Zoological Studies **49**(4): 564-576 (2010)

Zoological Studies

Population Genetics of the Spotted Seahorse (*Hippocampus kuda*) in Thai Waters: Implications for Conservation

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(Accepted December 15, 2009)

Thadsin Panithanarak, Ratima Karuwancharoen, Uthairat Na-Nakorn, and Thuy Thi Thu Nguyen (2010) Population genetics of the spotted seahorse (*Hippocampus kuda*) in Thai waters: implications for conservation. *Zoological Studies* **49**(4): 564-576. A population genetics approach was used to investigate the genetic diversity of the spotted seahorse (*Hippocampus kuda*) in Thai waters; specifically, the degree of genetic differentiation and species evolution was inferred from sequence analysis of 353 bp of the mitochondrial (mt)DNA control region. The data were then used to identify discrete populations in Thai waters for effective conservation and management. Spotted seahorses were collected from 4 regions on the east and west coasts of the Gulf of Thailand and a geographically separated region in the Andaman Sea. Of the 101 mtDNA sequences analyzed, 7 haplotypes were identified, 5 of which were shared among individuals from the east and west coasts of the Gulf of Thailand. The remaining haplotypes were restricted to individuals from the Andaman Sea. Nucleotide and haplotype diversities were similar within the Gulf of Thailand samples, whereas diversity was lower in the Andaman Sea sample. Genetic differentiation appeared between pairs of samples from the Gulf of Thailand and Andaman Sea (F_{ST}, $p < 0.0001$). A large genetic variance appeared among the 2 population groups (94.46%, $\Phi_{CT} = 0.94464$, $p < 0.01$). A Neighbor-joining tree indicated that individuals from the Gulf of Thailand and Andaman Sea formed 2 phylogenetically distinct groups, which were segregated into different population-based clades. While results reported here indicate that populations from the Gulf of Thailand and Andaman Sea should be treated as separate conservation units, a larger sample size from the Andaman Sea is required to confirm this genetic partitioning and low level of diversity observed in the present study. http://zoolstud.sinica.edu.tw/Journals/49.4/564.pdf

Key words: *Hippocampus kuda*, Mitochondrial DNA (mtDNA) control region, Spotted seahorses, Population genetics, Thailand.

Seahorses (genus *Hippocampus*) are in the family Syngnathidae, which also includes pipefish, pipehorses, and seadragons. Seahorses have a worldwide distribution inhabiting both temperate and tropical seas, with 33 species identified to date (Lourie et al. 1999 2004, Lourie and Randall 2003). Seven species are reported from Thailand: the spotted seahorse (*H. kuda*), three-spotted seahorse (*H. trimaculatus*), hedgehog seahorse (*H. spinosissimus*), Japanese seahorse (*H. mohnikei*), great seahorse (*H. kelloggi*), thorny seahorse (*H. histrix*), and tiger tail seahorse (*H. comes*) (Suvatti 1950, Thiemmedh 1968, Monkolprasit et al. 1987, Sukavisit 1989, Nateewathana et al. 1993, Monkolprasit et al. 1997, Chaiyapu 2003, Karuwancharoen and Poosuwan unpubl. data). Seahorse populations are in decline due to habitat destruction and overexploitation (IUCN 2004).

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Seahorse populations in Southeast Asian countries decreased by approximately 15%-50% over a 5 yr period (Vincent 1996). Of the 33 species identified, 1 is recognized as an endangered species (Knysna seahorse, *H. capensis*) and 9 are listed as vulnerable on the 2003 World Conservation Union (IUCN) *Red List of Threatened Animals*. The remaining species have not been categorized due to a lack of information (i.e., data deficient; IUCN 2007). The conservation and management of seahorse populations are of great international concern. For example, trade regulations under Appendix II of CITES (*Convention on International Trade in Endangered Species of Wild Fauna and Flora*, www.cites.org) have been legally recognized since May 2004, and are currently being enforced. However, additional non-regulatory steps may also benefit the conservation and management of seahorses, such as exploratory research concerning aquaculture and restocking. Restocking may introduce detrimental diseases and genetic abnormalities into natural populations. Alternatively, an in-depth analysis of population genetics that focuses on genetic diversity would provide a framework for future management and conservation measures, as an assessment of the genetic diversity of an endangered species would assist in modeling specific populations, assessing the current status, and predicting the rate of molecular evolution (Avise 1989, O'Brien 1994).

Monitoring the status of seahorses in Thai waters, particularly vulnerable species on the 2003 IUCN *Red List*, is possible via an analysis of the mitochondrial (mt)DNA control region. The mtDNA control region is sufficiently variable to use at the population level (Teske et al. 2003, Zhou et al. 2003, Guillen et al. 2005, Faria et al. 2008, Liao et al. 2008). The results from an mtDNA analysis can subsequently be applied to the development of seahorse conservation and management strategies, such as increasing natural seahorse abundances while avoiding impacts on genetic variations. A previous study on the population genetics of the endangered Knysna seahorse (*H. capensis*) revealed mtDNA differentiation among 3 populations occupying different habitats, which led to different and distinct conservation and management strategies for each South African population (Teske et al. 2003).

In Thai waters, preliminary analysis of the mtDNA control region of the spotted seahorse (*H. kuda*) in the Andaman Sea and along the east coast of the Gulf of Thailand suggests that 2 populations are present and likely have evolved independently of each other (Panithanarak 2006). Possible genetic differentiation between seahorse populations can be explained by their ecology, as most seahorse species exhibit site fidelity (Perante et al. 1998), a highly structured community (Vincent and Sadler 1995), and a low level of distribution (Lourie et al. 1999). Further, seahorse morphology constrains physical movement resulting in decreased mobility compared to other fish species. Nevertheless, long-distance colonization by a small number of founding individuals may be common in seahorses associated with the *H. kuda* complex (Teske et al. 2005). Another reason for genetic differentiation of Thai seahorse populations is isolation and independent evolution due to separation by the land barrier of the Malay Peninsula.

The present study investigated the population genetics of *H. kuda* in Thai waters. *Hippocampus kuda* is a valuable trade species in terms of traditional Chinese medicine, curios, and aquaria (IUCN 2004) and is distributed throughout Thai waters but is most abundant in the Gulf of Thailand (Karuwancharoen and Poosuwan after unpubl data). We examined the genetic diversity, genetic differentiation, and molecular evolution of spotted seahorses collected from 5 locations in Thai waters using sequence variations in the mtDNA control region. We focused on the identification of discrete populations or evolutionary significant units (ESUs) to facilitate future conservation and regulatory efforts as proposed by Moritz (1994).

