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# Phylogeny and taxonomy of endangered South and South-east Asian freshwater turtles elucidated by mtDNA sequence variation (Testudines: Geoemydidae: *Batagur*, *Callagur*, *Hardella*, *Kachuga*, *Pangshura*)

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Using DNA sequences of the mitochondrial cytochrome *b* gene, we investigated phylogeny and taxonomy of South and South-east Asian turtles of all species and subspecies of the genera *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura*. We found three major clades: (i) a moderately to well-supported clade containing all large riverine species assigned so far to *Batagur*, *Callagur* and *Kachuga*; (ii) a well-supported monophylum comprising the four *Pangshura* species; and (iii) *Hardella* that could constitute either the sister-taxon of *Pangshura* or of a clade comprising *Batagur*, *Callagur*, *Kachuga* and *Pangshura*. The genus *Kachuga* is clearly polyphyletic. Therefore, we recommend placing all *Batagur*, *Callagur* and *Kachuga* species in one genus. According to the International Code of Zoological Nomenclature *Batagur* Gray, 1856, being originally erected at higher rank, takes precedence over the simultaneously published name *Kachuga* Gray, 1856, and the younger name *Callagur* Gray, 1870, resulting in an expanded genus *Batagur*. Indonesian and Malaysian *Batagur baska* proved to be highly distinct from our sequences of this species from the Sundarbans (Bangladesh, adjacent India), suggesting that a previously unidentified species is involved. This finding is of high conservation relevance in the critically endangered *B. baska*. The currently recognized subspecies within *Hardella thurjii*, *Pangshura smithii* and *P. tentoria* do not correspond well with mtDNA clades. Considering that the two subspecies of *H. thurjii* are likely to be based only on individual ontogenetic differences, we propose abandoning the usage of subspecies within *H. thurjii*. In the Ghaghra River, Uttar Pradesh (India) we detected shared haplotypes in *P. smithii* and *P. tentoria*, implying that the unusual morphological characters of the Ghaghra River population of *P. tentoria* could be the result of interspecific hybridization.

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## Introduction

The genera *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura* belong to the family Geoemydidae and include 10 in part highly endangered South and South-east Asian turtle species (Table 1; van Dijk *et al.* 2000; IUCN 2006). Geoemydidae represent the sister group of land tortoises (Testudinidae; Gaffney & Meylan 1988; Shaffer *et al.* 1997) and comprise 65 small to large-sized species that occur, except the New World

genus *Rhinoclemmys*, in Asia, North Africa and Europe (Fritz & Havaš 2007). Most geoemydids are freshwater turtles; some are adapted to estuarine or terrestrial habitats (Ernst *et al.* 2000). *Batagur*, *Callagur* and *Hardella* are monotypic genera of large-sized, riverine species with maximum shell lengths of approximately 50–60 cm. *Kachuga* and *Pangshura* contain three and four species, respectively (Ernst *et al.* 2000; Das 2001; E. O. Moll, pers. comm.). Until Das (2001) removed

**Table 1** Currently recognized species and subspecies within *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura* according to Fritz & Havaš (2007).

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<i>Batagur</i> Gray, 1856
<i>Batagur baska</i> (Gray, 1831)
<i>Callagur</i> Gray, 1870
<i>Callagur borneoensis</i> (Schlegel & Müller, 1844)
<i>Hardella</i> Gray, 1870
<i>Hardella thurjii thurjii</i> (Gray, 1831)
<i>Hardella thurjii indi</i> Gray, 1870
<i>Kachuga</i> Gray, 1856
<i>Kachuga dhongoka</i> (Gray, 1835)
<i>Kachuga kachuga</i> (Gray, 1831)
<i>Kachuga trivittata</i> (Duméril & Bibron, 1835)
<i>Pangshura</i> Gray, 1856
<i>Pangshura smithii smithii</i> (Gray, 1863)
<i>Pangshura smithii pallidipes</i> (Moll, 1987)
<i>Pangshura sylhetensis</i> Jerdon, 1870
<i>Pangshura tecta</i> (Gray, 1831)
<i>Pangshura tentoria tentoria</i> (Gray, 1834)
<i>Pangshura tentoria circumdata</i> (Mertens, 1969)
<i>Pangshura tentoria flaviventer</i> Günther, 1864

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the small-sized *Pangshura* species *P. smithii*, *P. sylhetensis*, *P. tecta* and *P. tentoria* (maximum shell lengths 20–26.5 cm; Ernst *et al.* 2000) from *Kachuga*, these four species were placed for more than a century with the three large-sized *Kachuga* species (maximum shell lengths 48–58 cm; Ernst *et al.* 2000) into the genus *Kachuga* (Boulenger 1889; Siebenrock 1909; Smith 1931; Wermuth & Mertens 1961, 1977; Moll 1986, 1987; Ernst & Barbour 1989; Das 1991, 1995; Ernst *et al.* 2000). Like *Batagur*, *Callagur* and *Hardella*, the species of *Kachuga* are confined to rivers, while *Pangshura* species also occur in standing water bodies (Moll 1986, 1987).

All *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura* species are characterized by a more or less well-defined sexual dimorphism. Males are distinctly smaller sized than females; the most extreme size dimorphism occurs in *H. thurjii* with males reaching only approximately 17.5 cm maximum shell length, whereas females may have straight-line shell lengths of up to 61 cm (Ernst *et al.* 2000). Among the large-sized species, *B. baska*, *C. borneoensis*, *K. kachuga* and *K. trivittata* share another striking sexual dimorphism. Males have conspicuously coloured heads and necks, and in part also shells, a character state that is most pronounced during the breeding season (Theobald 1876; Anderson 1879; Boulenger 1889; Klingelhöffer & Mertens 1944; Moll 1980, 1986; Moll *et al.* 1981; Ernst & Barbour 1989; Ernst *et al.* 2000).

Harvesting of eggs, overexploitation of turtles for food and habitat alteration endanger all the large riverine species seriously and have brought *B. baska*, *C. borneoensis*, *K. kachuga* and especially *K. trivittata* to the fringe of extinction. Since the late 1960s, restocking programs are conducted for *B. baska*

and *C. borneoensis* and similar efforts are currently attempted for *H. thurjii*, *K. dhongoka*, *K. kachuga* and *K. trivittata* (van Dijk *et al.* 2000; Kuchling *et al.* 2006; E. O. Moll, pers. comm.). Understanding genetic differentiation of these turtles would significantly contribute to more powerful conservation strategies. However, few data on genetic variation are available until now and no previous study focused on geographical variation.

Using a patchy taxon sampling, a first molecular hypothesis for *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura* was established by Spinks *et al.* (2004), providing evidence that these genera represent a monophyletic group. Based on a complete taxon sampling of all species and subspecies, here we use sequence variation of a highly informative mitochondrial marker, the cytochrome *b* gene: (i) to reconstruct their phylogeny; (ii) to investigate geographical variation within most taxa; and (iii) to test whether the subspecies within *H. thurjii*, *P. smithii* and *P. tentoria* correspond with distinct mtDNA clades.

