Genetic Variability of the Critically Endangered Softshell Turtle, *Rafetus swinhoei*: A Preliminary Report

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ABSTRACT

The critically endangered softshell turtle, *Rafetus swinhoei*, is on the verge of extinction due to anthropogenic threats. However, taxonomic status of populations throughout its range has not been evaluated thoroughly. This project aims to fill this gap of knowledge by sampling all available specimens in museums and collections around the world. Using forensic methods and a phylogenetic approach, the project attempts to reveal the population structure and genetic diversity among these populations. The results of this study will in turn be helpful to the formulation of conservation measures for this species, especially future captive breeding programs by identifying genetically distinct populations. In this report, we present our preliminary results showing the deep divergence between *R. swinhoei* and *R. euphraticus*, and that genetic divergence of *R. swinhoei*'s populations within Vietnam is not high, although sequencing errors may confound precise interpretation. For future research, more samples from other parts of its range, especially samples from China, should be analyzed in order to fully understand population differentiation and structure of this poorly known species.

INTRODUCTION

The giant softshell turtle species *Rafetus swinhoei* is the world's rarest and probably the largest softshell species. Existing records, few of them recent, indicate a relictual, deeply discontinuous range, with specimens known from the Tai Hu Lake and Suzhou area, west of Shanghai, China, and with others from the Red River in Yunnan, in southern China and also in the same river system in northern Vietnam. There are also reports of specimens from Thanh Hoa Province, Vietnam; these would be the southernmost records of the species (Iverson, 1992; Pritchard, 2001; pers. obs.).

The species may once have been more abundant, but it was already rare in the 1870's, when Pierre-Marie Heude collected some specimens from the Tai Hu Lake area (Heude, 1880), but the trenchant division of the range into eastern and southern sections is probably ancient. Currently, there are only four captive specimens in China and one in Vietnam, although fishermen's accounts indicate that some wild animals existed as recently as 10-20 years ago. Search for museum specimens was conducted by PCHP on several trips to China and Vietnam, and over twenty individuals were located, most of them decades old, and nearly all with labels misidentifying them as species of *Pelochelys*.

As a species on the brink of extinction, comprehensive conservation and restoration efforts are urgent, and these efforts need to include both field surveys and potential captive breeding efforts. Furthermore, in view of the huge gap in the range of the species, it is important that genetic comparisons be made between the southern and the eastern populations, to determine if they are distinct. It was recently observed by Wen Cheng *et al*. (2007) of Peking University that the Yunnan *Rafetus* were "probably different from the *Rafetus* from the Yangtze in many respects".

There has been some controversy over the taxonomy of this species, beginning when Heude (1880) ignored Gray's description of *Oscaria swinhoei* (Gray, 1873) and allocated five species names within the genus *Yuen* to a group of *swinhoei* from the eastern population (Meylan and

Webb, 1988). However, after careful examination of the type specimen in the British Museum of Natural History, Meylan and Webb (1998) confirmed the validity of this species. More recently, Ha Dinh Duc (2000) described a new species of this genus, i.e. *Rafetus leloii*, based on specimens collected from Hoan Kiem Lake in downtown Hanoi, but the comparisons with Chinese material were flawed and Farkas and Webb (2003) sunk *leloii* into the synonymy of *swinhoei*. While only a limited number of specimens are available for documenting morphological variations in this species, molecular data might be able to provide resolution for this problem. In fact, recent molecular studies have successfully revealed high levels of genetic variations in some cryptic turtle species in Asia (Guiking *et al*., 2002; Stuart and Parham, 2004; Torsten *et al*., 2006).

To clarify these nomenclatorial and taxonomic problems, we attempt to sequence DNA from all museum specimens available throughout the range of this species to document genetic diversity within this group. Our sample will include as many as possibly of the known preserved and skeletal specimens in collections and museums in China, Vietnam, UK, Austria, and the USA (see below). In this report, we present our preliminary results including, sequences of the *R. swinhoei*'s sample from Ha Tay, the position of this species in the molecular phylogeny of all softshell species, and molecular divergence between this and other specimens of *R. swinhoei* in Vietnam.

MATERIAL AND METHODS

Materials:

Following is the list of specimens that we will target for this study:

A. Vietnam specimens

1. Juvenile. Vienna Natural History Museum no. NMW 30911. Entire specimen, in alcohol. Collected Hanoi, Bac-Phan Prov., Vietnam, 1914. Total leathery disc length 380 mm. Rediscovered and described by Farkas (1992).

