

COMPARATIVE MORPHOLOGICAL AND DNA ANALYSIS OF SPECIMENS OF GIANT FRESHWATER SOFT-SHELLED TURTLE IN VIETNAM RELATED TO HOAN KIEM TURTLE

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SUMMARY

Giant soft-shelled turtle has been observed and several specimens collected in different sites on the Song Hong (Red River), Song Ma (Ma River) and Song Da (Da River) in Northern Vietnam. Morphological comparison of the specimens revealed that the skulls are different to that of *Rafetus swinhoei* described before by having a wide and blunt outer appearance. Samples from skeletons and remains were collected from all specimens. One tissue sample of *Rafetus swinhoei* (GRAY, 1873) was provided by the Naturhistorisches Museum Wien, Austria. Fragments of mitochondrial cytochrome B, NADH4 and 16S rRNA genes were cloned and sequenced. No registered nucleotide sequences were found to be similar. Phylogenetic analysis showed the described turtle to form a unique group which is close to *Rafetus euphraticus* and *R. swinhoei*. Combined with morphological comparison of the specimens as well as with the distribution records, it is possible to conclude that the giant freshwater soft-shelled turtle of Vietnam is a new species that has not been previously taxonomically identified. Therefore, the new species is named as *Rafetus vietnamensis*.

Key words: giant soft-shelled freshwater turtle, DNA analysis, new species, *Rafetus vietnamensis*

INTRODUCTION

The giant soft-shelled turtle living in Hoan Kiem lake situated in Hanoi City, Vietnam has been previously reported (Ha Dinh Duc, 2000; Peter, 1994; Niekisch *et al.*, 1997; Heinselman, 2000; Pritchard, 2001). However, classification of this species is not yet finalized.

There are two published documents mentioning the classification of Hoan Kiem Lake Turtle. In the Vietnam Red Data Book the Hoan Kiem Lake Turtle was listed as endangered under the name *Pelochelys bibroni* (Red Data Book of Vietnam, 2000) and the "Conservation International" organisation classified it as *Rafetus swinhoei* (*Profiles of Turtles in Trouble*, 1999). One of the main reasons for this dispute is the lack of sufficient data generated from systematic study on a reasonable number of dead or living specimens.

In this paper we will report data on the morphological study of several newly discovered specimens kept in different sites of Vietnam. Additionally we present data from DNA analysis of these samples to support the taxonomical

classification of the giant soft-shelled turtle of Vietnam.

METHODS

Specimen discovery and biological sampling

According to information provided by local authorities field visits were organized to identify the existing specimens and to interview local people. Pictures of the sites and their surrounding areas, specimens and their owners and the related peoples were taken. Samples from the skeletal specimens were collected using a dental hand borer. Sampled materials were kept in a desiccator at 4°C until use.

Specimens and pictures were used by professional taxonomical drawers to generate black and white figures. Detailed measurement of the specimens was carried out by museum zoologists for morphological description.

DNA and data analysis

DNA extraction was carried out according to Kaestle (2000) with some modifications. A typical amplification reaction contained 1X PCR Buffer, 2,5

mM MgCl₂, 1 U Taq polymerase; 250 µM each of dNTPs; 10 pmol each of specific primers; 2-7 µl of DNA in 25 µl total volume. PCR products were separated on a 1% agarose gel.

The PCR products were cloned into the Topo TA Cloning Vector. The QIAGEN miniprep kit was then used to prepare recombinant plasmids for sequencing. Sequencing was performed with an automated DNA sequencer ABI Prism® 3100 Avant. Data was analyzed using DNASTar, BioEditor version 5.0.9 software.

RESULTS AND DISCUSSION

Formal Description

Family Tryonychidae, Subfamily Trionychinae, Tribe Trionychini, Genus *Rafetus*.