## Materials and methods

### Sampling

Most turtles used for sampling are long-term captives from the live collection of Peter and Reiner Praschag in Graz, Austria. These turtles will be or have been deposited into the collections of natural history museums upon their natural death (Appendix 1). Voucher photographs of all used turtles are housed in the Museum of Zoology Dresden and the Natural History Museum Vienna. Most specimens were personally collected from the 1970s to the early 1990s by Peter and Reiner Praschag or shipped by animal dealers in Dhaka (Dacca), Bangladesh and Mumbai (Bombay), India to Europe during this time. Of *C. borneoensis* and *K. trivittata* only specimens without locality data were available for study. Five samples of *B. baska* originate from turtles kept in the Prague Zoo. In addition to our known-locality samples, we used GenBank sequences that were from individuals of unknown geographical provenance to enlarge sample size and to test how variation of these sequences, which could originate from turtles collected in other regions, corresponds to our known-locality samples (Appendix 1). Tissue samples were either obtained by clipping off a tiny piece of the webbing of the toes of live turtles or by dissection of carcasses (thigh muscle). Samples were preserved in ethanol, and stored at –20 °C until processing. Remaining tissue and DNA samples are permanently kept at –80 °C in the tissue sample collection of the Museum of Zoology Dresden.

### Laboratory procedures

Total genomic DNA was extracted from samples by overnight incubation at 37 °C in lysis buffer (6% DTAB, 5 M NaCl, 1 M Tris–HCl, 0.5 M EDTA, pH 8.0) including 0.5 mg of

proteinase K (Merck), and subsequent purification following the DTAB method (Gustincich *et al.* 1991). DNA was precipitated from the supernatant with 0.2 volumes of 4 M LiCl and 0.8 volumes of isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Two fragments (overlapping by approximately 300 bp), together comprising almost the complete *cyt b* gene and the adjacent portion of the tRNA-Thr gene, were amplified using the two primer pairs mt-c-For2 5'-TGA GG(AGC) CA(AG) ATA TCA TT(CT) TGA G-3' plus mt-f-na3 5'-AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC CAA TG-3' or mt-a-neu3 5'-CTC CCA GCC CCA TCC AAC ATC TC(ACT) GC(ACT) TGA TGA AAC TTC G-3' plus mt-E-Rev2 5'-GC(AG) AAT A(AG)(AG) AAG TAT CAT TCT GG-3'. PCR was performed in a 50  $\mu$ L volume (50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of *Taq* DNA polymerase (Bioron), 10 pmol dNTPs (Eppendorf) and 10 pmol of each primer. After initial denaturing for 5 min at 95 °C, 35–40 cycles were performed with denaturing 1 min at 95 °C, annealing 1 min at 55 °C, and primer extension for 2 min at 72 °C, followed by a final elongation of 10 min at 72 °C. PCR products were purified by precipitation under the following conditions: 1 volume PCR product (30  $\mu$ L), 1 volume 4 M NH<sub>4</sub>Ac (30  $\mu$ L) and 12 volumes EtOH (100%; 360  $\mu$ L). DNA was pelleted by centrifugation (15 min at 16060 g) and the pellet washed with 70% ethanol. The pellet was dissolved in 20  $\mu$ L H<sub>2</sub>O. PCR products were sequenced with the primers mt-c-For2 and mt-E-Rev2 on an ABI 3130 sequencer (Applied Biosystems) or on an ABI 3730XL sequencer (Applied Biosystems). DNA extraction and sequencing of samples 3113–3115 and 3094 were repeated, whereby the primer mt-a-neu3 was replaced by the primer *CytbG* (5'-AAC CAT CGT TGT (AT)AT CAA CTA C-3'; Spinks *et al.* 2004). None of the sequences contained internal stop codons, and nucleotide frequencies corresponded to those known for coding mtDNA; we therefore conclude that we amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

#### Phylogenetic and population genealogy analyses

GenBank sequences of *Geoclemys hamiltonii* (Gray, 1831) and *Morenia ocellata* (Duméril & Bibron, 1835) were included as outgroups (Appendix 1), according to the findings of Spinks *et al.* (2004). Sequences were aligned with CLUSTALW using default parameters as implemented in MEGA 3.0 (Kumar *et al.* 2004). For the ingroup species, 691 of 1067 aligned sites were constant, 67 characters were variable but parsimony-uninformative, and 309 variable characters were parsimony-informative.

Data were analysed under the optimality criteria Maximum Parsimony (MP; equal weighting), Maximum Likelihood (ML), and the cluster algorithm Neighbor-Joining (NJ; with

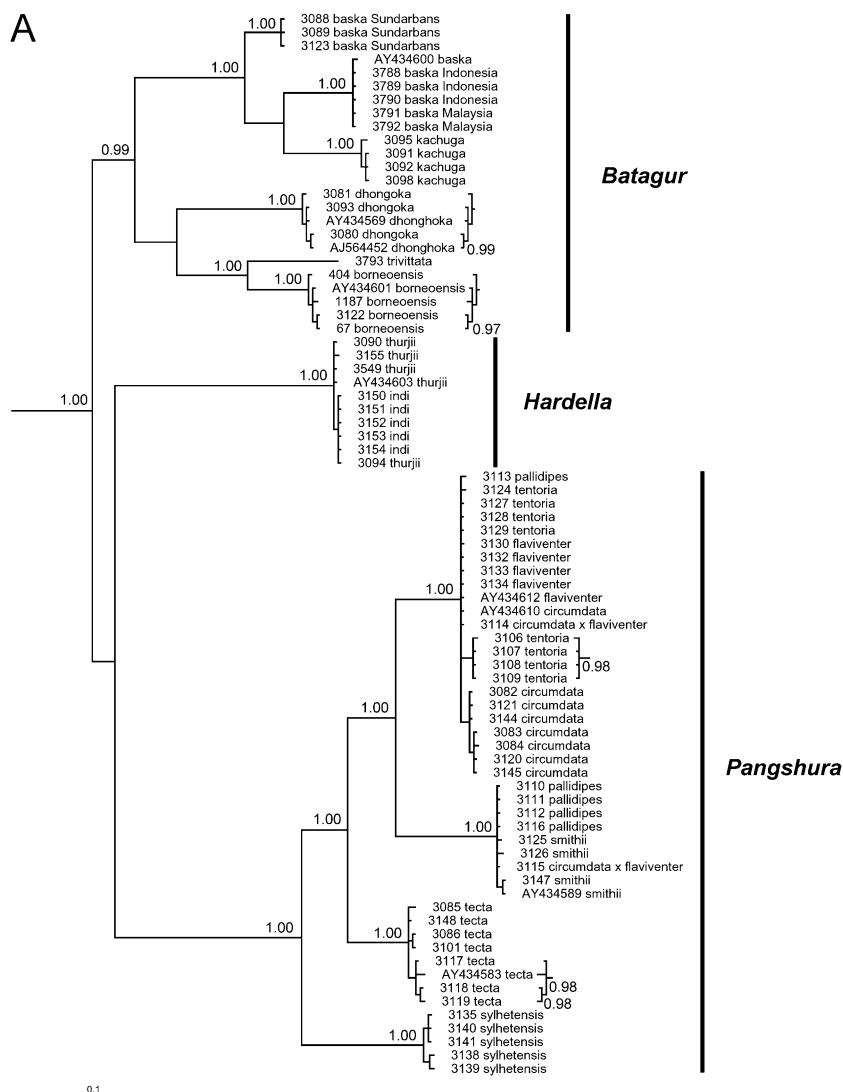
model corrected maximum likelihood distances) as implemented in PAUP\* 4.0b10 (Swofford 2002), as well as Bayesian inference of phylogeny as implemented in MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). Bayesian analysis (BA) was performed using four chains of 1 000 000 generations sampling every 100 generations and with the first 1000 generations discarded as burn-in (with which only the plateau of the most likely trees was sampled). The best evolutionary model for the data (ML calculation and ML distances) was established by hierarchical likelihood testing using MODELTEST 3.06 (best-fit model: TrN + I + G; Posada & Crandall 1998). Under ML we calculated trees without using a starting tree; parameters: Lset Base = (0.3163 0.3532 0.1019), Nst = 6, Rmat = (1.0000 14.0790 1.0000 1.0000 16.6295), Rates = Gamma Shape = 3.1376, Pinvar = 0.5722. Bootstrap support values were calculated with PAUP\* 4.0b10 for MP with nreps = 1000 and NJ with nreps = 100 000 based on ML distances, as well as for ML with GARLI 0.95 with the settings 'bootstrapreps = 100' and 'genthreshfortopterm = 5000'. These bootstrap settings are advised in the manual of the program (Zwickl 2006).