2. Soft parts and viscera of an adult female, originally from Vietnam, that died after many years in the Berlin Zoo, catalogued in the Berlin Zoologische Museum as ZMB 36437 and 36438 (head and visceral components being listed separately). Estimated total leathery shell length 60cm + (Niekisch *et al*., 1997). Viscera include ovaries containing a total of 130 unshelled ova each about 2 cm in diameter.

3. An entire, mounted large adult specimen (leathery disc length ca. 965 mm; total stretched length including head and neck ca. 1448 mm) in the Ngoc Son Temple on an island in Hoan Kiem Lake, Hanoi, Vietnam. The label on the display case indicates that the turtle was found dead in Hoan Kiem Lake in 1968; it also gives some distinctly exaggerated data on the dimensions and age of the specimen.

4. Mounted skeleton of an adult, with somewhat bizarre errors in placement of the plastral bones, sacrum, etc, also from Hoan Kiem Lake, in the collection of the Hanoi Museum (curator: Nguyen Duc Hong). Bony disc length approximately 600 mm.

5. Mounted large adult specimen, with skull removed and head reconstructed, on exhibit in the Hoa Binh municipal museum, Vietnam. Collected alive near Hoa Binh city by Nguyen Hoy Son and his son Nguyen Duc Viet in 1993; died after a few days. Leathery disc length 1095 mm, bony disc length 633 mm; straight head width 170 mm.

6. Bony carapace of subadult, Specimen T91 in University of Hanoi Collection. Ma Song River. Bony disc length 446 mm.

7. Skull of subadult, posteriorly damaged, in private collection of Ha Dinh Duc, University of Hanoi. Obtained from confiscated specimen brought to Hanoi Railway Station from Vinh, 1995. Leathery carapace length 620 mm.

8. Nearly complete skeleton, subadult, collection of Chelonian Research Institute (PCHP 6877). Caught in Ao Chau Swamp, Phu Tho Province, Vietnam; caught 1982, obtained from fisherman by PCHP August 1999. Bony disc length 476 mm.

9. Carapace, partial plastron, skull etc. Juvenile. Collection of Chelonian Research Institute (PCHP 6876). Caught in a pond at Dam Ben, near Ha Hoa (Phu Tho Province), Vietnam. Caught August 1999; obtained by PCHP November 2000. Bony disc length ca. 350 mm (nuchal bone missing).

10. Skull and lower jaw of adult, total length 22.8 cm. Chelonian Research Institute (PCHP 8366). Vietnam: Yen Bai Province: Minh Guan Commune. Donated by Mr. Bun, Sept. 2004 (originally collected 1981).

11. Tissue sample from an individual in Ha Tay, Ha Noi. Collected by Tim McCormack in 2008.

B. China specimens

12. Holotype. Juvenile. British Museum (Natural History) no. 1946.1.22.9. Skull extracted, catalogued separately (BMNH 1947.3.6.13). Purchased and forwarded to BMNH by Consul Robert Swinhoe. Total leathery disc length 330 mm. Described as a new species, *Oscaria swinhoei*, by J.E.Gray in 1873. Type locality: "Neighborhood of Shanghai."

13. A subfossil skull and carapace bones from Zhejiang Province (Luojiajiao Relics, Tongxiang County), as well as a mounted skeleton and a stuffed animal, that provided the type material for the species *Pelochelys taihuensis* (Zhang, 1984). Now known to be a synonym of *Rafetus swinhoei* (Farkas, 1992).

14. A fossilized skull and plastral fragments from Upper Pleistocene marine deposits near Taiwan, and described as *Trionyx liupani* (Tao, 1986). Considered referable to *Rafetus swinhoei* (Farkas, 1992).

15. A skull and jaw of an adult from Tai Hu lake (near Shanghai); died in Suzhou Zoo. In personal, collection of Dr. Zhao Ken Tang, Suzhou Railway Teachers College (retired). Total length of skull 21.3 cm; basicranial length 16.0 cm. Maximum straight width 12.3 cm.

16. Mounted entire adult specimen, attached to wooden board, in care of Prof. Huang Gong Quin, Chief Veterinarian of Suzhou Zoo; inspected at China Tiger Breeding Center, south of Suzhou. Originally from Tai Hu Lake, died in Suzhou Zoo (actually killed by a rock-throwing zoo visitor in 1995). Leathery disc length 86.0 cm, width across 8th costals 17.7 cm, width nuchal callosity 30.7 cm, bony disc length 56.3 cm, width 64.5 cm. Head width 14 cm.