MATERIALS AND METHODS

Sample collection

Seahorse samples were collected at 3 sites on the east coast of the Gulf of Thailand: the Bangsaen coast, Chonburi Province (n = 40), Samed I., Rayong Province (n = 29), and Chang Is., Trat Province (n = 11). Samples were also obtained from a site on the west coast of the Gulf of Thailand (the coast of Cha-um, Phetchaburi Province, n = 20), and from the Andaman Sea (Aow Po, Phuket Province, n = 6) (Fig. 1). Collection sites were based on a taxonomic study of seahorses (Pisces: Hippocampinae) in Thai waters (Karuwancharoen and Poosuwan unpubl. data) and on information provided by local fishermen. Live seahorses were kept in captivity at the aquaculture unit of the Institute of Marine Science, Burapha Univ., Chonburi after clipping a sample

from the dorsal fin (1 × 1 mm in size). A study by Lourie (2003) revealed that fin-clipping had no effect on seahorse growth or survival rates. In some cases where live sample collection was not possible, dried or ethanol-preserved specimens, or both either from the collection sites or from the reference collection of the Institute of Marine Science, Burapha Univ., Chonburi were used.

DNA extraction, amplification, and sequencing

DNA was extracted from small pieces of dorsal fins (ca. 1-2 mm^2), pectoral fins (ca. 1-2 mm^2), or both. DNA was extracted with a DNA extraction kit, QIAamp DNA mini kit (Qiagen, Hilden, Germany). The extraction protocol followed the manufacturer's suggestions. DNA was amplified with the HCAL2 forward primer (5'-CACACTTTCATCGACGCTT-3') and HCAH2 reverse primer (5'-TCTTCAGTGTTATGCTTTA-3') (Teske et al. 2003). These primers were designed to amplify a DNA fragment around the right domain of the mitochondrial (mt)DNA control region (~533 bp). In addition, the primers were suitable for DNA amplification of small pieces of tissue, including fins from young seahorses and dead specimens (Teske et al. 2003).

A polymerase chain reactions (PCR) was performed in a 50 μL volume following Teske et al. (2003). The reaction contained 1 ng/ml DNA, 0.2 μM each of dNTPs (Promega, Madison, WI, USA), buffer consisting of 100 mM NaCl, 0.1 mM EDTA, and 20 mM Tris-HCl (pH 8.0), and 0.4 μM each of the primers (Bioservice Unit, Bangkok, Thailand), 2.5 mM $MgCl_2$, and 1 unit *Taq* DNA polymerase (Promega).

The PCR consisted of an initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Then the reaction was completed with a final extension at 72°C for 10 min.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen). The protocol for PCR purification followed the manufacturer's instructions. Sequencing reactions were performed using the reverse primer, HCAH2 (see Teske et al. 2003). DNA sequencing of all specimens was carried out by the sequencing service, Macrogen (Seoul, Korea).

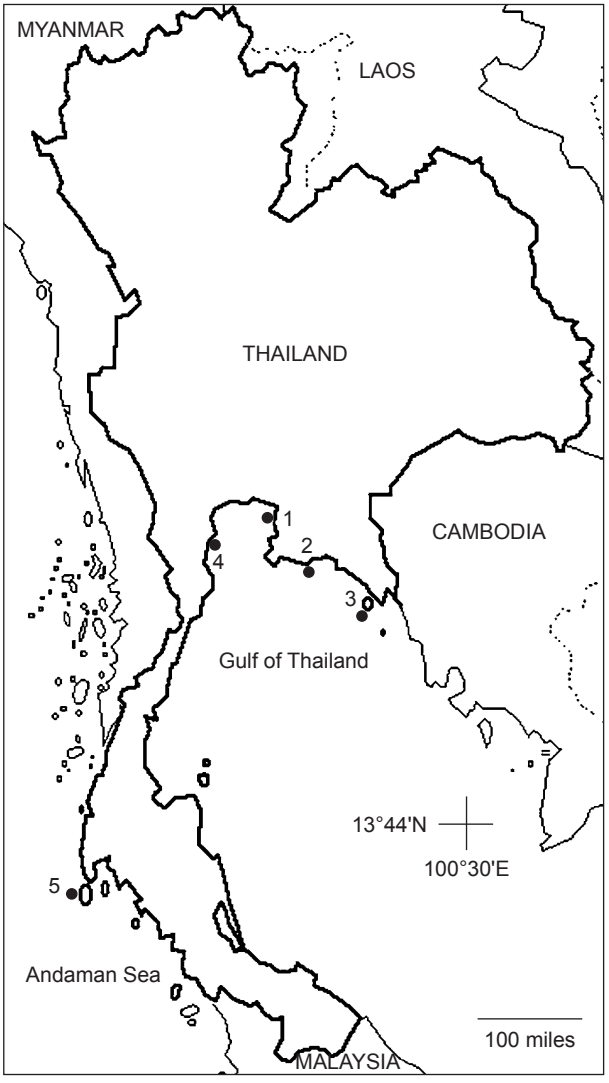

Fig. 1. Map illustrating sample collection sites of spotted seahorses in Thai waters (in the Gulf of Thailand and Andaman Sea). Numbers indicate the collection sites: **1**, Bangsaen coast, Chonburi Province; **2**, Samed I., Rayong Province; **3**, Chang Is., Trat Province; **4**, coast of Cha-um, Phetchaburi Province; **5**, Aow Po, Phuket Province.

Data analysis

Nucleotide sequences were manually checked for accuracy and were compared and aligned with the mtDNA control region sequences of the *H. kuda* complex (accession nos.: AY149664-AY149678, Teske et al. 2005) using Clustal X (Thompson et al. 1997). New sequences were deposited in GenBank (accession nos.: EU717698-EU717704).

Statistical analysis

For the Thai population analysis, the genetic diversity, level of genetic differentiation, and population structure were investigated as follows.

First, nucleotide (π) and haplotype diversities (h) of spotted seahorses in Thai waters were calculated using DnaSP 4.0 (Rozas et al. 2003). Second, the level of genetic differentiation among populations was estimated with F_{ST} (molecular distance: pairwise difference) using Arlequin 3.1 (Excoffier and Schneider 2005). p values for the pairwise F_{ST} tests were adjusted with the Bonferroni correction for multiple tests (Hochberg 1988). Finally, the population structure was investigated using an analysis of molecular variance (AMOVA; Michalakis and Excoffier 1996) using Arlequin. The population structure or the partitioning of genetic variance using AMOVA (distance method: pairwise difference) was analyzed on 3 hierarchical levels: within each sampled population, among populations sampled within the Gulf of Thailand and Andaman Sea, and between populations of the Gulf of Thailand and Andaman Sea.

