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Naming one of the world's rarest chelonians, the southern *Batagur*

PETER PRASCHAG[1], ROBERT S. SOMMER[2], COLIN MCCARTHY[3], RICHARD GEMEL[4] & UWE FRITZ[2,5]

[1]*Am Katzelbach 98, A-8054 Graz, Austria. E-mail: peter@praschag.at*
[2]*Museum of Zoology (Museum für Tierkunde), Natural History State Collections Dresden, Königsbrücker Landstr. 159, D-01109 Dresden, Germany. E-mails: robert.sommer@snsd.smwk.sachsen.de, uwe.fritz@snsd.smwk.sachsen.de*
[3]*Reptile and Amphibian Group, Department of Zoology, The Natural History Museum, Cromwell Road, London SW5 7BD, UK. E-mail: c.mccarthy@nhm.ac.uk*
[4]*Naturhistorisches Museum Wien, Burgring 7, A-1010 Wien, Austria. E-mail: richard.gemel@nhm-wien.ac.at*
[5]*Corresponding author. E-mail: uwe.fritz@snsd.smwk.sachsen.de*

Abstract

Using mtDNA sequences of historical museum specimens, including the herein designated lectotype of *Tetraonyx affinis* Cantor 1847 and topotypic specimens of *Trionyx (Tetraonyx) cuvieri* Gray 1831 and *Tetronyx longicollis* Lesson 1834, we demonstrate that the name *Batagur affinis* (Cantor 1847) has to be used for a recently identified critically endangered terrapin species from Southeast Asia. Further, we provide evidence that *Batagur baska* (Gray 1830) historically was distributed from north-easternmost India and Bangladesh to at least the Ayeyarwady and Bago estuaries in Myanmar while *B. affinis* occurs in the southern Malay Peninsula and Sumatra. The taxonomic allocation of the extant and extirpated *Batagur* populations in the northern Malay Peninsula, Cambodia and southern Vietnam remains unclear. A museum specimen from the mid-19th century suggests that *B. baska* once also occurred in the Indus Delta of southern Pakistan.

Key words: Southeast Asia, South Asia, lectotype designation, taxonomy, endangered species

Introduction

Batagur baska (Gray 1830), a large estuarine terrapin reaching a shell length of approximately 60 cm, is one of the most critically endangered chelonians of the world (IUCN, 2007; Kalyar *et al.*, 2007). Historically, it occupied a range extending from the Brahminy-Baitarini Delta (Orissa) and the Sundarbans Region (north-easternmost India, Bangladesh) through the Ayeyarwady (Irrawaddy) River mouth in Myanmar and the Malay Peninsula (southern Thailand, Malaysia) to Sumatra, Cambodia and southern Vietnam; however, it was extirpated in much of its former range (Moll, 1980; Das, 1991, 1995, 2001; Ernst *et al.*, 2000; Platt *et al.*, 2003; Kalyar *et al.*, 2007). *Batagur baska* is more or less confined to estuaries, mangrove belts and inshore beds of marine vegetation. During the reproductive season adult terrapins may travel far upstream to reach nesting beaches that are often located well above tidal influence (Kalyar *et al.*, 2007). In a recent paper, Praschag *et al.* (2007) demonstrated that *B. baska* actually consists of two genetically well-differentiated species. While it is clear that the name *B. baska* (Gray 1830), with type locality of "India", has to be restricted to the species occurring in north-eastern India and Bangladesh, there are several candidates available for naming the second species from Indonesia and Malaysia (Praschag *et al.*, 2007). As national and international conservation measures are significantly influenced by zoological nomenclature, it is crucial to determine its valid name. To accomplish this goal, here we use mtDNA sequence data of historical museum specimens, including a syntype

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of *Tetraonyx affinis* Cantor 1847 and topotypic specimens of *Trionyx (Tetraonyx) cuvieri* Gray 1831 and *Tetronyx longicollis* Lesson 1834. Further, we use these data for delineating the range of the two *Batagur* species.