17. Mounted adult in Fudan University collection, Shanghai; originally from Tai Hu Lake. Specimen prepared pre-1949 by Tang Jhi Min, father of present curator (retired 1987). Leathery disc length 83.2 cm, midline bony disc length 50.0 cm, max. carapace length 51.0 cm, max disc width 54.6 cm, width across eighth pleural bones 14.8 cm. Head width 12.7 cm. Head to tail tip 143 cm.

18. Mounted adult male in Zhe Jiang Provincial Museum (Huangzou), labeled *Pelochelys cantorii,* catalogued as no. 54001 (1954); actually *R. swinhoei*. Leathery carapace length 64.5 cm, midline disc length 40.0 cm, max. bony disc length 42.5 cm; width across pleural bones 15.0 cm. Neural bones were ankylosed; presumably an old individual.

19. Adult bony carapace, labeled *Pelochelys bibroni*, in Shanghai Natural History Museum, Pudong. Disc length 565 mm, width 581 mm (648 across widest span of rib tips).

20. Small adult female, mounted, with external label "*Pelochelys bibroni* Owen. Shanghai Natural History Museum No. 6018 1532." An old printed label concealed within the inguinal region read: "Musée Heude. *Pelochelys guierryana* H. Localité_____ Donateur_____." This specimen thus appears to be one of the original "lost" Heude specimens, although the species name does not feature in Heude (1880); see discussion. Leathery disc length 65.5 cm, bony disc length 42.7 cm, bony disc width 36.0 cm.

21. Adult male, mounted, Shanghai Natural History Museum. No. 2000 x 076. Leathery disc length 57.5 cm, bony disc length 38.5 cm, bony disc width 37.5 cm. Locality: "Jiang-Su."

22. Adult male, mounted. Yunnan. Shanghai Natural History Museum 76-3-13-1. Leathery disc length 71.2 cm, bony disc length 46.5 cm, bony disc width 45.0 cm. (May be the only voucher specimen from the southwestern enclave in China, i.e. the Yunnan section of the Red River.)

23. Adult male, mounted. Shanghai Natural History Museum, "ex. British Museum, Asian Part." Leathery disc length 86.0 cm, bony disc length 54.2 cm, bony disc width 54.5 cm.

Methods:

Molecular data:

Museum samples (bone, dried tissues) were extracted following protocols specified in Torsten *et al*. (2006) and Le *et al*. (2007). These protocols are highly effective and allow to successfully sequence even nuclear genes from bone (Le *et al*., 2007) or mitochondrial fragments from the 180 years old type specimen (Torsten *et al*., 2006). Specifically, the sequencing procedure included:

Bone was sampled from small and relatively unimportant parts, such as digit and tail vertebrae, to minimize the damage to morphological characters of the specimens. This practice also has the advantage that the bone is small enough for immediate extraction without further manipulation, (e.g., drilling and grinding). Due to the risk of contamination on the surface of the bone, the sample was first cleaned with 10% chlorox, and then placed on a clean surface to dry. The clean bone was then decalcified by incubation at 55^{o}C in 1 ml of 0.5 M EDTA for 24h. After decalcification, the bone will be washed with 1 ml of 10 mM Tris to remove remaining EDTA (Austin *et al*., 2002). At this point the bone was ready for extraction using DNeasy Kit (Qiagen).

The extraction procedure followed the manufacturer's instructions for animal tissues. For the incubation step, the lysis usually takes up to 48 h in order for the bone to become completely digested. During this step, the extraction was checked every 12 hours to monitor the progress. If the lysis occurred slowly, more proteinase K was added (usually in 20 µl increments). A negative control was used in every extraction. DNA obtained from bones was amplified by HotStarTaq^{TM} mastermix or HotStar Taq (Qiagen), since this Taq performs well on samples with low-copy targets and the Taq is highly specific. For HotStarTaq mastermix, the PCR volume consisted of 21 µl (5 µl water, 2 µl of each primer, 10 µl of HotStarTaq mastermix, and 2 µl of DNA or higher depending on the quantity of DNA in the final extraction solution). For HotStar Taq, the PCR volume ranged from 21 to 22 µl (2 µl of dNTP, 2 µl of each primer, 2 µl of buffer 10; 12 µl of water, and 1 to 2 µl of DNA depending on the quantity of DNA in the final extraction solution). PCR conditions are: 95^{o}C for 15 min to activate the Taq; with 42 cycles at 95^{o}C for 30 s, 45^{o}C for 45 s, 72^{o}C for 60 s; and a final extension of 6 min. In case PCR reactions did not work, the PCR products will be used as template for new PCR reactions.