Phylogenetic relationships between Thai and *H. kuda* complex (Teske et al. 2005) haplotypes were estimated with Neighbor-joining (NJ) phylogenetic trees (Saitou and Nei 1987). Because of the large dataset, other methods, e.g., maximum-parsimony and maximum-likelihood methods, were not applicable. The most appropriate DNA substitution model for the dataset was selected using MODELTEST 3.06 (Posada and Crandall 1998). The model selected by hierarchical likelihood ratio tests (hLRTs) was the HKY+I+G model (Hasegawa et al. 1985) with the frequency of invariable sites set to 0.6240 and a gamma correction of 0.8367. For the Akaike information criterion (AIC), the model selected was the TrN+I+G model (Tamura and Nei 1993) with the frequency of invariable sites set to 0.6438 and a gamma correction of 0.9650. NJ trees were constructed using PAUP* 4.0 beta 10 (Swofford 2002) with 1000 bootstrap replicates (Felsenstein 1985). The trees were rooted with *H. fisheri* from Hawaii, *H. ingens* from the eastern Pacific coast of Mexico, and *H. reidi* from the Gulf of Mexico, as they represent 3 geographically distant seahorse species closely related to Thai and *H. kuda* complex haplotypes in this study (Teske et al. 2005).

RESULTS

Of the 106 seahorses analyzed, 5 failed to produce a sequence due to amplification failure or a low quality of the PCR product. The mtDNA control region sequences ranged 369-472 bp, although sequences were truncated to 353 bp, based upon alignment with the *H. kuda* complex (Teske et al. 2005) prior to the analyses.

Genetic diversity

The analysis of the 101 spotted seahorse mtDNA control region sequences (353 bp) yielded 19 variable sites (3 indels, 12 transitions, and 4 transversions; Fig. 2) that defined 7 haplotypes (kudaTH1, kudaTH2, kudaTH3, kudaTH4, kudaTH5, kudaTH6, and kudaTH7; Table 1). The most common haplotype was kudaTH1, which was observed in 61% of the seahorses examined (Table

	0	0	0	0	1	1	1	1	2	2	2	2	2	3	3	3	3	3	3
	5	8	9	9	0	4	6	6	3	5	5	6	7	3	3	4	4	5	5
	0	8	2	3	9	1	6	9	6	6	7	6	2	5	7	2	8	0	2
kudaTH4	G	G	T	T	C	A	C	C	G	A	A	A	T	A	A	G	C	C	C
kudaTH5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
kudaTH1	A	A	A	.	T	-	.	T	A	G	T	G	C	-	.	A	A	T	.
kudaTH7	A	A	A	.	T	-	-	T	A	G	T	G	C	-	.	A	A	T	.
kudaTH2	A	A	A	.	T	-	.	T	A	G	T	G	C	-	.	A	A	T	T
kudaTH3	A	A	A	C	T	-	.	T	A	G	T	G	C	-	.	A	A	T	.
kudaTH6	A	A	A	.	.	-	.	T	A	G	T	G	C	-	.	A	A	.	.

Fig. 2. Variable sites within Thai spotted seahorse haplotypes. Numbers above the nucleotides indicate the nucleotide position (from 1 to 353 based upon alignment with the previously reported *Hippocampus kuda* complex; Teske et al. 2005). Dots indicate that the nucleotide is identical to kudaTH4. Dashes indicate a deletion or insertion compared to kudaTH4. kudaTH3 was identical to kudaPH2 and kudaPH6 (Teske et al. 2005). kudaTH4 was identical to kudaID5 (Teske et al. 2005).

1) and was observed at the highest frequency among specimens from the east and west coasts of the Gulf of Thailand (i.e., Bangsaen coast, Samed I., Chang Is., and the Cha-um coast; Table 2). Five haplotypes (kudaTH1, kudaTH2, kudaTH3, kudaTH6, and kudaTH7) were limited to the Gulf of Thailand; specifically, kudaTH1, kudaTH2, and kudaTH3 were observed among all 4 Gulf of Thailand collection sites, while kudaTH6 was observed in three of the 4 Gulf of Thailand sites, except the Chang Is., and kudaTH7 was the only haplotype observed among specimens from the Bangsaen coast. In contrast, haplotypes kudaTH4 and kudaTH5 were observed only among specimens from the Andaman Sea (Aow Po). Haplotypes of seahorses from the Gulf of Thailand (kudaTH1, kudaTH2, kudaTH3, kudaTH6, and kudaTH7) differed by 1-3 bp, and those in

Table 1. *Hippocampus kuda* complex (Teske et al. 2005) haplotypes and Thai haplotypes from this study. Details include sample collection sites, the number of specimens (numbers in parentheses are the number of specimens/collection site). kuda, *H. kuda*; fusc, *H. fuscus*; TH, Thailand; PH, the Philippines; ID, Indonesia; TW, Taiwan; FJ, Fiji; ZA, South Africa; MZ, Mozambique; MY, Malaysia; IN, India; TZ, Tanzania; EG, Egypt

Haplotype	Collection site (number of specimens)
Thai haplotypes	
kudaTH1	Bangsaen (23), Samed (20), Chang (6), Cha-um (13)
kudaTH2	Bangsaen (5), Samed (1), Chang (1), Cha-um (3)
kudaTH3[1]	Bangsaen (7), Samed (5), Chang (3), Cha-um (2)
kudaTH4[2]	Aow Po (5)
kudaTH5	Aow Po (1)
kudaTH6	Bangsaen (1), Samed (2), Cha-um (2)
kudaTH7	Bangsaen (1)
H. kuda complex haplotypes	
kudaPH1-kudaPH8[3]	Tayabas Bay, Quezon, the Philippines
kudaID1-kudaID7	North Sulawesi, northeastern Indonesia
kudaTW	Taiwan
kudaFJ	Fiji
kudaZA1-kudaZA3	KwaZulu/Natal, South Africa
kudaMZ	Inhaca Is., Mozambique
kudaMY1-kudaMY10[4]	Pulai Estuary, Johor, Malaysia
kudaIN1-kudaIN11	India
kudaTZ	Pemba, Tanzania
fuscEG1-fuscEG5[5]	Gulf of Suez, Red Sea, Egypt

According to the 353 bp mtDNA control region based upon alignment with the *H. kuda* complex (Teske et al. 2005). [1]kudaTH3 is identical to kudaPH2 and kudaPH6. [2]kudaTH4 is identical to kudaID5. [3]kudaPH2 is identical to kudaPH6. [4]kudaMY1 is identical to kudaMY3. [5]fuscEG1 is identical to fuscEG2 and fuscEG6.

Table 2. Relative frequencies of the spotted seahorse haplotypes observed in Thai waters. Numbers in parentheses indicate the number of specimens per population

Haplotype	Relative frequency				
	Bangsaen (37)	Cha-um (20)	Chang (10)	Samed (28)	Aow Po (6)
kudaTH1	0.622	0.650	0.600	0.714	0
kudaTH2	0.135	0.150	0.100	0.036	0
kudaTH3	0.189	0.100	0.300	0.179	0
kudaTH4	0	0	0	0	0.833
kudaTH5	0	0	0	0	0.167
kudaTH6	0.027	0.100	0	0.071	0
kudaTH7	0.027	0	0	0	0

seahorses from the Andaman Sea (kudaTH4 and kudaTH5) differed by 1 bp (Fig. 2). Conversely, haplotypes restricted to either the Gulf of Thailand or the Andaman Sea differed by 13-17 bp (Fig. 2).