Material and methods

The two *Batagur* species previously lumped within *B. baska* differ significantly in their mitochondrial cytochrome *b* gene (cyt *b*; uncorrected *p* distance of 4.22%; Praschag *et al.*, 2007), which is why we decided to sequence and compare the most informative part of this marker to determine the taxonomic allocation of historical museum specimens.

Muscle or connective tissue was removed from inside of seven historical museum specimens of *B. baska* sensu lato using sterile equipment (Table 1). Isolation and amplification of mtDNA were carried out inside ultraviolet-sterilized PCR Enclosures (HeraSafe KSP9, Thermo) in a clean room for ancient DNA, which was irradiated with UV light at least 6 h before and after every working step. In this clean room no *Batagur* samples were studied before. Soft tissue residues were washed three times (at intervals of 2 h, 2 h and 12 h) with 1.5 ml of GTE buffer (100 mM glycine, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and for 1 min in 100% ethanol, 5 min in 70% ethanol and 10 min in sterile water. Samples were gently vortexed for 5 s after their placement into a new wash. The washed tissue samples were dried and incubated at 56°C for 15 h in 300 µl of TNES buffer (10 mM Trizma Base, 100 mM NaCl, 10 mM EDTA, 2% sodium lauryl sulphate [SDS, 39 mM] DTT) with additions of 60 µl of proteinase K. The remaining extraction procedure followed the DNeasy Tissue Kit blood and tissue 50 (Qiagen, Cat. No. 69564) protocol for animal tissues, with modifications after Kearney & Stuart (2004). For PCR, 13.25 µl of water, 0.25 µl of Ampli *Taq* Gold (Applied Biosystems) and 2.5 µl of buffer (including $MgCl_2$), 2 µl BSA, 1 µl dNTP and 5 µl of DNA template were used. All PCRs contained a negative control (all PCR reagents except the DNA template). Four primer pairs (Table 2) were designed to amplify approximately 320 bp of the most informative part of the cyt *b* gene in four fragments of 90–120 bp overlapping by 30–45 bp after primer sequences were trimmed. PCR conditions were: 95°C for 5 min, 95°C for 1 min, 50°C for 1 min, 72°C for 2 min, 72°C for 10 min for 39 cycles. PCR products were electrophoretically separated using a 1% low melt agarose gel dyed in ethidium bromide and visualized under UV light. PCR products were sequenced in both directions by direct double-strand cycle sequencing using 2 µl Big Dye buffer (5x), 1µl Big Dye T-Mix, 1 µl of primer (5 pmol/µl), 5 µl water and 1–5 µl of the PCR product with 25 cycles (96°C for 10 s, 50°C for 5 s, 60°C for 4 min). Cycle sequencing products were precipitated with ethanol and 3 M NaAc and sequenced with an ABI 3130 sequencer (Applied Biosystems).

Cyt *b* sequences of historical specimens were compared with the data from Spinks *et al.* (2004) and Praschag *et al.* (2007) using BioEdit 7.0.5.2 (Hall, 1999), and a parsimony network was calculated for a 320 bp long alignment using TCS 1.21 (Clement *et al.*, 2000). We also included the sequences of *B. kachuga* from Praschag *et al.* (2007) in the alignment; *B. kachuga* is a species allied to the two other *Batagur* species but occurs unlike these estuarine species in inland rivers. In addition, *B. kachuga* has five claws on the fore feet while the two estuarine *Batagur* species have only four claws.

Results

Three of the samples from historical museum specimens failed to amplify; the other four yielded clear sequences. The sequence from a syntype of *Tetraonyx affinis* Cantor 1847 (BMNH 1947.3.4.31) is identical with a haplotype previously identified from five fresh samples from Indonesian and Malaysian *Batagur* (Praschag *et al.*, 2007); the other three sequences from museum specimens originating in Myanmar and Pakistan are highly distinct. One of these sequences, from Pegu = Bago, Myanmar (BMNH 1867.12.30.59), is identical with the sequences of *B. baska* from the Sundarbans published by Praschag *et al.* (2007); the other two differ

in one (NHMW 1841, Indus Delta, Pakistan) or two nucleotides (BMNH 1887.3.30.7, Rangoon = Yangon, Myanmar) from this haplotype.