PCR products were visualized using electrophoresis through a 2% low melting-point agarose gel (NuSieve GTG, FMC) stained with ethidium bromide. For reamplification reactions, PCR products were excised from the gel using a Pasteur pipette, and the gel plug was melted in 300 µl sterile water at 73^{o}C for 10 min. The resulting gelpurified product was used as a template in 42.2µl reamplification reactions with all PCR conditions similar to those used for mitochondrial genes. PCR products were cleaned using PerfectPrep^{TM} PCR Cleanup 96 plate (Eppendorf) or

using glass milk and 70% ethanol, and cycle sequenced using ABI prism big-dye terminator according to manufacturer recommendation. Sequences were generated in both directions on an ABI 3100 Genetic Analyzer.

Primers from Engstrom *et al*. (2004) were used to amplified two mitochondrial gene regions, Cytochrome b (Cytb) and NADH dehydrogenase subunit 4 (ND4) and one nuclear intron R35. Two mitochondrial genes have been shown to be useful in addressing the relationships at species or below species levels (Spinks and Shaffer, 2005; Stuart and Parham, 2004; Torsten *et al*., 2006; Fritz *et al*., 2005). Specifically, the primers used in this study included: GLUDGE, CB3, CB534, Tcytbthr, ND4 672, Hist, R35 EX1, and R35 EX2

Phylogenetic analyses:

Sequences were aligned using ClustalX v1.83 (Thompson *et al*., 1997) with default settings for complete alignment. Data were then analyzed using maximum parsimony (MP) as implemented in PAUP*4.0b10 (Swofford, 2001). For maximum parsimony analysis, heuristic analyses will be run with 100 random taxon addition replicates using the tree-bisection and reconnection (TBR) branch swapping algorithm in PAUP, with no upper limit set for the maximum number of trees saved. Bootstrap support (BP) (Felsenstein, 1985) will be evaluated using 1000 pseudoreplicates and 100 random taxon addition replicates. Gaps in sequence alignments will be treated as a fifth character state (Giribert and Wheeler, 1999).

RESULTS AND DISCUSSION

The final matrix subjected to analyses consists of 2933 aligned characters: cytb (1140), ND4 (732), and R35 (1061). The results from the bootstrap analysis show that the cladogram is generally strongly supported with only 27% of nodes receiving low bootstrap values (<70%) (see Figure 1). Similar to Engstrom *et al*. (2004), two subfamilies Trionychinae and Cyclanorbinae are strongly supported (BP = 100%). However, within Trionychinae, the clade Apalonia as defined by Engstrom *et al*. (2004) containing two genera *Rafetus* and *Apalone* is not indicated in two most parsimonious cladograms, although it is weakly supported in the bootstrap consensus tree (BP=60). Rather, in our most parsimonious trees, *Rafetus* is placed as sister to the clade Amydona. Within this clade, the major difference between our results and those of Engstrom *et al*. (2004) are the positions in the clade comprising *Nilssonia* and *Aspideretes*. Our results for this group are also different from those analyzed by Praschag *et al*. (2007). Finally, between this study and that of Engstrom *et al*. (2004) the positions of three *Chitra* species are not identical.

Importantly, our study found that there is a large divergence between two species of *Rafetus* (7.8% in ND4 and 8.8% in cytb). This result shows that two species of *Rafetus* might have been isolated for a very long period of time. The two species also have highly disjunct distributions with *R. swinhoei* occurring in eastern Asia while *R. euphraticus* occurring in Tigris-Euphrates river basin in southeastern Syria, Iraq, and southwestern Iran (Iverson, 1992). The collision of Indian subcontinent into Eurasia and the rise of the Himalaya around 50-55 million years ago

could separate the distribution of the two taxa (Sterling *et al*., 2006). However, more studies, especially molecular estimation based on fossil calibration, can shed light on the age of this split.