Genetic diversity indices for samples from the Bangsaen coast, Samed I., Chang Is., and Cha-um coast populations are shown in table 3. Haplotype and nucleotide diversities for samples from the Gulf of Thailand were similar. Samples from the Chang Is. yielded the greatest haplotype diversity (h = 0.60 ± 0.13, n = 10), whereas those from the Cha-um coast yielded the greatest nucleotide diversity (π = 0.0024 ± 0.0007, n = 20). The smallest haplotype and nucleotide diversities were observed in samples from Aow Po, the only population sampled from the Andaman Sea (h = 0.33 ± 0.22, π = 0.0009 ± 0.0006, n = 6).

Genetic differentiation

Pairwise F_{ST} values between pairs of samples ranged -0.043-0.961. The Aow Po population significantly differed from the other populations ($p < 0.0001$, Table 4), whereas other populations were genetically similar.

The results suggest that populations within the Gulf of Thailand were genetically similar to each other, and that they significantly differed from the population sampled in the Andaman Sea. The AMOVA revealed a significant structure between the Gulf of Thailand and Andaman Sea (variance = 94.46%, Φ_{CT} = 0.94464, $p < 0.01$, Table 5), whereas no significant structure was found among populations in the Gulf of Thailand (variance = -0.08%, Φ_{SC} = -0.01374, p = 0.74). A

Table 3. Genetic diversity indices and sample sizes (n) of the Bangsaen coast, Samed I., Chang Is., coast of Cha-um, and Aow Po populations. Indices include the number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and polymorphic (segregated) sites (S)

Site	*n*	H	*h*	π	S
Bangsaen	37	5	0.57 ± 0.08	0.0019 ± 0.0004	5
Samed I.	28	4	0.47 ± 0.10	0.0019 ± 0.0004	4
Chang Is.	10	3	0.60 ± 0.13	0.0019 ± 0.0004	2
Cha-um	20	4	0.56 ± 0.12	0.0024 ± 0.0007	4
Aow Po	6	2	0.33 ± 0.22	0.0009 ± 0.0006	1

Table 4. Population pairwise F_{ST} (molecular distance: pairwise difference) (below the diagonal) and F_{ST} p values (above the diagonal) among pairs of populations from the Bangsaen coast, Samed I., Chang Is., the coast of Cha-um, and Aow Po. Significant F_{ST} p values after the Bonferroni correction are shown in bold

	Bangsaen	Cha-um	Chang	Samed	Aow Po
Bangsaen		0.610	0.815	0.617	**< 0.0001**
Cha-um	-0.013		0.425	0.561	**< 0.0001**
Chang	-0.043	0.005		0.620	**< 0.0001**
Samed	-0.010	-0.014	-0.024		**< 0.0001**
Aow Po	0.949	0.942	0.961	0.952	

Table 5. Population structure of spotted seahorses in Thai waters using the AMOVA method. The partitioning of genetic variance into 3 hierarchical levels is shown in the table

Hierarchical level	*d.f.*	Percent (%) genetic variance	Fixation indices	*p* value
Within each sampled population	96	5.61	0.94387	< 0.01
Among populations sampled within the Gulf of Thailand and Andaman Sea	3	-0.08[a]	-0.01374	0.74
Between the Gulf of Thailand and Andaman Sea populations	1	94.46	0.94464	< 0.01

[a]It is possible by chance to have a slightly negative percent variance, if the expectation of the estimator is 0 (the Arlequin Forum, http://www.rannala.org/gsf).

slight genetic structure was observed within each population (variance = 5.61%, Φ_{ST} = 0.94387, $p < 0.01$).

Phylogenetic relationships

NJ trees based on the HKY+I+G and TrN+I+G models were topologically similar. A representative NJ tree based on the HKY+I+G model is shown in figure 3. In this tree, *H. fuscus* (fuscEG1-fuscEG6) was paraphyletic to *H. kuda* from the Indian Ocean as it is a closely related species from the Red Sea (Teske et al. 2005). Thai haplotypes were segregated into 2 groups. The 1st group comprised haplotypes of seahorses from the Gulf of Thailand (kudaTH1, kudaTH2, kudaTH3, kudaTH6, and kudaTH7) and were closely related to haplotypes from Pacific Ocean populations in the Philippines, Fiji, and Taiwan, (kudaPH1-kudaPH8, kudaFJ, and kudaTW, respectively) with high bootstrap support (84%). The 2nd group included haplotypes of seahorses from the Andaman Sea (kudaTH4 and kudaTH5) that clustered with haplotypes from Indian Ocean populations, specifically Indonesian haplotypes (kudaID1-kudaID5, and kudaID7) (Fig. 3).

DISCUSSION

Analysis of the partial mtDNA control region of spotted seahorses in Thai waters revealed clear genetic differentiation between individuals from the Gulf of Thailand and Andaman Sea. Further phylogenetic analysis also showed evidence of independent evolution of populations in the Gulf of Thailand and Andaman Sea. These data suggested that populations from the Gulf of Thailand and Andaman Sea should be treated as separate conservation units. We could not rule out that the sample size of the Andaman Sea in this study was smaller than the others, and the unequal geographical sampling may have biased the conclusions that could be drawn. As a consequence, a larger sample size is required to confirm the low level of diversity observed in the Andaman Sea, as well as the distinct differences noted between individuals collected from the Gulf of Thailand and Andaman Sea. The results are discussed in greater detail below with an emphasis on seahorse conservation.

Genetic diversity and conservation

Genetic diversities of populations in the Gulf of Thailand were relatively similar, with haplotype and nucleotide diversities ranging 0.47-0.60 and 0.0019-0.0024, respectively. Genetic diversity in Andaman Sea seahorses was much lower than that in Gulf of Thailand ones (h = 0.33, π = 0.0009). Compared to the endangered Knysna seahorse, *H. capensis* of South Africa, which occupies a different range (Teske et al. 2003), the genetic diversity of Thai populations of *H. kuda* was relatively low (with the exception of the Swartvlei population of southern Africa (h = 0.48, Table 6)). The low genetic diversity of Thai populations reflects a small number of haplotypes and a modest number of differences among Gulf of Thailand haplotypes (1-3 bp) and between the Andaman Sea haplotypes (1 bp), as well as low haplotype and nucleotide diversities in the Andaman Sea population. The low diversity in the Andaman Sea might signify either low genetic diversity or a small sample size. The relatively low genetic diversity of Gulf of Thailand populations suggests that the population size tends to be smaller than the existing size even though the capture and trade of seahorses are regulated. However, the genetic results indicated that Gulf of Thailand populations are not fragmented, suggesting that current gene flow among populations is high enough to minimize inbreeding and the loss of genetic diversity. Therefore, to conserve the present genetic diversity of Thai spotted seahorse populations, regulatory enforcement of species capture and trade is essential to prevent population declines or fragmentation throughout its range.