In the parsimony network, the haplotypes of the three *Batagur* species are not connected if 90–95% probability thresholds are used. If a connection is enforced, the minimum distances between the haplotypes of all three species are similar. *Batagur baska* and the *Batagur* species from Indonesia and Malaysia differ by at least 20 mutational steps, *B. baska* and *B. kachuga* by a minimum of 21 steps and the *Batagur* species from Indonesia and Malaysia differs from *B. kachuga* by 19 steps (Fig. 1).

TABLE 1. Historical museum specimens of *Batagur baska* sensu lato used for mtDNA extraction and sequencing. Abbreviations: BMNH—The Natural History Museum, London; NHMW—Naturhistorisches Museum Vienna. GenBank accession numbers are given for mtDNA sequences.

Specimen	Locality	Condition	Year collected	mtDNA sequence	Remarks
BMNH 1856.5.6.5	Moulmein [Mawlamyine, Myanmar]	Dried shell, adult	By 1856	Failed to amplify	
BMNH 1866.3.25.10	India	Dried shell, adult	By 1866	Failed to amplify	
BMNH 1867.12.30.59	Pegu [Bago, Myanmar]	Dried shell, adult	By 1867	AM922507	Topotype of *Tetronyx longicollis* Lesson 1834
BMNH 1887.3.30.7	Rangoon [Yangon, Myanmar]	Dried shell, adult	By 1887	AM922508	Topotype of *Trionyx (Tetraonyx) cuvieri* Gray 1831 and *Tetronyx longicollis* Lesson 1834
BMNH 1946.1.22.44	Pinang [Penang, Malaysia]	Juvenile in alcohol	By 1847	Failed to amplify	Syntype of *Tetraonyx affinis* Cantor 1847
BMNH 1947.3.4.31	Pinang [Penang, Malaysia]	Juvenile in alcohol	By 1847	AM922509	Syntype of *Tetraonyx affinis* Cantor 1847
NHMW 1841	Indus Delta, Sindh [Pakistan]?	Stuffed subadult male	By 1857	AM922510	

TABLE 2. Primer sequences used for amplification and sequencing four overlapping cyt *b* fragments of historical museum specimens of *Batagur baska* sensu lato.

Primer	Sequence (5' to 3')
Batagur-F1	CTTACATCGGCAACACCC
Batagur-R1	GTTTGATCCGGTTTGRTGG
Batagur-F2	TTCACATTTCACTTCCTACTC
Batagur-R2	GCCTAGTAGGTCTTTGTATG
Batagur-F3	GGATCAAACAAYCCTACAG
Batagur-R3	TGAGAATAGTGATAGGCTTA
Batagur-F4	CTTCTCATACAAAGACCTACT
Batagur-R4	AATATCATTCTGGTTTGATGTG

Discussion and conclusions

Our genetic data of historical museum specimens confirm the previous finding of Praschag *et al.* (2007) that *Batagur baska* sensu lato consists of two genetically highly distinct species. According to anecdotal evidence, males of the two species differ significantly in their sexually dimorphic breeding coloration. Males of the northern species were described as having a waxy-blue nose, deep black heads and necks, passing into a rich crimson on the base of the neck, and brilliant rosy carmine-coloured forelimbs during the mating season; their iris being greenish yellow then (Anderson, 1879; Rashid & Swingland, 1997). In contrast, the skin and shell

of Malaysian males becomes uniform jet black, without any trace of blue or red, and their iris turns to immaculate white during breeding (Moll, 1980). Other morphological differences between the two species need to be investigated.