We also compared the sequences obtained in this study with those produced by Le Tran Binh *et al*. (2003) and uploaded on GenBank with the numbers: AJ607407, AJ607408, AJ608763, AJ608764, AJ608765, AJ608766. These authors took samples from three locations, including Hoan Kiem Lake, Ha Tay, and Thanh Hoa. For cytb, we found 19 mismatches in a total of 510 base pairs. However, these mismatches likely result from sequencing errors as 14 of the mismatches located at the very end of the sequences. In addition, in all of the 14 mismatches and some others, it is clear that haplotypes from three different locations in Le Tran Binh *et al*. (2003) are identical. This indicates that the real divergences among these geographic samples are much smaller than shown in the mismatch analysis. Similarly, in ND4, there are 21 mismatches in a total of about 700 base pairs, but some could be due to artifact. In conclusion, we hypothesize that the divergences between these specimens fall within species limit, although this problem clearly requires more studies. Moreover, molecular comparison between Chinese specimens, including the type, and specimens from Vietnam should be conducted before genetic variability and population structure within this group can be clarified.

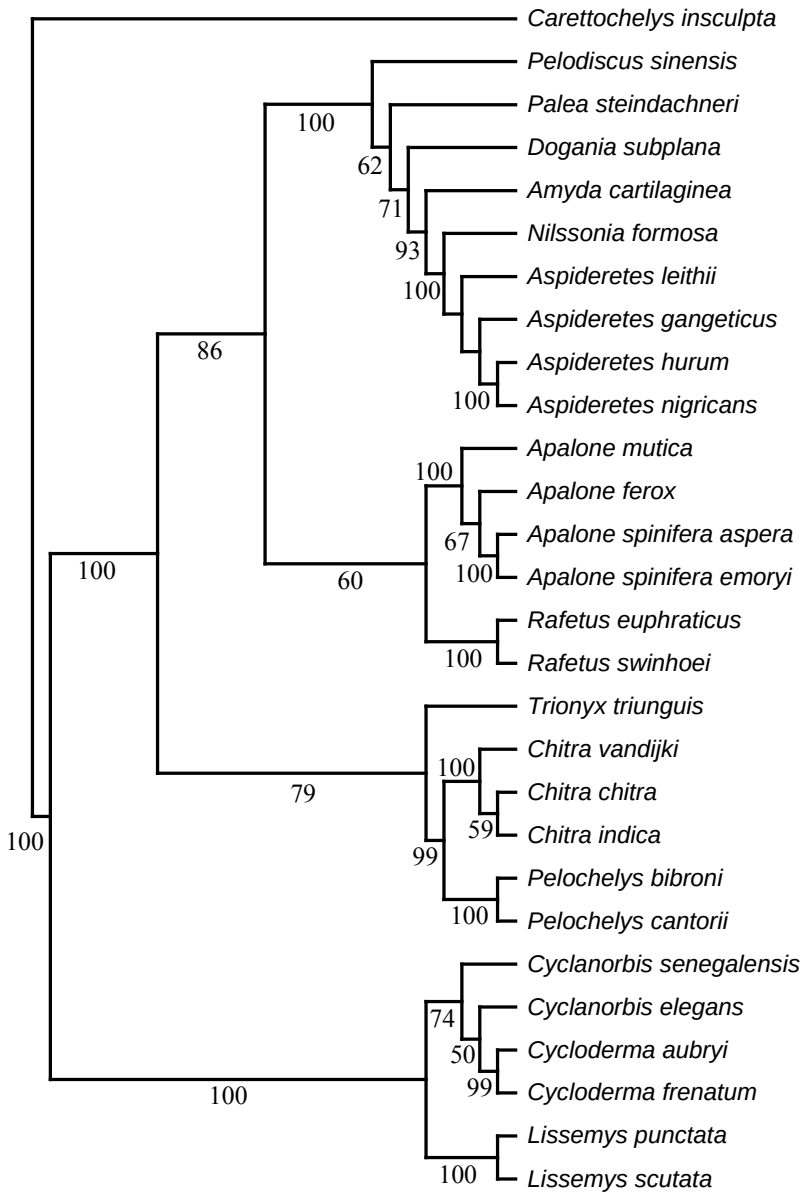

Figure 1. Bootstrap consensus tree based on 1,000 pseudo-replicates. In two most parsimonious trees, the genus *Rafetus* is grouped with the clade containing *Pelodiscus sinensis* and others. Of 2933 total characters, 1537 are constant, 431 variable, and 965 parsimony informative. The length of the shortest tree is 4038 (CI = 0.49, RI = 0.59).

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