Implications of the population structure and evolution on conservation

An investigation of the genetic differentiation of Thai spotted seahorse populations revealed 7 haplotypes. Five were restricted to Gulf of Thailand populations and 2 were restricted to a population within the Andaman Sea. Importantly, none of these haplotypes was shared between the 2 regions. When compared to previously identified *H. kuda* complex haplotypes (Teske et al. 2005), 2 haplotypes observed among Thai-collected seahorses were identical to haplotypes observed among seahorses collected from the Philippines and North Sulawesi, Indonesia, i.e., kudaTH3 was identical to kudaPH2 and kudaPH6 (the Philippines), and kudaTH4 was identical

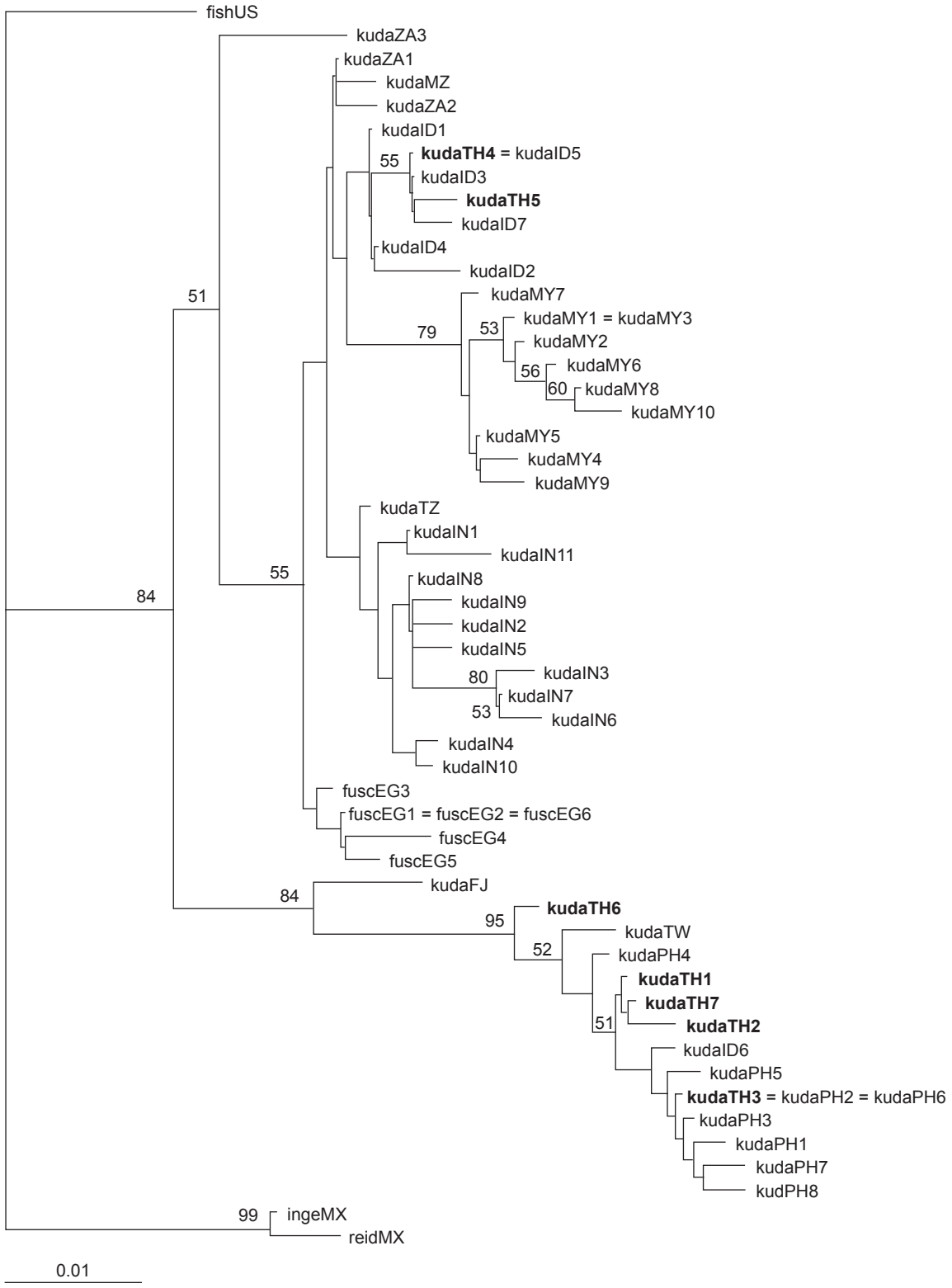

Fig. 3. A representative Neighbor-joining tree based on the HKY+I+G model (Hasegawa et al. 1985) selected by Modeltest 3.7 (Posada and Crandall 1998). The tree illustrates phylogenetic relationships among Thai haplotypes (kudaTH1-kudaTH7 shown in bold) and *Hippocampus kuda* complex haplotypes (Teske et al. 2005). Numbers indicate bootstrap support (%). Designated outgroups included fishUS (*H. fisheri*, Hawaii), ingeMX (*H. ingens*, Mexico), and reidMX (*H. reidi*, Mexico).

to kudaID5 (Indonesia). Genetic differentiation between the regions was also evident in pairwise F_{ST} comparisons between the Gulf of Thailand and Andaman Sea populations (Table 4), as well as in the large genetic variance between populations in the Gulf of Thailand and Andaman Sea (variance = 94.46%, Φ_{CT} = 0.94464, $p < 0.01$). Clear genetic differentiation between the 2 regions and site-specific haplotypes indicate that gene flow between these regions is limited. This study supports the independent evolution of Gulf of Thailand and Andaman Sea populations, as Gulf of Thailand populations clustered together with Pacific Ocean populations, indicating a shared lineage. On the other hand, the Andaman Sea population clustered with Indian Ocean populations, indicating a close relationship with Indonesian populations (Fig. 3). Phylogenetic relationships of spotted seahorses were previously described using variations in the non-coding mtDNA control region (Teske et al. 2005) and the protein coding-region of the cytochrome *b* gene (Lourie et al. 2005). Both studies buttress the findings reported here and support the conclusion that populations in the Gulf of Thailand and Andaman Sea have evolved independently of one another.