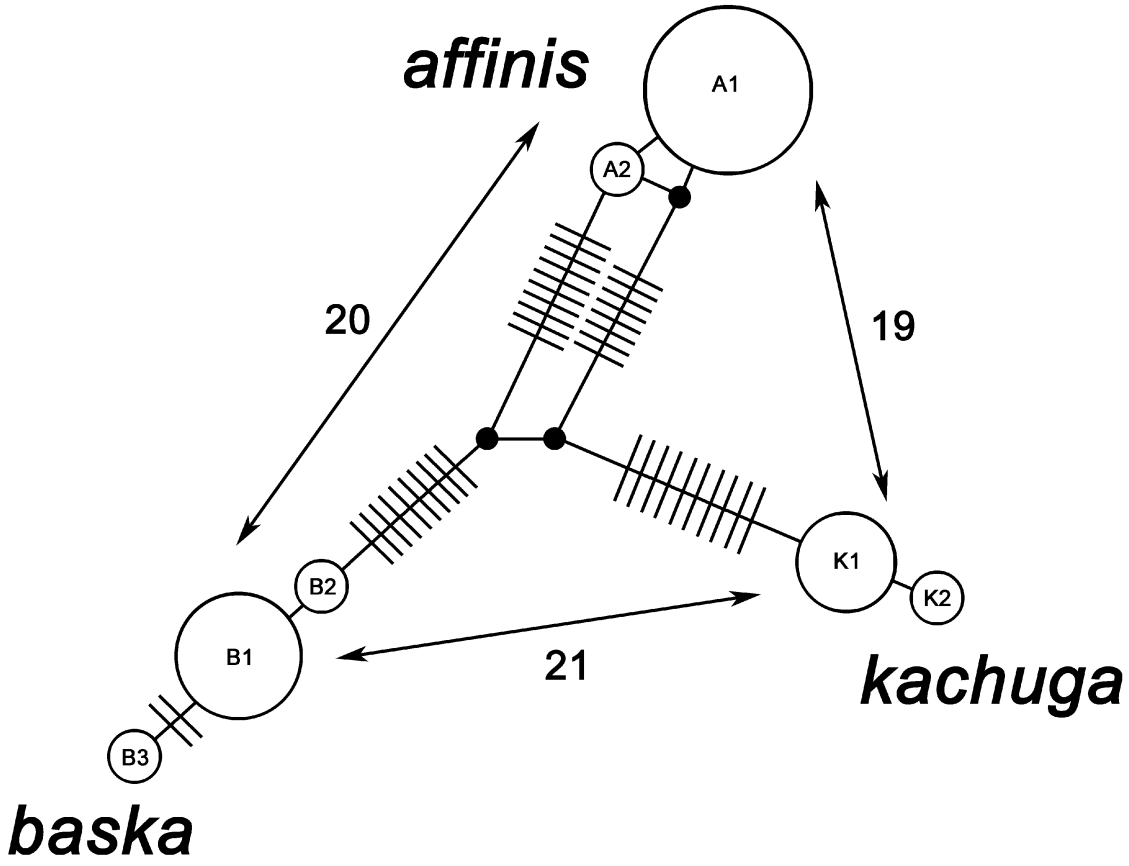

FIGURE 1. Parsimony network of mtDNA haplotypes of three *Batagur* species based on an alignment of 320 bp (partial cyt *b* gene). Symbol size, approximate haplotype frequency. Each line joining haplotypes indicates one nucleotide substitution except when hatches across lines are present; then each hatch indicates one step. Missing node haplotypes are shown in black. Arrows, minimum number of mutational steps between the three species. Haplotype frequencies are A1 (n = 6): AM691750-AM691754 (GenBank accession numbers, Praschag *et al.*, 2007), BMNH 1947.3.4.31 (this study); A2 (n = 1): AY434600 (Spinks *et al.*, 2004); B1 (n = 4): AM495267-AM495269 (Praschag *et al.*, 2007), BMNH 1867.12.30.59 (this study); B2 (n = 1): NHMW 1841 (this study); B3 (n = 1): BMNH 1887.3.30.7 (this study); K1 (n = 3): AM495284-AM495285, AM495287 (Praschag *et al.*, 2007); K2 (n = 1): AM495286 (Praschag *et al.*, 2007).