Diagnosis

The giant fresh water soft-shelled turtle differs significantly from all the described genera in its body size, living conditions, appearance, skull morphology and DNA sequence. The diagnosis focused on identification of the species by using the specific characteristics of the wide and blunt appearance of the skull with a blunt curved maxillary arch, the giant size of the body, its habitat and especially the DNA sequence comparisons. Special consideration is focused on the DNA analysis of the *Rafetus swinhoei* specimen collected in the Northern Vietnam in 1914 and kept in Naturhistorischem Museum Wien, Austria.

Etymology

The full name “giant freshwater soft-shelled turtle” reflects the three main characteristics of this turtle: (1) the shell is covered by a leathery flap forming skin, instead of a hard protective covering. (2) The suitable living environment of this turtle is limited to the areas of freshwater, not brackish or marine water. In the last ten years the giant soft-shelled turtles have been reported to be caught or seen in many swamp areas belonging to three main rivers Song Hong, Song Ma and Song Da in Northern Vietnam. And (3) among the freshwater soft-shelled turtles this species seems to be the largest one: The observed body size of the adult turtles ranges from 1500-2200 mm and the weight varies around 150 kg (120-220 kg).

Hoan Kiem Lake situated in central of Hanoi and the Hoan Kiem Turtle was observed and

reported to be unique here. Therefore the name *Rafetus hoankiemensis* was given in an article in the Vietnam Investment Review from January 2000. Whether this name is scientifically accepted needs to be discussed by experienced zoologists. Another name *Rafetus leloii* given by Ha Dinh Duc in an article in the Archeological Journal in Vietnamese (Ha Dinh Duc, 2000) is related to the Tale of the Sword of King Le Loi who established and ruled the Le Dymnasty from 1428 to 1433.

Type species

Rafetus leloii (HA), *R. hoankiemensis* suggested by VIR(2000)

Rafetus vietnamensis sp. nov.

Holotype

The Hung Ky specimen (HK1) is the most representative for all specimens because of its completeness (Figure 1) and will be considered the holotype.

Description

The HK1 and two other entire specimens kept in Ngoc Son Temple (NS1) and Hoa Binh Museum (QL1) allowed us to measure the following parameters:

The total body length from the muzzle to the end of tail is estimated at about 1500 – 2000 mm and body weight about at 170-220 kg at the adult stage (3). The head size of these turtles is significantly larger and the snout is less protruded than that of *Pelochelys bibroni* and *Rafetus swinhoei*. The head is situated on a long neck with a heavy double holed proboscis nose.

The following morphological charactersitics were observed:

1. The external appearance of Vietnam turtle fits the main characteristic of soft-shelled turtle in lacking the hard outer protective shell. Pictures of living turtles in the Quynh Lam swamp (1993) and Hoan Kiem lake (2002) clearly showed these turtles have a low profile shell with an elongated shape that is covered by a leathery skin resulting in a flap like appearance. The skin color of these turtles on the upper side is uniformly grey-green without any texture, but changes slightly under different ecological and environmental regimes (food, natural sunlight and water quality). The Hoan Kiem lake turtle for instance is uniformly murky gray-green

(Fig. 2a), while the Quynh Lam swamp turtle changes to more khaki-green (Fig. 2b). The skin color of the under side is always pinkish.

2. The total body length from the muzzle to the end of tail is estimated at 1700 - 2000 mm and body weight at 120-220 kg in the adult stage.

3. The head size of these turtles is significantly

larger and the snout is less protruded than that of *Pelochelys bibroni* and *Rafetus swinhoei*. The head is situated on a long neck with a heavy double holed proboscis nose. Generally, the skull of these turtles makes a wide and blunt outer appearance with blunt curved maxillary arches. These turtles cannot retract their head back into the carapace (Fig. 3).