Genetic differentiation between Gulf of Thailand and Andaman Sea populations may have been initiated as early as the Pleistocene (2.4 Ma~10,000 yr ago) similar to that seen between other marine species in Southeast Asia (McMillan and Palumbi 1995), as evidenced in a phylogenetic study of seahorses (Casey et al. 2004). Diversification of spotted seahorses could be explained by the Pleistocene isolation of marine basins hypothesis (McManus 1985). The observed population fragmentation may be associated with lowered sea levels during the Pleistocene ice ages (120 m below present levels at the last glacial maximum) and formation of land bridges among mainland Asia, Borneo, and other Western Indonesian Is. and the Philippine islands which would have isolated the Indian and Pacific Ocean populations. Low dispersal over a long period would limit subsequent movement of individuals leading to the existence of private haplotypes within *H. kuda* of the Gulf of Thailand and Andaman Sea as evidenced in this study. Regardless of geographic barriers, characteristics unique to seahorses may have facilitated the genetic divergence of Thai populations. Seahorses have limited dispersal compared to other marine fishes (Lourie et al. 1999). Juveniles are unlikely to disperse long distances because they are fully developed and begin feeding immediately, unlike other marine fishes with pelagic larvae (Lourie et al. 1999). In addition, most seahorse species exhibit site fidelity and a highly structured community (Vincent and Sadler 1995).

Genetic divergence between populations of marine organisms in the Gulf of Thailand and Andaman Sea was reported for several other species including the orange-spotted grouper (*Epinephelus coioides*) (Antoro et al. 2006), tropical abalone (*Haliotis assinina*) (Tang et al. 2004), and giant tiger shrimp (*Penaeus monodon*) (Klinbunga et al. 1999 2001). Genetic differentiation between Gulf of Thailand (Nakornsrithammarach) and Andaman Sea (Trang) populations of orange-spotted groupers was attributed to a geographic barrier, the Malaysian Peninsula (Antoro et al. 2006), a conclusion consistent with the Pleistocene isolation of marine basins hypothesis of McManus (1985).

The findings of the present study reveal how the study of population genetics can help in

Table 6. Genetic diversity indices of the endangered Knysna (*Hippocampus capensis*) of South Africa (Teske et al. 2003) and sample sizes (*n*) of Knysna (1-3), Swartvlei, and Keurbooms. Indices include the number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), and polymorphic (segregated) sites (S)

Site	*n*	H	*h*	π	S
Knysna					
1	30	9	0.73	0.00348	8
2	30	7	0.76	0.00299	5
3	30	8	0.84	0.00425	7
Swartvlei	30	3	0.48	0.00461	5
Keurbooms	18	8	0.75	0.00458	7

the conservation and management of vulnerable and endangered marine species. Genetic differentiation between populations of spotted seahorses in the Gulf of Thailand and Andaman Sea, and their independent evolution indicate that Thai populations are segregated into at least 2 populations and should be treated as 2 separate conservation units. For instance, increasing the abundance of the Andaman Sea population via restocking with individuals collected from the Gulf of Thailand would not be appropriate, as it could lead to outbreeding depression of wild populations.

Acknowledgments: We would like to thank the following people for providing specimens and help with sample collection: our colleagues at the Institute of Marine Science, Burapha Univ. (Chanvit Suphaphanyapong, Sukrudee Deebugkam, Prattana Khemtong, Dr. S. Putchakarn, Dr. V. Muthuwan, Dr. S. Sawatpeera, and V. Boonchauyleaw), Samrauy Likhasithiphan (Eastern Marine Fisheries Research and Development Center, EMDEC, Rayong), Suchat Saengchan (Andaman Sea Fisheries Research and Development Center, AFDEC, Pang-nga Station), Thadsapol Krajangdara and Wanlee Singthongyam (Andaman Sea Fisheries Research and Development Center, AFDEC, Phuket), and Samart Dechsathit (Krabi Coastal Fisheries Research and Development Center, Krabi). We also thank the Asia Research Center, Chulalongkorn Univ. for financial support.

REFERENCES

Antoro S, U Na-Nakorn, W Koedprang. 2006. Study of genetic diversity of orange-spotted grouper, *Epinephelus coioides*, from Thailand and Indonesia using microsatellite markers. Mar. Biotechnol. **8:** 17-26.

Avise JC. 1989. A role for molecular genetics in the recognition and conservation of endangered species. Trends Ecol. Evol. **4:** 279-281.

Casey SP, HJ Hall, HF Stanley, ACJ Vincent. 2004. The origin and evolution of seahorses (genus *Hippocampus*): a phylogenetic study using the cytochrome *b* gene of mitochondrial DNA. Mol. Phylogenet. Evol. **30:** 261-272.

Chaiyapu M. 2003. Taxonomy of pipefishes and seahorses (PISCES: SYNGNATHIDAE) found in Thai waters. MS thesis, Chulalongkorn Univ., Bangkok, Thailand. (in Thai)

Excoffier LG, S Schneider. 2005. Arlequin vers. 3.0: an integrated software package for population genetics data analysis. Evolut. Bioinform. Online **1:** 47-50.

Faria PJ, NMR Guedes, C Yamashita, P Martuscelli, CY Miyaki. 2008. Genetic variation and population structure of the endangered Hyacinth Macaw (*Anodorhynchus hyacinthinus*): implications for conservation. Biodivers. Conserv. **17:** 765-779.

Felsenstein JW. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:** 783-791.

Guillen AKA, GM Barrett, O Takenaka. 2005. Genetic diversity among African great apes based on mitochondrial DNA sequences. Biodivers. Conserv. **14:** 2221-2233.

Hasegawa M, K Kishino, T Yano. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22:** 160-174.

Hochberg Y. 1988. A sharper Bonferroni procedure for multiple tests of significance. Biometrika **75:** 800-802.

IUCN. 2004. 2004 IUCN red list of threatened species. Available at http://www.iucnredlist.org/

IUCN. 2007. 2007 IUCN red list of threatened species. Available at http://www.iucnredlist.org/

Klinbunga S, DJ Penman, BJ McAndrew, A Tassanakajon. 1999. Mitochondrial DNA diversity in three populations of the giant tiger shrimp *Penaeus monodon*. Mar. Biotechnol. **1:** 113-121.

Klinbunga S, D Siludjai, W Wudthijinda, A Tassanakajon, P Jarayabhand, P Menasveta. 2001. Genetic heterogeneity of the giant tiger shrimp (*Penaeus monodon*) in Thailand revealed by RAPD and mitochondrial DNA RFLP analyses. Mar. Biotechnol. **3:** 428-438.

Liao TY, TY Wang, HD Lin, SC Shen, CS Tzeng. 2008. Phylogeography of the endangered species, *Sinogastromyzon puliensis* (Cypriniformes: Balitoridae), in southwestern Taiwan based on mtDNA. Zool. Stud. **47:** 383-392.

Lourie SA. 2003. Fin-clipping procedure for seahorses. Fisheries Centre, Univ. of British Columbia: Project Seahorse Technical Bulletin 3 (vers. 1.1), pp 1-4.