Especially on the subspecies and population levels, dichotomous phylogenetic analyses may be misleading due to persisting ancestral haplotypes. Such genealogies are often multifurcated and need to be depicted using algorithms allowing for reticulations (Posada & Crandall 2001). Therefore, we calculated parsimony haplotype networks as implemented in TCS 1.21 (Clement *et al.* 2000) where appropriate.

## Results

### Phylogenetic analyses

All tree-building methods reveal *Pangshura* as perfectly supported monophylum with bootstrap or posterior probability values of 100% (Fig. 1). All other species are located outside of this *Pangshura* clade; BA and ML suggest with weak support *Hardella* as sister-taxon of *Pangshura*. Under MP and NJ (not shown), *Hardella* constitutes with weak support the sister of *Pangshura* plus all other investigated taxa. *Kachuga* is clearly polyphyletic and its three species appear with the other large riverine taxa (*B. baska*, *C. borneoensis*) in a moderately to well-supported monophylum. It includes two weakly to well-supported subclades; one containing the sequences of *B. baska* and *K. kachuga*, and the other *C. borneoensis*, *K. dhongoka* and *K. trivittata*. Within the first, well-supported subclade, the sequences of *B. baska* from the Sundarbans (sample numbers 3088–3089, 3123) are highly distinct from six other *B. baska* sequences. Five of these sequences originate from Indonesian and Malaysian turtles (3788–3092) and the sixth is a sequence of unknown geographical provenance downloaded from GenBank (AY43600; Spinks *et al.* 2004). The Sundarban *B. baska* sequences are consistently placed as sister-taxon of *K. kachuga* plus the Indo-Malaysian *B. baska*. The Indo-



**Fig. 1** A–C. Phylogenetic relationships of *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura* as revealed by analysis of a 1067 bp long mtDNA fragment (partial cytochrome *b* gene). On the right, recommended generic assignments. —A. Bayesian tree. —B. Maximum Likelihood tree. —C. Strict consensus of 128 parsimony trees (758 steps; CI = 0.5884, RI = 0.9413). For all trees, support values are presented at nodes. ML tree includes ML bootstrap values (top) and NJ bootstrap values using ML distances (bottom) only at crucial nodes; dashes indicate that the respective branch is not supported. BA, posterior probabilities greater than 0.95; ML, MP and NJ, bootstrap values greater than 50 except in ML tree, where in some cases lower values are included for clarity. Numbers preceding species names are MTD T or accession numbers and refer to the Appendix 1. Branch lengths for the BA and ML trees proportional to the mean number of substitutions per site; branch lengths for the MP tree arbitrary. Outgroup taxa (*Geoclemys hamiltonii*, *Morenia ocellata*) removed for clarity.

Malaysian sequences of *B. baska* differ by uncorrected average *p* distances of 4.22% from the Sundarban *B. baska* and 5.05% from *K. kachuga*; these distances are of approximately the same magnitude as the sequence divergence between the Sundarban *B. baska* and *K. kachuga* (Table 2). Within the second subclade, *K. trivittata* is with high support sister of *C. borneoensis*; *K. dbongoka* is suggested as sister-taxon of *C. borneoensis* + *K. trivittata*.

The two subspecies of *H. thurjii* are only badly supported. *Hardella t. thurjii* sequences from Uttar Pradesh (India) and Bangladesh are paraphyletic with respect to the sequences of the Indus River subspecies *H. t. indi*.

Within *Pangshura*, all species correspond with well-supported clades. *Pangshura tecta* and *P. sylhetensis* are the successive sister-taxa of *P. tentoria* + *P. smithii*. One sequence originating from a turtle collected in the Ghaghra River

(Uttar Pradesh, India) that was morphologically identified as an intergrade between *P. tentoria circumdata* and *P. tentoria flaviventer* (sample number 3115) is nested within *P. smithii* however; another intergrade (3114) as well as a turtle identified as *P. smithii pallidipes* (3113) from the same locality occur in the *P. tentoria* clade. Within *P. tecta*, sequences from Bangladeshi turtles (sample numbers 3117–3119) plus a GenBank sequence of unknown origin (AY34583) are suggested as a moderately to well-supported clade (ML: 79, MP: 82, NJ: 85, BA: 0.98) that is either embedded within a polytomy comprising sequences from Pakistani and Indian turtles or those sequences are with weak support sister to the Bangladeshi sequences. The currently recognized subspecies of *P. smithii* and *P. tentoria* generally do not correspond well with mtDNA clades. Within *P. smithii* there is no phylogenetic differentiation at all paralleling the two recognized subspecies *P. s. smithii* and