Figure 2: A giant freshwater soft-shelled turtle caught in Quynh Lam swamp, Hoa Binh by local fishermen on April 23, 1993. According to the newspaper record this turtle weighed 125 kg and was kept in an Aquarium for two weeks before dying. The stuffed specimen is now in Hoa Binh Museum and the skull was kept by

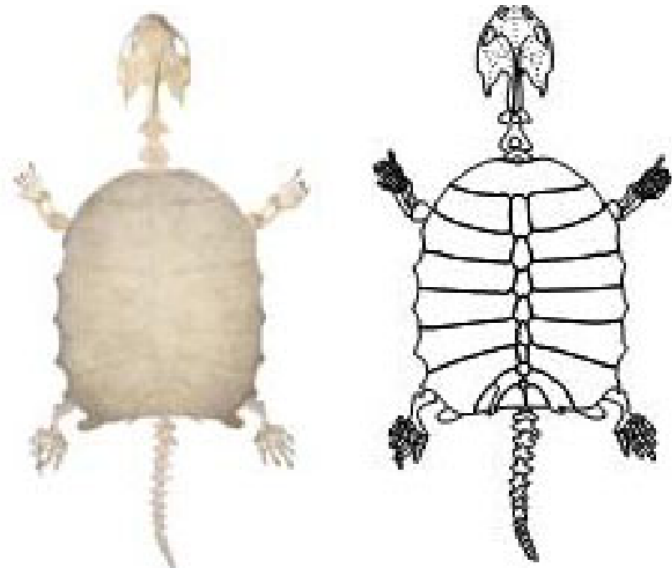


Figure 1: Skeleton of the holotype of the giant freshwater soft-shelled turtle kept in Hung Ky Pagoda, Hanoi. Morphological parameters are: carapace disk - 580x640 mm; skull size - 150x236 mm; total skeleton length (muzzle to tail) -1686 mm. 1B is a drawing of 1A.

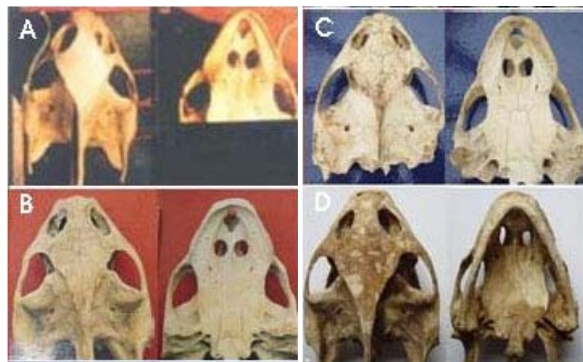


Figure 3. Skulls of three found specimens HK1 from Hanoi (B); QP1 from Thanh Hoa (C) and QL1 from Hoa Binh (D) compared with that of *Rafetus swinhoei* from Shanghai, China (A).



Figure 4: Distribution records of giant freshwater soft-shelled turtles in Vietnam. The number of records reported by local authorities in the period from 1967-2003 are 1 for Nga Ba Van Yen, 1 for Tran Yen, 1 for Ao Chau, more than 6 for Dam Long, more than 3 for Suoi Hai, 3 for Quynh Lam, 5 for Dinh Cu, more than 14 for Quang Phu, 4 for Hoan Kiem Lake, totally 38. Most of the records were reported by authentic local turtle fishing men during the 2003 survey.

Distribution

For a long period (until 1990) the soft-shelled turtle was reported to be seen only in Hoan Kiem Lake with stuffed specimens preserved in Ngoc Son Temple, Hanoi (1967) and Hoa Binh Museum (1997). An entire skeleton was kept in Hung Ky Temple. In 2003 one skull was discovered in Quang Phu, Thanh Hoa, 160 km South of Hanoi and a rear part (1/4) of a carapace was found in Suoi Hai, Son Tay. Data collected by interviews of several “turtle fishing men” and local authorities revealed that the soft-shelled turtles are recorded at least in 9 locations of swampies connected to Song Hong (Red River), Song Ma (Ma River), Song Chu (Chu River) and

Song Da (Da River) in Northern Vietnam (Fig. 4). This gives a total known range of about 16000 km².

Phylogenetic DNA analysis

Fragments of 16S rRNA, cytochrome B (cyt b) and nicotinamide adenine dehydrogenase 4 (ND4) genes of 369, 510 and 708 base pairs respectively, were cloned using specific primers and total DNA, isolated from the skeleton and tissue samples, as templates. Sequencing data from these partial fragments were submitted to EMBL Nucleotide Sequence Database under the accession numbers AJ607405; AJ607406; AJ607407; AJ607408; AJ608763; AJ608764; AJ608765 and AJ608766.