Lourie SA, SJ Foster, EWT Cooper, ACJ Vincent. 2004. A guide to the identification of seahorses. Project Seahorse and TRAFFIC North America. Washington DC: Univ. of British Columbia and World Wildlife Fund.

Lourie SA, DM Green, ACJ Vincent. 2005. Dispersal, habitat differences, and comparative phylogeography of Southeast Asian seahorses (Syngnathidae: *Hippocampus*). Mol. Ecol. **14:** 1073-1094.

Lourie SA, JE Randall. 2003. A new pygmy seahorse, *Hippocampus denise* (Teleostei: Syngnathidae) from the Indo-Pacific. Zool. Stud. **42:** 284-291.

Lourie SA, ACJ Vincent, HJ Hall. 1999. Seahorse: an identification guide to the world's species and their conservation. London: Project Seahorse.

McManus JW. 1985. Marine speciation, tectonics and sea level changes in Southeast Asia. *In*: Fifth International Coral Reef Congress, Tahiti **4:** 133-138.

McMillan WO, SR Palumbi. 1995. Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. Proc. R. Soc. Lond. B **260:** 229-236.

Michalakis Y, L Excoffier. 1996. A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics **142:** 1061-1064.

Monkolprasit S, S Sontirat, S Vimollohakarn, T Songsirikul. 1997. Check list of fishes in Thailand. Bangkok: Office of Environmental Policy and Planning.

Monkolprasit S, S Vimollohakarn, T Songsirikul. 1987. Fish of Thailand. Bangkok: Faculty of Fisheries, Kasetsart Univ. (in Thai)

Moritz C. 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. Mol. Ecol. **3:** 401-411.

Nateewathana A, C Aungtonya, R Sirivejabandhu. 1993. Revised checklist of fishes in the reference collection of Phuket Marine Biological Center, Department of Fisheries, Thailand. Phuket Mar. Biol. Center Special Publ. **12:** 9-39.

O'Brien SJ. 1994. Genetic and phylogenetic analyses of endangered species. Annu. Rev. Genet. **28:** 467-489.

Panithanarak T. 2006. Population genetics of spotted seahorses (*Hippocampus kuda*) in eastern coast of the Gulf of Thailand and the Andaman Sea. Final Report. Chonburi, Thailand: Institute of Marine Science, Burapha Univ. (in Thai)

Perante NC, MG Pajaro, ACJ Vincent. 1998. Demographics of the seahorse *Hippocampus comes* seahorses in the central Philippines. *In* B Morton ed. Proceedings of the Third International Conference on the Marine Biology of the South China Sea. Hong Kong: Hong Kong Univ. Press, pp. 439-448.

Posada D, KA Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:** 817-818.

Rozas J, JC Sánchez-DelBarrio, X Messeguer, R Rozas. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics **19:** 2496-2497.

Saitou N, M Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.

Sukavisit P. 1989. Check list fishes of Thailand. Bangkok: Department of Fisheries. (in Thai)

Suvatti C. 1950. Fauna of Thailand. Bangkok: Department of Fisheries.

Swofford DL. 2002. PAUP*-phylogenetic analysis using parsimony (*and other methods), vers. 4.0b10. Sunderland, MA: Sinauer Associates.

Tamura K, M Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. **10:** 512-526.

Tang S, A Tassanakajon, S Klinbunga, P Jarayaphand, P Menasveta. 2004. Population structure of tropical abalone (*Haliotis assinina*) in coastal waters of Thailand determined using microsatellite markers. Mar. Biotechnol. **6:** 604-611.

Teske PR, MI Cherry, CA Matthee. 2003. Population genetics of the endangered Knysna seahorse, *Hippocampus capensis*. Mol. Ecol. **12:** 1703-1715.

Teske PR, H Hamilton, PJ Palsboll, CK Choo, H Gabr, SA Lourie et al. 2005. Molecular evidence for long-distance colonization in an Indo-Pacific seahorse lineage. Mar. Ecol.-Prog. Ser. **286:** 249-260.

Thiemmedh J. 1968. Fishes of Thailand: their English, scientific and Thai names. Kasetsart Univ. Fish. Res. Bull. **4:** 8-149.

Thompson JD, TJ Gibson, F Plewniak, F Jeanmougin, DG Higgins. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24:** 4876-4882.

Vincent ACJ. 1996. The international trade in seahorses. Cambridge, UK: TRAFFIC International.

Vincent ACJ, LM Sadler. 1995. Faithful pair bonds in the wild seahorses, *H. whitei*. Anim. Behav. **50:** 1557-1569.

Zhou JF, QJ Wu, YZ Ye, JG Tong. 2003. Genetic divergence between *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* as assessed by mitochondrial DNA analysis, with emphasis on origin of European domestic carp. Genetica **119:** 93-97.

Appendix 1. Details of spotted seahorse specimens in this study and haplotypes identified