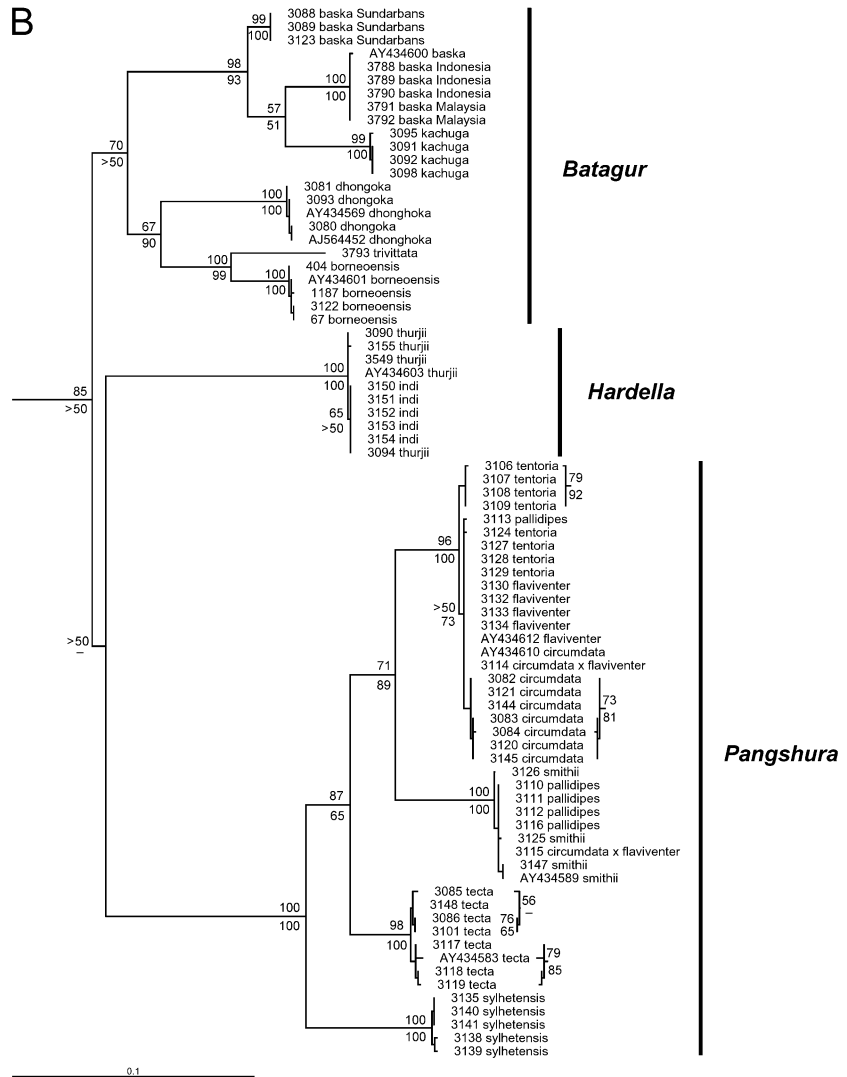


Fig. 1 Continued.

*P. s. pallidipes*. In *P. tentoria*, the phylogenetically most distinctive subspecies is *P. t. circumdata* of which nearly all sequences occur in a moderately supported clade with bootstrap values of 73 (ML), 75 (MP) and 81 (NJ); however, under BA posterior probabilities are below 0.95. Four sequences of *P. t. tentoria* from the Mahanadi River (Orissa, India; sample numbers 3106–3109) occur in a similarly supported clade (bootstrap support of 79 under ML, 90 under MP, 92 under NJ; posterior probability of 0.98 under BA) that is under ML sister of all other sequences within *P. tentoria*, including the clade of *P. t. circumdata*.

**Network analyses of population genealogy**

*Batagur baska*. The three *B. baska* sequences from the Sundarbans represent the same haplotype, which is highly distinct from the GenBank sequence AY434600 of *B. baska* and a third

haplotype that was found in our five *B. baska* samples from Indonesia and Malaysia. This haplotype differs by one mutation step from the GenBank sequence. Using TCS, the Sundarban haplotype is not connected with the two other *B. baska* haplotypes if 90%–95% criteria are applied for the network probability. If a connection is enforced, the Sundarban haplotype is separated by 44 and 45 mutation steps from the GenBank haplotype and the haplotype of the Indonesian and Malaysian samples, respectively, a distance that is of a similar degree to the differentiation when the *B. baska* haplotypes and *K. kachuga* haplotypes are compared. Within *K. kachuga*, two haplotypes occur that differ from one another by two mutations steps. These two *K. kachuga* haplotypes are separated from the *B. baska* haplotype from the Sundarbans by 54 mutation steps each and from the *B. baska* GenBank sequence and the Indo-Malaysian *B. baska* haplotype by 52 or 53 mutation steps.





**Table 2** Uncorrected average *p* distances (percentages) between investigated species and subspecies and their outgroups. On the diagonal the within-taxon divergence is given in bold. Sequences of putative hybrids of *Pangshura smithii* and *P. tentoria* (Ghaghra River, samples 3113–3115) not included.

	G.	M.	B.	'B.	C.	H. t.	H. t.	K.	K.	K.	P. s.	P. s.	P.	P. t.	P. t.	P. t.
	<i>hamiltonii</i>	<i>ocellata</i>	<i>baska</i>	<i>baska'</i>	<i>borneoensis</i>	<i>thurjii</i>	<i>indi</i>	<i>dhongoka</i>	<i>kachuga</i>	<i>trivittata</i>	<i>smithii</i>	<i>pallidipes</i>	<i>sylhetensis</i>	<i>tecta</i>	<i>tentoria</i>	<i>circumdata</i>
<i>Geoclemys hamiltonii</i>	—															
<i>Morenia ocellata</i>	15.70	—														
<i>Batagur baska</i> (Sundarbans)	12.71	13.08	0													
' <i>Batagur baska</i> ' (Indo-Malaysia)	13.59	14.24	4.22	0.03												
<i>Callagur borneoensis</i>	13.26	13.99	8.27	9.40	0.13											
<i>Hardella thurjii thurjii</i>	14.17	13.37	10.91	11.66	10.44	0.08										
<i>Hardella thurjii indi</i>	14.22	13.43	10.97	11.72	10.40	0.10	0									
<i>Kachuga dhongoka</i>	13.52	13.95	8.28	10.16	7.12	11.69	11.74	0.10								
<i>Kachuga kachuga</i>	13.81	13.60	4.97	5.05	8.95	10.89	10.97	9.19	0.09							
<i>Kachuga trivittata</i>	13.63	13.80	8.72	9.46	5.13	11.02	10.97	8.11	9.56	—						
<i>Pangshura smithii smithii</i>	14.81	15.73	11.37	12.29	13.29	13.57	13.61	12.38	12.15	13.73	0.27					
<i>Pangshura smithii pallidipes</i>	14.79	15.64	11.34	12.34	13.17	13.47	13.50	12.33	12.18	13.59	0.17	0				
<i>Pangshura sylhetensis</i>	15.35	15.21	11.70	12.44	12.82	13.26	13.29	12.20	11.90	12.75	9.18	0.19				
<i>Pangshura tecta</i>	15.37	14.60	11.06	11.50	12.31	13.10	13.14	11.73	11.54	12.89	6.77	6.79	0.37			
<i>Pangshura tentoria tentoria</i>	14.73	14.71	11.20	11.72	13.27	13.00	13.05	12.61	12.13	13.03	5.59	5.60	6.13	0.32		
<i>Pangshura tentoria circumdata</i>	14.59	14.57	11.35	11.62	13.42	12.94	12.99	13.06	12.05	13.19	5.74	5.75	6.03	0.56	0.15	
<i>Pangshura tentoria flaviventer</i>	14.62	14.69	11.19	11.63	13.29	12.89	12.94	12.75	12.06	13.06	5.69	5.69	5.98	0.26	0.31	0

**Table 3** Occurrence and frequency of haplotypes in samples of *Pangshura smithii* and *P. tentoria*. Sequence numbers refer to samples deposited in the tissue collection of the Museum of Zoology Dresden (MTD T) or are accession numbers.