Although we succeeded only in obtaining sequence data for four museum specimens, the distribution of both species has become clearer than before (Fig. 2). One species, to which the name *Batagur baska* (Gray 1830), with type locality of India, should be applied (Praschag *et al.*, 2007), was historically distributed from the north-eastern Indian subcontinent to at least the Ayeyarwady and Bago River mouths in Myanmar. It is unclear how far south this northern species ranged historically. The old records for the north-western Malay Peninsula could refer to *B. baska* sensu stricto as well.

The second species previously lumped together with *B. baska* sensu stricto occupies the southern part of the range. Genetically verified records from Malaysia and Indonesia (Praschag *et al.*, 2007; this study) suggest that the southern Malay Peninsula and Sumatra, the only Indonesian region in which *B. baska* sensu lato occurs, are inhabited by the southern *Batagur*. It seems possible that the historical ranges of the two species met or overlapped in the north-western Malay Peninsula.

The taxonomic allocation of the relatively remote Cambodian remnant population (Platt *et al.*, 2003) and of the extinct southern Vietnamese *Batagur* remains unclear.

Emys baska was described by the British zoologist John Edward Gray in 1830 with the type locality "India" (Gray, 1830–1832), which is to be identified with what is now north-eastern India and Bangladesh. It was placed in the genus *Batagur* by Gray (1856), and over the subsequent decades the usage of this name combination stabilized to cover the large dark estuarine turtle ranging from Orissa to Sumatra and Vietnam (e. g., Boulenger, 1889; de Rooij, 1915; Smith, 1931; Bourret, 1941). In the synonymy of *B. baska* sensu lato several names exist that could refer to the southern *Batagur* species, namely *Trionyx (Tetraonyx) cuvieri* Gray 1831, *Tetronyx longicollis* Lesson 1834, *Tetraonyx affinis* Cantor 1847, and *Batagur baska ranongensis* Nutaphand 1979. Two other names, *Emys tetraonyx* Temminck & Schlegel 1835 and *Tetraonyx lessonii* Duméril & Bibron 1835, are merely replacement names (*Emys tetraonyx* Temminck & Schlegel 1835 for *Tetronyx longicollis* Lesson 1834; *Tetraonyx lessonii* Duméril & Bibron 1835 for *Emys batagur* Gray 1831 with type locality of India, a junior synonym of *Emys baska* Gray 1830, plus *Tetronyx longicollis* Lesson 1834; Fritz & Havaš, 2007).

