seahorse has an endangered status in the IUCN Red List, whereas the West African seahorse, tiger tail seahorse, thorny seahorse, great seahorse, yellow seahorse, hedgehog seahorse, and three-spot seahorse have vulnerable status. All the sampled seahorse species were noted to be under some kind of threat.

The *cyt b* sequence acted as a good genetic marker to reveal relationships among seahorse species and was also used in many studies on seahorse population genetics [30–32].

Fig. 2 – Pictures of dried seahorses of different *Hippocampus* species: (A) *H. algiricus* (TS20MO-1); (B) *H. capensis* (TS16MO-1); (C) *H. comes* (left to right: TS12MO-2, TS10MO-2, and TS09MO-1); (D) *H. histrix* (TS09MO-2); (E) *H. kelloggi* (left to right: TS18MO-1, TS05MO-1, and TS09MO-4); (F) *H. kuda* (left to right: TS19MO-1, TS10MO-4, and TS12MO-1); (G) *H. spinosissimus* (left to right: TS02MO-1, TS21MO-1, and TS17MO-3); and (H) *H. trimaculatus* (left to right: TS06MO-2, TS13MO-1, and TS04MO-1).

However, the wide distribution of *cyt b* haplotypes observed in tiger tail seahorse, three-spot seahorse, and yellow seahorse may hinder it from discriminating genetic structures among different populations [21]. Therefore, it was difficult to deduce the native habitats of these dried seahorses based solely on *cyt b* sequences.

Distributions of these sampled seahorses are incompatible with official documents, which recorded that, in the most recent 4 years, the imported dried seahorses were mostly from Asia; however, native habitats of both the Knysna seahorse and West African seahorse are in Africa. Except for smuggling, this inconsistency can be explained by the fact that China is a primary consumer and a major exporter of dried seahorses, so it may re-export dried seahorses.

Molecular forensics is a potent tool that can be used to eliminate smuggling of endangered creatures and their products, and disclose the real exploitation of certain organisms, which would help enforce the law and promote legislation. A good example of this is a recent DNA barcode study on shark, which demonstrated that up to 22 shark species are consumed in Taiwan, 56% of which have a status ranging from endangered to vulnerable (unpublished data). Results of this seahorse study revealed that seahorses face as serious a fishing pressure as do sharks. Threatened seahorse species are traded, and young individuals are also fished. Even though many seahorse species can be raised artificially and aquaculture techniques for rearing them are being developed [33–36], authorities should take more measures to supervise the species composition of imported desiccated seahorses and manage the utilization of these organisms until the production from aquaculture can satisfy the demands of the TCM market.

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