Taxon	Locality	Sequence	Haplotype
<i>Pangshura smithii smithii</i>	Pakistan: Indus River System	MTD T 3147	S2
<i>Pangshura smithii smithii</i>	India: Assam: Tezpur: Brahmaputra River	MTD T 3125	S4
<i>Pangshura smithii smithii</i>	India: Assam: Tezpur: Brahmaputra River	MTD T 3126	S3
<i>Pangshura smithii smithii</i>	Unknown	AY434589	S2
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3110	S1
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3111	S1
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3112	S1
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3113	T5
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3116	S1
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	MTD T 3124	T4
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	MTD T 3127	T1
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	MTD T 3128	T1
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	MTD T 3129	T1
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	MTD T 3106	T3
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	MTD T 3107	T2
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	MTD T 3108	T2
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	MTD T 3109	T2
<i>Pangshura tentoria circumdata</i>	Pakistan: Indus River System	MTD T 3144	T6
<i>Pangshura tentoria circumdata</i>	Pakistan: Indus River System	MTD T 3145	T7
<i>Pangshura tentoria circumdata</i>	India: Madhya Pradesh: Chambal River	MTD T 3120	T7
<i>Pangshura tentoria circumdata</i>	India: Madhya Pradesh: Chambal River	MTD T 3121	T6
<i>Pangshura tentoria circumdata</i>	India: Uttar Pradesh: Gompti River	MTD T 3082	T6
<i>Pangshura tentoria circumdata</i>	India: Uttar Pradesh: Gompti River	MTD T 3083	T7
<i>Pangshura tentoria circumdata</i>	India: Uttar Pradesh: Gompti River	MTD T 3084	T8
<i>Pangshura tentoria circumdata</i>	Unknown	AY434610	T1
<i>Pangshura tentoria flaviventer</i>	Nepal: Sapta Khosi River	MTD T 3132	T1
<i>Pangshura tentoria flaviventer</i>	Nepal: Sapta Khosi River	MTD T 3133	T1
<i>Pangshura tentoria flaviventer</i>	Nepal: Sapta Khosi River	MTD T 3134	T1
<i>Pangshura tentoria flaviventer</i>	Bangladesh: Sunargon Market	MTD T 3130	T1
<i>Pangshura tentoria flaviventer</i>	Unknown	AY434612	T1
<i>Pangshura tentoria circumdata</i> × <i>Pangshura tentoria flaviventer</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3114	T1
<i>Pangshura tentoria circumdata</i> × <i>Pangshura tentoria flaviventer</i>	India: Uttar Pradesh: Ghaghra River	MTD T 3115	S1

of both species are separated by a minimum of 58 mutation steps. Topology in the subnets remains identical then; however, a loop connecting the two subnets equally parsimonious over three haplotypes of the *P. smithii* subnet occurs only when the connection is enforced.

Within *P. smithii*, the most common haplotype S1 is confined to *P. s. pallidipes*, while the three other haplotypes S2–S4, different by one to two mutation steps, represent samples of *P. s. smithii* (Table 3). Under coalescence theory, S1 is ancestral to S2–S4.

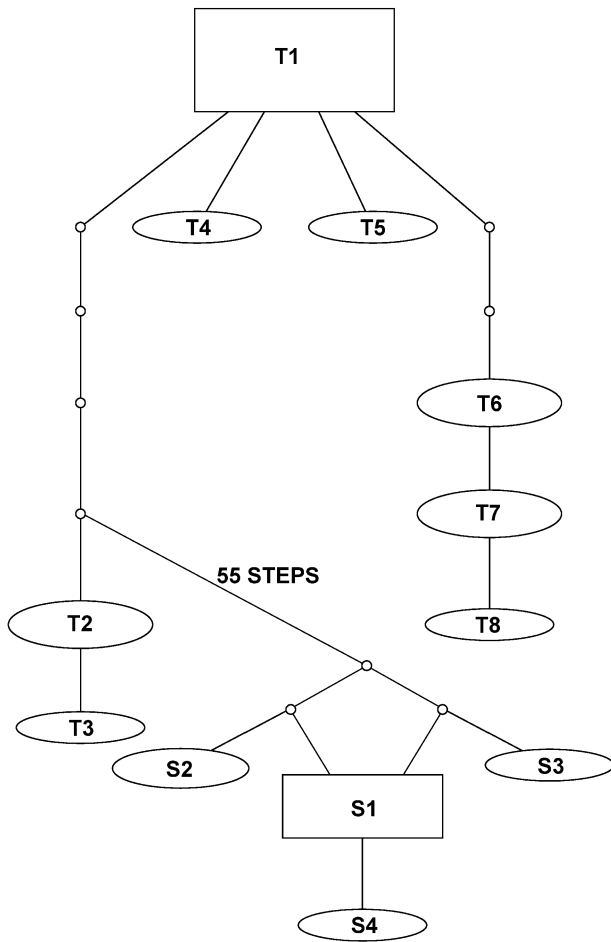
Most sequences of *P. t. circumdata* (Indus River system, Pakistan: 3144–3145; Chambal River, Madhya Pradesh, India: 3120–3121; Gompti River, Uttar Pradesh, India: 3082–3084) correspond to three unique haplotypes (T6–T8), differing in three to five mutation steps from the most common haplotype occurring in *P. tentoria* (T1); one GenBank sequence of *P. t. circumdata* without locality data (AY434610) is identical with T1 however. T1 is ancestral to all other *tentoria* haplotypes (Fig. 2). Besides AY434610 and the above mentioned

intergrade between the *P. t. circumdata* and *P. t. flaviventer* (3114) from the Ghaghra River, T1 occurs in *P. t. tentoria* from the Brahmaputra River (3127–3129) and in *P. t. flaviventer* from the Sapta Khosi River in Nepal (3132–3134), from Bangladesh (3130); also a GenBank sequence of *P. t. flaviventer* (AY434612) represents this haplotype. All four sequences of *P. t. tentoria* from the Mahanadi River (3106–3109) represent the unique haplotypes T2 and T3 that differ in five to six mutation steps from T1.