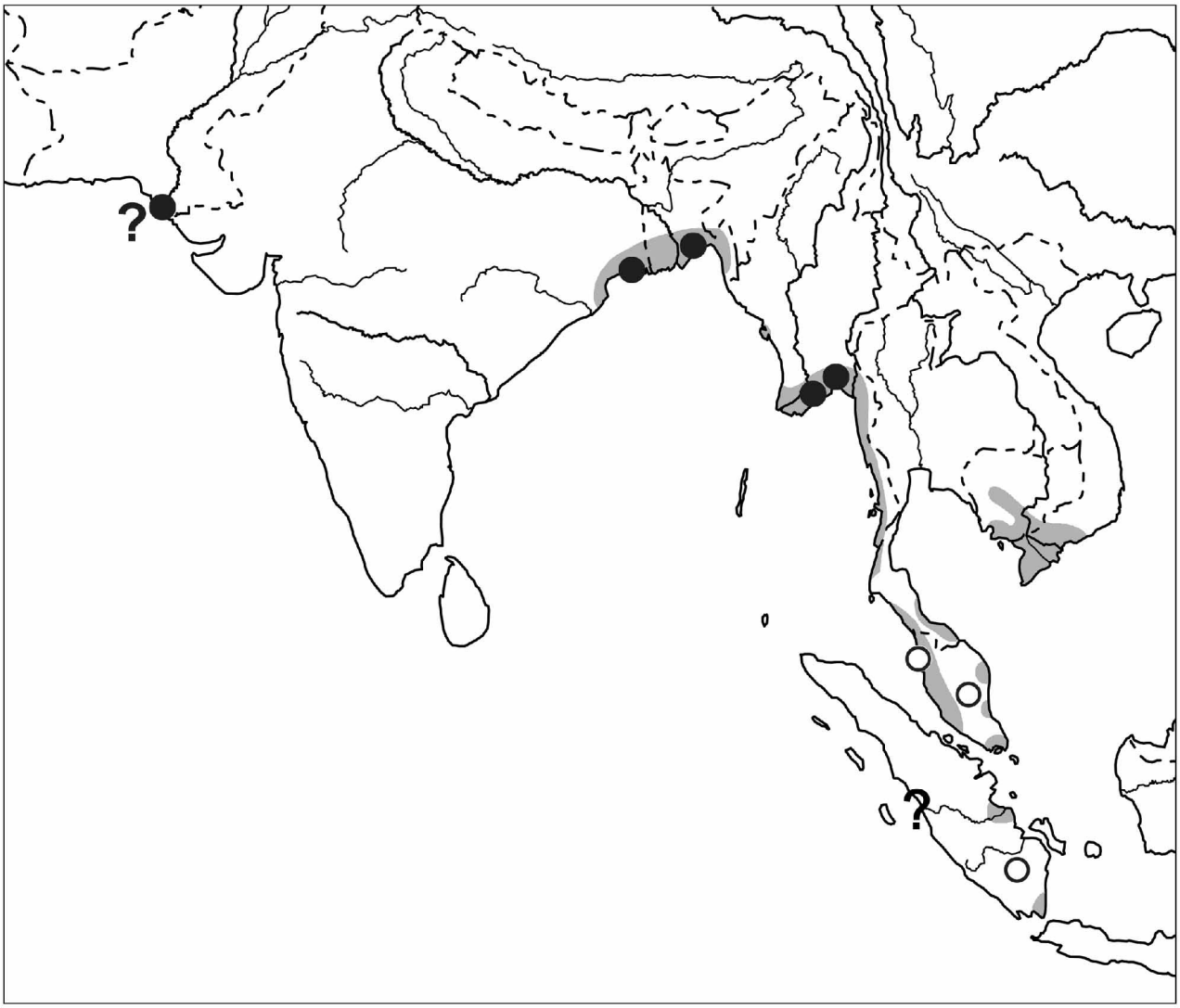

FIGURE 2. Approximate historical range of *Batagur affinis* and *B. baska* (distribution data from Smith, 1931; Bourret, 1941; Iverson, 1992; Das, 1991, 1995, 2001; Platt *et al.*, 2003; Kalyar *et al.*, 2007; van Dijk, Kaylar and Platt, pers. comm.). Collection sites of genetically verified specimens indicated (open circles: *B. affinis*, black circles: *B. baska*); symbols for imprecise localities from Praschag *et al.* (2007) in Sumatra and peninsular Malaysia placed inland.

Trionyx (Tetraonyx) cuvieri was described by Gray (1831) in the English version of Cuvier's "Animal Kingdom", based on a personal communication with Georges Cuvier. Because this book is rare in libraries and difficult to obtain, we reproduce the description here (Gray, 1831: 19, under the genus *Trionyx*; capitals