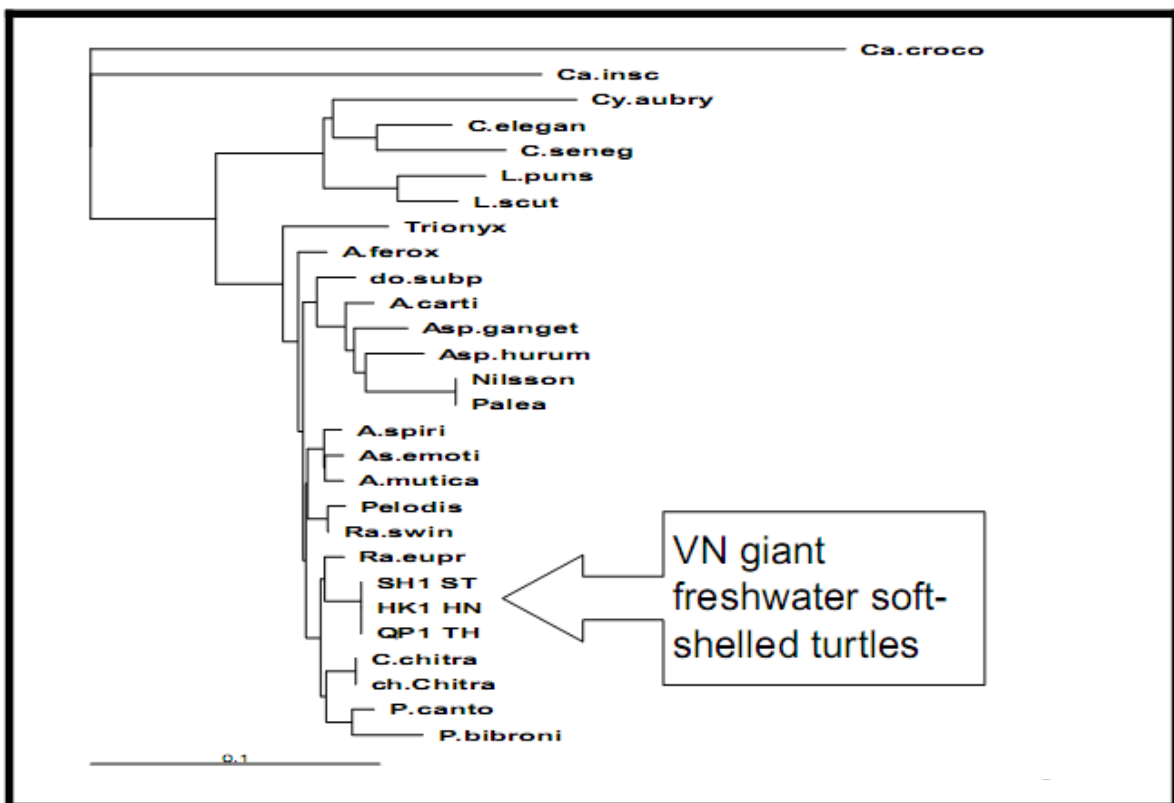


Figure 5. The phylogenetic trees 16S rRNA nucleotide sequences indicating a unique cluster of specimens of VN giant freshwater soft-shelled turtle separated from the other described *Tryonichidae* species. Samples from specimens found in Hung Ky Temple Hanoi (HK1 HN), Suoi Hai, Son Tay (SH1 ST) and Quang Phu , Thanh Hoa (QP1 TH). *Rafetus swinhoei*'s tissue kindly provided by Naturhistorisches Museum Wien, Austria. Data of *Caiman crocodilus* was used as out-group.