## Discussion

Based on an incomplete taxon sampling (one sequence each of *B. baska*, *C. borneoensis*, *H. t. thurjii*, *K. dbongoka*, *P. s. smithii*, *P. t. circumdata*, *P. t. flaviventer*, *P. tecta*), a first molecular hypothesis for relationships of the genera *Batagur*, *Callagur*, *Hardella*, *Kachuga* and *Pangshura* was presented by Spinks *et al.* (2004) using mtDNA (cytochrome *b*, 12S rRNA genes) and nDNA data (intron from the R35 neural transmitter gene). This investigation confirmed *Pangshura* as a clade distinct





**Fig. 2** TCS network for mtDNA haplotypes of *Pangsbura smithii* and *P. tentoria*. Connection of subnets enforced. Haplotypes with biggest outgroup probability shown as rectangles (outgroup probabilities of unconnected subnets under 90% and 95% criteria: S1 = 0.7500; T1 = 0.3871). Symbol size approximately corresponds to haplotype frequency; missing haplotypes, small circles. With exception of the line between the subnets symbolizing 55 mutation steps, each line between symbols and haplotypes indicates one mutation step. For haplotype frequencies and occurrence of haplotypes, see Table 3, for further explanation, see text.

from the sole studied *Kachuga* species, *K. dbongoka*. The latter was found to be sister of *C. borneoensis* and both together being the sister group of *B. baska*. *Pangsbura* + *Hardella* were found to be the sister group of (*K. dbongoka* + *C. borneoensis*) + *B. baska*. Our phylogenetic analyses, based on a complete taxon sampling of all species and subspecies of *Pangsbura* and all taxa of the other genera, corroborate that *Pangsbura* is monophyletic (bootstrap and posterior probability support of 100%) and distinct from *Kachuga*, and thus support the recognition of *Pangsbura* as distinct genus.

Originally, *Pangsbura* was erected by Gray (1856) as a subgenus within *Batagur*, a genus that contained then the small-sized *Pangsbura* species as well as species that are now placed into the genera *Batagur*, *Kachuga* and *Morenia*. Already Günther (1864) elevated *Pangsbura* to full genus level, but Boulenger's (1889) influential 'Catalogue of the Chelonians and Crocodiles in the British Museum (Natural History)' relegated *Pangsbura* into the synonymy of *Kachuga*, while *Batagur* and *Callagur* were recognized as distinct genera. It was not before Moll's (1986) treatise of *Kachuga* that *Pangsbura* was acknowledged on the subgeneric level as being distinct from the large-sized species of *Kachuga*. Das (2001) elevated *Pangsbura* to full genus level without providing the rationale that led him to this decision.

In contrast to the well-established monophyly of *Pangsbura*, the genus *Kachuga* clearly is polyphyletic. Each of the studied *Kachuga* species groups with moderate to high support either with *B. baska* sensu lato or with *C. borneoensis* or, in the case of *K. dbongoka*, with a clade comprising *C. borneoensis* and *K. trivittata*. Moreover, sequences of *B. baska* from Indonesia and Malaysia proved to be highly distinct from sequences of this species from the Sundarbans, suggesting that a previously unidentified species is involved. *Batagur baska* is a rare and endangered estuarine species, distributed over a patchy range from the Sundarbans Region of north-easternmost India and neighbouring Bangladesh through the Ayeyarwady (Irrawaddy) River mouth in Myanmar and the Malay Peninsula (Malaysia, southern Thailand) to southern Vietnam, Cambodia and Sumatra, and is now extirpated in much of its former range (Moll 1980; Das 1991, 1995, 2001; Ernst *et al.* 2000; Platt *et al.* 2003). Due to their large size (maximum shell length approximately 60 cm; Ernst *et al.* 2000), adult *B. baska* are rare in scientific collections and it could easily be that morphological variation was therefore overlooked until now. Anecdotal observations on the sexual dimorphism of *B. baska* suggest that morphological variation exists, supporting the assumption that *B. baska* may be a collective term for at least two distinct, large estuarine turtle species. Males from the Sundarbans and presumably also from Myanmar were described as having a pale bluish nose, deep black heads and necks, passing into a rich crimson on the base of the neck, and brilliant rosy carmine-coloured forelimbs during breeding; 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). Our known-locality samples of *B. baska* from the Sundarbans Region, which may be identified with the type locality 'India' of *B. baska* (Gray 1831), indicate that the species from Indonesia and Malaysia needs to be nomenclaturally distinguished from *B. baska*. In the synonymy of *B. baska* exist several candidates for naming the other

species. Further research is needed however, as it is unclear to which species the descriptions of *Trionyx (Tetraonyx) cuvieri* Gray, 1831 (unknown type locality) and *Tetraonyx longicollis* Lesson, 1834 (type locality of Ayeyarwady River, Pegu, Myanmar) refer. The description of another possible candidate, *Tetraonyx affinis* Cantor, 1847 (type locality of Penang, Malaysia), was based on syntypes comprising specimens of *B. baska* sensu lato and *C. borneoensis* (Fritz & Havaš 2007), demanding the fixation of a lectotype.

All species in the clade comprising *B. baska* sensu lato, *C. borneoensis*, *K. dbongoka*, *K. kachuga* and *K. trivittata* are morphologically similar, large-sized riverine species with a similar natural history, and all except *K. dbongoka* share the for chelonians highly unusual sexual dichromatism, being important for mate choice, a character that may be regarded as a synapomorphy. Another shared sexually dimorphic character is that the costo-peripheral shell fontanelles in males never close but remain open throughout life (Moll 1980, 1986; E. O. Moll, pers. comm.). Obviously, the recognition of *Kachuga* besides the two monotypic genera *Batagur* and *Callagur* has rather historical than phylogenetic reasons and we conclude that a genus containing all species of the former genera *Batagur*, *Callagur* and *Kachuga* is a much more appropriate classification reflecting their phylogenetic relationships. Therefore, we propose placing all species into the genus *Batagur* Gray, 1856. This name takes precedence over the simultaneously published name *Kachuga* Gray, 1856 (because *Kachuga* was originally erected as subgenus of *Batagur*; ICZN 1999: Art. 61.2.1, precedence of taxon at higher rank) as well as over the younger name *Callagur* Gray, 1870 (ICZN 1999: Art. 23.1, principle of priority). We believe that this classification is superior to the phylogenetically also correct, but less parsimonious recognition of two genera, one for *B. baska* sensu lato plus *K. kachuga*, and another for *C. borneoensis* plus *K. dbongoka* and *K. trivittata* that would reflect the subclades within *Batagur* sensu lato. Nevertheless, it needs to be pointed out that the subclades are also supported by morphological evidence. The triturating surfaces of the jaws in *B. baska* sensu lato and *K. kachuga* have two denticulated ridges, in the other species one. Moreover, males of *C. borneoensis*, *K. dbongoka* and *K. trivittata* have striped shells while males of *B. baska* sensu lato and *K. kachuga* lack shell striping (Ernst *et al.* 2000; E. O. Moll, pers. comm.).