and italics as in original): "M. Cuvier informed me on his late visit to this country that there has been lately received at the Paris Museum a species of this genus, with 4 *claws* on *each foot*; it will form a section (*Tetraonyx*), and it may be indicated by the name of *Trionyx (Tetraonyx) Cuvieri*." This description was most likely indirectly based on *Batagur* specimens collected by Auguste Adolphe Marc Reynaud in Bengal and by Charles Paulus Bélanger in Burma (Ayeyarwady River). Their zoological collections were given to the Paris Museum in 1828 (October) and 1829, respectively, matching the time of the description of *Trionyx (Tetraonyx) cuvieri*. In the Paris Museum there are still three *Batagur* specimens from these collections present (MNHN 7967, MNHN 9094, MNHN 9095); a fourth specimen from Bengal which was formerly on display (MNHN 7993) could also belong to the Reynaud collection (Bour, pers. comm.). MNHN 7967 is the specimen figured in the original description of *Tetronyx longicollis* Lesson 1834; however, according to its label this specimen was collected in Bengal while the Ayeyarwady (Irravaddy = Irrawaddy) River and Bago (Pégou = Pegu) are mentioned by Lesson (1834), suggesting that his description was based on several specimens. Consequently, both names, *Trionyx (Tetraonyx) cuvieri* Gray 1831 as well as *Tetronyx longicollis* Lesson 1834, have composite type localities (Ayeyarwady River and Bengal; Ayeyarwady River, Bago and Bengal, respectively). Bengal, like the type locality "India" of *Emys baska* Gray 1830, is to be identified with northeastern India and Bangladesh, where the northern *Batagur* species, *B. baska* sensu stricto, occurs (Praschag *et al.*, 2007).

Two dried *Batagur* shells in the Natural History Museum London that yielded mtDNA sequences are topotypes of *Trionyx (Tetraonyx) cuvieri* Gray 1831 and *Tetronyx longicollis* Lesson 1834 and provide evidence that the northern species once occurred in all sites of their composed type localities. BMNH 1887.3.30.7 originated from Rangoon (Yangon) near the southern Ayeyarwady Delta and BMNH 1867.12.30.59 from Pegu (Bago) in Myanmar (Fig. 2). Accordingly, there is no doubt that *Trionyx (Tetraonyx) cuvieri* Gray 1831 and *Tetronyx longicollis* Lesson 1834 were based exclusively on specimens of the northern *Batagur* and are junior synonyms of *Emys baska* Gray 1830.

In contrast, the description of *Tetraonyx affinis* Cantor 1847 was based on two species. One of the three syntypes from "Pinang" = Penang, Malaysia is a *Batagur borneoensis* (BMNH 1946.1.22.47) while the two other specimens are *Batagur baska* sensu lato indeed. DNA amplification in the syntype BMNH 1946.1.22.44 failed but BMNH 1947.3.4.31 yielded the same haplotype as the five fresh *Batagur* samples from Indonesia and Malaysia studied by Praschag *et al.* (2007). This provides clear evidence that this specimen represents the southern *Batagur*. Therefore, BMNH 1947.3.4.31 is designated herewith as lectotype of *Tetraonyx affinis* Cantor 1847 (Fig. 3), enabling thereby the future usage of the name *Batagur affinis* (Cantor 1847) for the southern species.

The taxonomic allocation of the last name needing to be discussed, *Batagur baska ranongensis* Nutaphand 1979, must remain unclear because its type locality (estuaries of Ranong Province, Malay Peninsula, Thailand) lies between the verified distribution ranges of the two species.

Incidentally it should be noted that one of our museum specimens used for DNA sequencing could constitute the first record for a previous occurrence of *Batagur* in southern Pakistan (Fig. 2). In the collection of the Natural History Museum Vienna is a stuffed subadult male *B. baska* sensu stricto (NHMW 1841) bearing a historical label "Indus Delta in Sindh." It was collected by the brothers Hermann, Adolf and Robert Schlagintweit. The Schlagintweit brothers undertook in the mid of the 19th century extensive explorations in northern and central India on behalf of the British East India Company. In 1854, they arrived in Bombay and travelled over Madras to Calcutta, from where they followed the course of the Ganges River upstream over Varanasi, Allahabad and Fatehgarh to the Himalaya, where they entered also Tibet. From Simla (= Shimla, Himachal Pradesh, India) the Schlagintweit brothers independently crossed the Karakorum Massif to reach the upper Indus. They met again in 1856 in Rawalpindi, Punjab, where Robert Schlagintweit organized the transport of thousands of collected specimens over what is now southern Pakistan to Bombay, using a caravan of hundreds of camels, horses and people. During the journey, collecting was obviously continued. In 1857, the Schlagint-