Sample name	Catalog no.	Site	Collection date	Type of specimen	Haplotype	Comments
kuda_BS1	BIMS-F9130-1	Bangsaen	19/8/2003	preserved	kudaTH1	
kuda_BS2	BIMS-F9130-2	Bangsaen	19/8/2003	preserved	kudaTH1	
kuda_BS3	BIMS-F9130-3	Bangsaen	19/8/2003	preserved	kudaTH1	
kuda_BS7	BIMS-F9130-7	Bangsaen	19/8/2003	preserved	kudaTH3	
kuda_BS9	BIMS-F9130-9	Bangsaen	19/8/2003	preserved	kudaTH2	
kuda_BS10	BIMS-F9130-10	Bangsaen	19/8/2003	preserved	kudaTH1	
kuda_BS11	BIMS-F9130-11	Bangsaen	19/8/2003	preserved	kudaTH3	
kuda_BS14	BIMS-F9130-14	Bangsaen	19/8/2003	preserved	kudaTH3	
kuda_BS15	BIMS-F9130-15	Bangsaen	19/8/2003	preserved	kudaTH3	
kuda_BS16	BIMS-F9130-16	Bangsaen	19/8/2003	preserved	kudaTH1	
kuda_1	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_2	-	Bangsaen	27/1/2006	live	kudaTH6	
kuda_3	-	Bangsaen	27/1/2006	live	-	degraded sample
kuda_4	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_5	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_6	-	Bangsaen	27/1/2006	live	-	ambiguous sequence
kuda_7	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_8	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_9	-	Bangsaen	27/1/2006	live	kudaTH2	
kuda_10	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_11	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_12	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_13	-	Bangsaen	27/1/2006	live	kudaTH7	
kuda_14	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_15	-	Bangsaen	27/1/2006	live	kudaTH3	
kuda_16	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_17	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_18	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_19	-	Bangsaen	27/1/2006	live	kudaTH2	
kuda_20	-	Bangsaen	27/1/2006	live	kudaTH1	
kuda_21	-	Bangsaen	24/2/2006	live	kudaTH1	
kuda_22	-	Bangsaen	24/2/2006	live	kudaTH2	
kuda_23	-	Bangsaen	24/2/2006	live	-	PCR failed
kuda_24	-	Bangsaen	24/2/2006	live	kudaTH3	
kuda_25	-	Bangsaen	24/2/2006	live	kudaTH3	
kuda_26	-	Bangsaen	24/2/2006	live	kudaTH2	
kuda_27	-	Bangsaen	24/2/2006	live	kudaTH1	
kuda_28	-	Bangsaen	24/2/2006	live	kudaTH1	
kuda_29	-	Bangsaen	24/2/2006	live	kudaTH1	
kuda_30	-	Bangsaen	24/2/2006	live	kudaTH1	
kuda_RY1	BIMS-T0007-4	Samed	2006	preserved	kudaTH1	
kuda_RY2	BIMS-T0007-5	Samed	2006	preserved	kudaTH1	
kuda_RY3	BIMS-T0007-6	Samed	2006	preserved	kudaTH1	
kuda_RY4	BIMS-T0007-7	Samed	2006	preserved	kudaTH1	
kuda_RY5	BIMS-T0007-8	Samed	2006	preserved	kudaTH1	
kuda_RY6	BIMS-T0007-9	Samed	2006	preserved	kudaTH1	
kuda_RY7	BIMS-T0007-10	Samed	2006	preserved	kudaTH1	
kuda_RY8	BIMS-T0007-11	Samed	2006	preserved	kudaTH1	
kuda_RY9	BIMS-T0007-12	Samed	2006	preserved	kudaTH1	
kuda_RY10	BIMS-T0007-13	Samed	2006	preserved	kudaTH1	
kuda_RY11	BIMS-T0007-14	Samed	2006	preserved	kudaTH1	
kuda_RY12	BIMS-T0007-15	Samed	2006	preserved	kudaTH1	
kuda_RY13	BIMS-T0007-16	Samed	2006	preserved	kudaTH1	

Appendix 1. (continue)

Sample name	Catalog no.	Site	Collection date	Type of specimen	Haplotype	Comments
kuda_RY14	BIMS-T0007-17	Samed	2006	preserved	kudaTH1	
kuda_RY15	BIMS-T0007-18	Samed	2006	preserved	kudaTH1	
kuda_RY16	BIMS-T0007-19	Samed	2006	preserved	kudaTH1	
kuda_RY17	BIMS-T0007-20	Samed	2006	preserved	kudaTH6	
kuda_RY18	BIMS-T0007-21	Samed	2006	preserved	kudaTH1	
kuda_RY19	-	Samed	24/11/2006	live	kudaTH2	
kuda_RY20	-	Samed	24/11/2006	live	kudaTH6	
kuda_RY21	-	Samed	24/11/2006	live	kudaTH1	
kuda_RY22	-	Samed	24/11/2006	live	kudaTH1	
kuda_RY23	-	Samed	24/11/2006	live	kudaTH1	
kuda_RY24	-	Samed	24/11/2006	live	kudaTH3	
kuda_RY25	-	Samed	24/11/2006	live	-	sampling error
kuda_Y1	-	Samed	24/11/2006	live	kudaTH3	
kuda_Y2	-	Samed	24/11/2006	live	kudaTH3	
kuda_Y3	-	Samed	24/11/2006	live	kudaTH3	
kuda_Y4	-	Samed	24/11/2006	live	kudaTH3	
kuda_TR1	BIMS-F8014-1	Chang	9/12/2003	preserved	kudaTH1	
kuda_TR3	BIMS-F8014-3	Chang	9/12/2003	preserved	kudaTH3	
kuda_TR4	BIMS-F8014-4	Chang	9/12/2003	preserved	kudaTH1	
kuda_TR5	BIMS-F8014-5	Chang	9/12/2003	preserved	kudaTH1	
kuda_TR6	BIMS-F8014-6	Chang	9/12/2003	preserved	kudaTH3	
kuda_TR7	BIMS-F8014-7	Chang	9/12/2003	preserved	kudaTH1	
kuda_TR11	BIMS-F8014-11	Chang	9/12/2003	preserved	kudaTH1	
kuda_TR12	BIMS-F8014-12	Chang	9/12/2003	preserved	kudaTH1	
kuda_TR13	BIMS-F8014-13	Chang	9/12/2003	preserved	kudaTH2	
kuda_TR14	BIMS-F8014-14	Chang	9/12/2003	preserved	-	PCR failed
kuda_TR20	-	Chang	22/3/2007	live	kudaTH3	
kuda_PBR1	-	Cha-um	2006	live	kudaTH1	
kuda_PBR2	-	Cha-um	2006	live	kudaTH1	
kuda_PBR3	-	Cha-um	2006	live	kudaTH1	
kuda_PBR4	-	Cha-um	2006	live	kudaTH1	
kuda_PBR5	-	Cha-um	2006	live	kudaTH1	
kuda_PBR6	-	Cha-um	2006	live	kudaTH1	
kuda_PBR7	-	Cha-um	2006	live	kudaTH2	
kuda_PBR8	-	Cha-um	2006	live	kudaTH6	
kuda_PBR9	-	Cha-um	2006	live	kudaTH1	
kuda_PBR10	BIMS-T0008-10	Cha-um	2006	preserved	kudaTH1	
kuda_PBR11	-	Cha-um	29/11/2006	live	kudaTH1	
kuda_PBR12	-	Cha-um	29/11/2006	live	kudaTH1	
kuda_PBR13	-	Cha-um	29/11/2006	live	kudaTH2	
kuda_PBR14	-	Cha-um	29/11/2006	live	kudaTH3	
kuda_PBR15	-	Cha-um	29/11/2006	live	kudaTH1	
kuda_PBR16	-	Cha-um	29/11/2006	live	kudaTH3	
kuda_PBR17	-	Cha-um	29/11/2006	live	kudaTH2	
kuda_PBR18	-	Cha-um	29/11/2006	live	kudaTH1	
kuda_PBR19	BIMS-T0008-19	Cha-um	29/11/2006	preserved	kudaTH1	
kuda_PBR20	BIMS-T0008-20	Cha-um	29/11/2006	preserved	kudaTH6	
kuda_PK1	-	Aow Po	30/5/2005	live	kudaTH4	
kuda_PK2	-	Aow Po	30/5/2005	live	kudaTH4	
kuda_PK3	-	Aow Po	30/5/2005	live	kudaTH5	
kuda_PK4	-	Aow Po	30/5/2005	live	kudaTH4	
kuda_PK5	-	Aow Po	30/5/2005	live	kudaTH4	
kuda_PK6	-	Aow Po	30/5/2005	live	kudaTH4	