Our investigation provides a stable phylogenetic hypothesis for all *Pangshura* species, with *P. smithii* + *P. tentoria* being sister species and *P. tecta* and *P. sylhetensis* their successive sister-taxa. Uncertainties exist with respect to the phylogenetic position of *H. thurjii* that is suggested either as sister-taxon of *Pangshura* (BA, ML) or sister to a clade containing *Pangshura* + *Batagur* sensu lato (MP, NJ).

Within *Pangshura* and *Hardella* we found only weak support for the currently recognized subspecies; only most

sequences of *P. t. circumdata* correspond with a moderately supported clade within *P. tentoria*. On the other hand, a similar degree of mtDNA differentiation also occurs in the population of *P. t. tentoria* from the Mahanadi River that is not paralleled by a morphological segregation, while no differentiation was found between other populations of *P. t. tentoria* and *P. t. flaviventer*. Ancestral polymorphism (incomplete lineage sorting) could contribute to the lacking congruence between morphologically defined subspecies and mtDNA clades. However, at least the validity of the two subspecies of *H. thurjii* is also only very weakly supported from morphology, and purported differences exist only in the extent of carapace keeling. The subspecies *H. t. thurjii* from the Ganges and Brahmaputra drainage system has only a weak vertebral keel, whereas *H. t. indi* from the Indus River system is said to have two additional weak lateral keels on the pleural scutes (McDowell 1964; Ernst & Barbour 1989; Das 1991, 1995, 2001; Ernst *et al.* 2000). Such differences typically relate to different ontogenetic stages in geoemydid terrapins, with older individuals losing the lateral keels. Moreover, the supposed character state of *H. t. indi* seems to be entirely based on an observation by McDowell (1964), who studied only three specimens of *H. t. indi* of unknown age, and the original description of *H. t. indi* is contradictory ('thorax [= shell] less obscurely three-keeled' [than in *H. t. thurjii*]; Gray 1870: 58). Considering that *H. t. indi* has no unique haplotypes and that haplotypes within *H. thurjii* differ at best in two mutation steps (corresponding with an uncorrected *p* distance of only 0.1% for the two subspecies; Table 2), a continued usage of subspecies within *H. thurjii* seems not appropriate. Morphological differences between the subspecies of *P. smithii* and *P. tentoria* are much more pronounced, although only colouration and pattern characters are concerned. Both of the latter species comprise a subspecies, *P. s. pallidipes* and *P. t. flaviventer*, each of which is characterized by a pale colouration, including a uniform yellow instead of an intensely spotted plastron. A third subspecies within *P. tentoria*, *P. t. circumdata*, differs from *P. t. tentoria* and *P. t. flaviventer* by the presence of a distinct pink to reddish ring at the pleuro-marginal juncture of the carapace, distinct neck stripes and a red postorbital mark (Mertens 1969; Moll 1987).

Moll (1987) assigned pale-coloured *P. tentoria* from the Ghaghra River (Uttar Pradesh, India) to an intergrade population between *P. t. circumdata* and *P. t. flaviventer* because pink shell and head markings may also occur there. *Pangshura smithii* and *P. tentoria* live in this river syntopically (Moll 1987; own observation of Peter and Reiner Prasad). While we are able to confirm that *P. tentoria* from this river morphologically agree well with Moll's appraisal, we discovered there shared haplotypes in *P. smithii* and *P. tentoria*. Incomplete lineage sorting seems unlikely as explanation when the highly distinct and otherwise strictly species-specific haplotypes are

considered, differing in at least 58 mutation steps (Fig. 2) and an uncorrected average *p* distance of 5.68%, compared to 0.14% within *P. smithii* haplotypes and 0.16% within *P. tentoria* haplotypes. This suggests not only that interspecific hybridization may happen in syntopically occurring *P. smithii* and *P. tentoria*, but that the pale colouration of *P. tentoria* in the Ghaghra River may rather be the result of hybridization with the pale coloured *P. smithii pallidipes*, and not of subspecific intergradation with also pale coloured *P. t. flaviventer*.

## Conclusions

Our mtDNA data underline that phylogeny and diversity in South and South-east Asian turtles is badly understood and further sampling, especially in the large riverine species is in dire need, also for developing effective conservation strategies. The large riverine turtles from South and South-east Asia constitute a species complex that should be assigned to the genus *Batagur* Gray, 1856 and that comprises besides *B. baska* (Gray, 1831) the species *B. borneoensis* (Schlegel & Müller, 1844), *B. dbongoka* (Gray, 1835), *B. kachuga* (Gray, 1831) and *B. trivittata* (Duméril & Bibron, 1835), as well as one additional species allied to *B. baska* and *B. kachuga* occurring in Indonesia and Malaysia. *Hardella* and *Pangsbura* represent two distinct genera with one and four well-supported species, respectively. Distinctness of the subspecies within *H. thurjii*, *P. smithii* and *P. tentoria* is badly supported by mtDNA data however. Because definite diagnostic morphological characters are lacking, we suggest abandoning the recognition of the two subspecies within *H. thurjii*. Further, we provide evidence for hybridization of *P. smithii pallidipes* and *P. tentoria* in the Ghaghra River (Uttar Pradesh, India). This underlines the necessity to use nuclear genomic markers in future studies.

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## Appendix 1

Studied samples and sequences downloaded from GenBank. Abbreviations: AMCC, Ambrose Monell Cryo Collection at the American Museum of Natural History, New York; MTD D, Museum of Zoology Dresden, Herpetological Collection; MTD T — Museum of Zoology Dresden, Tissue Collection; NHMW, Natural History Museum Vienna, Herpetological Collection; ZSI, Zoological Survey of India, Kolkata (Calcutta). GenBank sequences were published by Barth *et al.* (2004) and Spinks *et al.* (2004).