A phylogenetic tree was constructed from the deduced amino acid sequence of cytochrome B and NADH4 and the nucleotide sequence of 16S rRNA. *Caman crocodiulus*, used as an out-group always

clustered in a unique group. The three samples from the freshwater soft-shelled turtles of Vietnam form a unique group (Fig 4A: 16S; 4B: CytB and 4C: NADH4). This group locates close to that of *Rafetus*

euphraticus. The tissue sample provided by the Naturhistorisches Museum Wien originated from a specimen of *Rafetus swinhoi* collected from Tonkin Vietnam in May 12, 1914, described as *Trionyx swinhoi* (Gray, 1873) and later corrected by Farkas (1992) as *Rafetus swinhoi* (GRAY) (Farkas, 1992) also appears separately in the phylogenetic tree. This indicates that the new species could be one of the genus *Rafetus*, but is not *Rafetus swinhoi*. Furthermore it is to note that data from all different specimens from Hanoi, Thanh Hoa and Hoa Binh, representative for Red River (Song Hong), Ma River (Song Ma) and Da River (Song Da), respectively, are the same. This finding revealed that the giant freshwater soft-shelled turtles found in Northern Vietnam are identical and unique. Significant differences have been found by comparing the ND4 partial sequences of these turtles with that of *Pelochelys bibronii* (Owen 1853) and *P. cantorii* (Fig. 4d). This evidence proves that the giant freshwater soft-shelled turtle of Vietnam is definitively not *Pelochelys bibronii* (Owen, 1853) as described previously in the Red Data Book of Vietnam (2000). These results indicating that the giant soft-shelled freshwater turtle of Vietnam is a new undescribed species.

There is a question whether *Rafetus swinhoi* is already extinct in Vietnam or the world? The previously mentioned specimen collected by Gray in Hanoi, Tonkin, 12/5/. In the current list of Endangered Categories of Endangered Species in China *Rafetus swinhoi* is listed under the category of "extinct in China" and was not been reported in the last 120 years (<http://monkey.ioz.ac.cn/bwg-cciced/english/cesis/reptilia>). Even the specimen in Shanghai Zoo was reported to be collected from the riverhead area of Red River of Vietnam (Meylan, 1987).

Based on all these facts it could be concluded that most common location of this giant freshwater soft-shelled turtle is Vietnam and the most reasonable scientific name for it is *Rafetus vietnamensis*. (The two above mentioned names *hoankiemensis* and *leleoi* are likely more related to the Swort Legend of Hoan Kiem Lake written by Le Loi rather than taxonomy).

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SO SÁNH HÌNH THÁI VÀ PHÂN TÍCH DNA MŨI RÙA LÊN MAI M M N C NG T C A VI T NAM G N V I RÙA H HOÀN KI M

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TÓM TẮT

Rùa lên mai m m đã được quan sát và thu thập mẫu vật nhiều địa điểm khác nhau trên Sông Hồng, Sông Mã, Sông Đà miền Bắc Việt Nam. Mẫu của rùa *Rafetus swinhoei* (GRAY, 1873) đã được cung cấp bởi Bộ tàng Naturhistorischem Wien, Áo. Phân tích hình thái học của các mẫu vật thu thập cho thấy rùa lên mai m m của Việt Nam khác với rùa *Rafetus swinhoei* đã được mô tả trước đây là có hình dáng và bên ngoài nhẵn. Các gen của cytochrome B ty thể, NADH4 và 16S rRNA gene đã được nhân bản, giải trình tự và không có trình tự đồng nhất nào đã được công bố trên ngân hàng gen. Phân tích phát sinh loài cho thấy rùa lên mai m m của Việt Nam tạo thành một nhóm riêng biệt, gần với *Rafetus euphraticus* và *R. swinhoei*. Kết quả so sánh hình thái của mẫu vật cũng như phân bố địa lý của loài rùa này có thể kết luận rằng rùa lên mai m m được công nhận là một loài mới, đặc trưng nghiên cứu phân loại. Kết quả phân tích của chúng tôi có thể đặt tên rùa lên mai m m của Việt Nam là *Rafetus vietnamensis*.

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