weit Collection was shipped from Bombay to Europe. More than 14,000 specimens were kept for years in the Jägersburg castle in Forchheim, southeast Germany (Wolkenhauer, 1918). Later, the specimens were dispersed over different collections, among others the Zoological State Collection Munich (Zoologische Staatssammlung München), from where the Natural History Museum Vienna obtained in 1902 the *Batagur*, later catalogued as NHMW 1841, in exchange. As is obvious from the itinerary of the Schlagintweit brothers, there exists the possibility of locality confusion as their route led over the lower Brahmaputra and Ganges region, from where *B. baska* is well-known. However, taking into account the large-scale extirpation of these large estuarine terrapins and the rarity of historical records, a former occurrence in the Indus Delta seems possible, especially when it is considered that several other freshwater turtle species are distributed in the Indus as well as in the Ganges systems (Iverson, 1992; Ernst *et al.*, 2000).

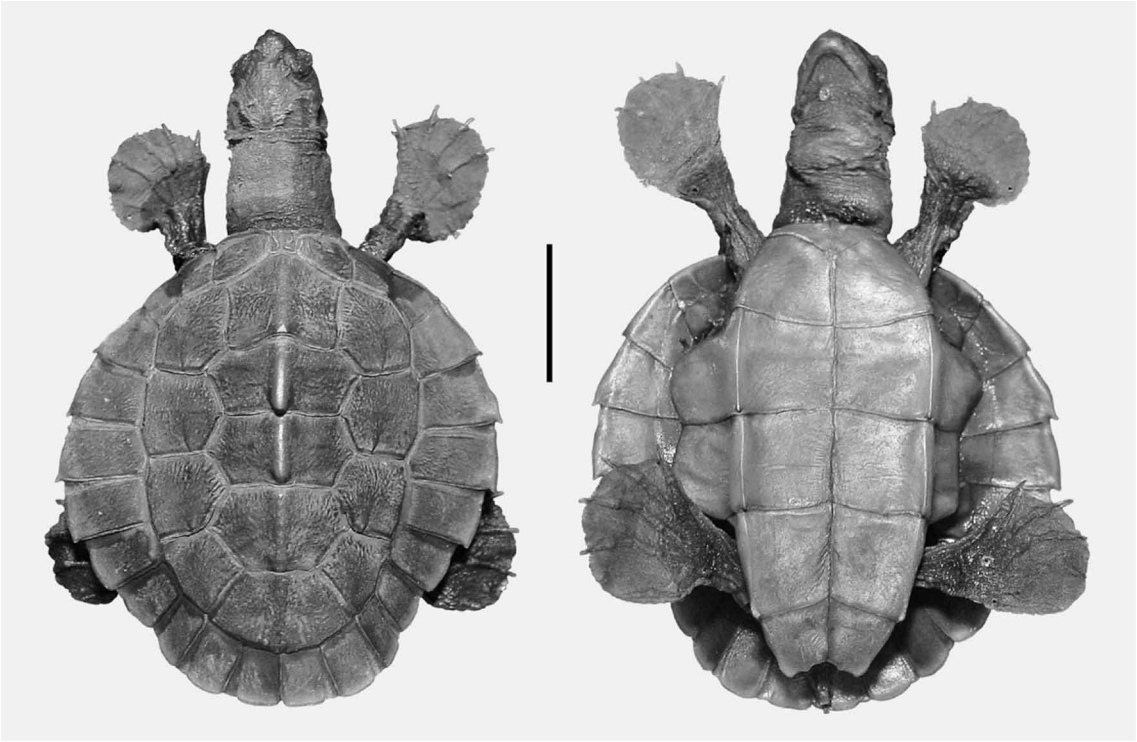

FIGURE 3. Dorsal and ventral aspect of the herein designated lectotype of *Tetraonyx affinis* Cantor 1847 (BMNH 1947.3.4.31). Scale bar: 2 cm. – Photos: C. McCarthy.

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