Species	Origin	MTD T	Museum specimen	Accession number
<i>Batagur baska</i>	India: West Bengal: Sundarbans	3088	—	AM495267
<i>Batagur baska</i>	India: West Bengal: Sundarbans	3089	—	AM495268
<i>Batagur baska</i>	Bangladesh: Sundarbans	3123	—	AM495269
' <i>Batagur baska</i> '	Indonesia (confiscated specimen)	3788	—	AM691750
' <i>Batagur baska</i> '	Indonesia (confiscated specimen)	3789	—	AM691751
' <i>Batagur baska</i> '	Indonesia (confiscated specimen)	3790	—	AM691752
' <i>Batagur baska</i> '	Malaysia	3791	—	AM691753
' <i>Batagur baska</i> '	Malaysia	3792	—	AM691754
<i>Callagur borneoensis</i>	Unknown	3122	—	AM495270
<i>Callagur borneoensis</i>	Unknown	67	MTD D 43137	AM691755
<i>Callagur borneoensis</i>	Unknown	404	MTD D 44004	AM691756
<i>Callagur borneoensis</i>	Unknown	1187	MTD D 45366	AM691757
<i>Hardella thurjii thurjii</i>	India: Uttar Pradesh: Gompti River	3090	—	AM495271
<i>Hardella thurjii thurjii</i>	India: Uttar Pradesh: Gompti River	3094	—	AM495272
<i>Hardella thurjii thurjii</i>	India: West Bengal: Kolkata (Calcutta)	3549	ZSI	AM495273
<i>Hardella thurjii thurjii</i>	Bangladesh: Dhaka (Dacca)	3155	—	AM495274
<i>Hardella thurjii indi</i>	Pakistan: Indus River System	3150	NHMW 29289 : 6	AM495275
<i>Hardella thurjii indi</i>	Pakistan: Indus River System	3151	NHMW 29289 : 1	AM495277
<i>Hardella thurjii indi</i>	Pakistan: Indus River System	3152	NHMW 29289 : 3	AM495278
<i>Hardella thurjii indi</i>	Pakistan: Indus River System	3153	NHMW 29289 : 2	AM495279
<i>Hardella thurjii indi</i>	Pakistan: Indus River System	3154	NHMW 29289 : 4	AM495280
<i>Kachuga dhongoka</i>	India: Uttar Pradesh: Chambal River	3080	—	AM495281
<i>Kachuga dhongoka</i>	India: Uttar Pradesh: Chambal River	3081	—	AM495282
<i>Kachuga dhongoka</i>	India: Uttar Pradesh: Chambal River	3093	—	AM495283
<i>Kachuga kachuga</i>	India: Uttar Pradesh: Chambal River	3091	—	AM495284
<i>Kachuga kachuga</i>	India: Uttar Pradesh: Chambal River	3092	—	AM495285
<i>Kachuga kachuga</i>	India: Uttar Pradesh: Chambal River	3095	—	AM495286
<i>Kachuga kachuga</i>	India: West Bengal: Howrah Market	3098	—	AM495287
<i>Kachuga trivittata</i>	Unknown	3793	AMCC 164926	AM691758
<i>Pangshura smithii smithii</i>	Pakistan: Indus River System	3147	NHMW 33310 : 3	AM495288
<i>Pangshura smithii smithii</i>	India: Assam: Tezpur: Brahmaputra River	3125	—	AM495289
<i>Pangshura smithii smithii</i>	India: Assam: Tezpur: Brahmaputra River	3126	—	AM495290
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	3110	—	AM495291
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	3111	—	AM495292
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	3112	—	AM495293
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	3113	—	AM495294
<i>Pangshura smithii pallidipes</i>	India: Uttar Pradesh: Ghaghra River	3116	—	AM495295
<i>Pangshura sylhetensis</i>	India: Assam: Jia Bhoroli River	3135	—	AM495296
<i>Pangshura sylhetensis</i>	India: Assam: Jia Bhoroli River	3138	—	AM495297
<i>Pangshura sylhetensis</i>	India: Assam: Jia Bhoroli River	3139	—	AM495298
<i>Pangshura sylhetensis</i>	India: Assam: Jia Bhoroli River	3140	—	AM495299
<i>Pangshura sylhetensis</i>	India: Assam: Jia Bhoroli River	3141	—	AM495300
<i>Pangshura tecta</i>	Pakistan: Indus River System	3148	NHMW 33311 : 1	AM495301
<i>Pangshura tecta</i>	India: Maharashtra: Pune	3101	—	AM495302
<i>Pangshura tecta</i>	India: Uttar Pradesh: Gompti River	3085	—	AM495303
<i>Pangshura tecta</i>	India: Uttar Pradesh: Gompti River	3086	—	AM495304
<i>Pangshura tecta</i>	Bangladesh: Sunargon Market	3117	—	AM495305
<i>Pangshura tecta</i>	Bangladesh: Sunargon Market	3118	—	AM495306
<i>Pangshura tecta</i>	Bangladesh: Sunargon Market	3119	—	AM495307
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	3124	—	AM495308
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	3127	—	AM495309
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	3128	—	AM495310
<i>Pangshura tentoria tentoria</i>	India: Assam: Tezpur: Brahmaputra River	3129	—	AM495311
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	3106	—	AM495312



Appendix 1 *Continued.*

Species	Origin	MTD T	Museum specimen	Accession number
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	3107	—	AM495313
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	3108	—	AM495314
<i>Pangshura tentoria tentoria</i>	India: Orissa: Mahanadi River	3109	—	AM495315
<i>Pangshura tentoria circumdata</i>	Pakistan: Indus River System	3144	NHMW 33312 : 1	AM495316
<i>Pangshura tentoria circumdata</i>	Pakistan: Indus River System	3145	NHMW 33312 : 2	AM495317
<i>Pangshura tentoria circumdata</i>	India: Madhya Pradesh: Chambal River	3120	—	AM495318
<i>Pangshura tentoria circumdata</i>	India: Madhya Pradesh: Chambal River	3121	—	AM495319
<i>Pangshura tentoria circumdata</i>	India: Uttar Pradesh: Gompti River	3082	—	AM495320
<i>Pangshura tentoria circumdata</i>	India: Uttar Pradesh: Gompti River	3083	—	AM495321
<i>Pangshura tentoria circumdata</i>	India: Uttar Pradesh: Gompti River	3084	—	AM495322
<i>Pangshura tentoria flaviventer</i>	Nepal: Sapta Khosi River	3132	—	AM495323
<i>Pangshura tentoria flaviventer</i>	Nepal: Sapta Khosi River	3133	—	AM495324
<i>Pangshura tentoria flaviventer</i>	Nepal: Sapta Khosi River	3134	—	AM495325
<i>Pangshura tentoria flaviventer</i>	Bangladesh: Sunargon Market	3130	—	AM495326
<i>Pangshura tentoria circumdata</i> × <i>Pangshura tentoria flaviventer</i>	India: Uttar Pradesh: Ghaghra River	3114	—	AM495327
<i>Pangshura tentoria circumdata</i> × <i>Pangshura tentoria flaviventer</i>	India: Uttar Pradesh: Ghaghra River	3115	—	AM495328
GenBank sequences				
'Batagur baska'	Unknown	—	—	AY434600
<i>Callagur borneoensis</i>	Unknown	—	—	AY434601
<i>Hardella thurjii thurjii</i>	Unknown	—	—	AY434603
<i>Kachuga dhongoka</i>	Unknown	—	MTD D 42577	AJ564452
<i>Kachuga dhongoka</i>	Unknown	—	—	AY434569
<i>Pangshura smithii smithii</i>	Unknown	—	—	AY434589
<i>Pangshura tentoria circumdata</i>	Unknown	—	—	AY434610
<i>Pangshura tentoria flaviventer</i>	Unknown	—	—	AY434612
<i>Pangshura tecta</i>	Unknown	—	—	AY434583
<i>Geoclemys hamiltonii</i>	Unknown	—	—	AY434573
<i>Morenia ocellata</i>	Unknown	—	—	AY434605