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Further specimens and phylogenetic position of the recently described leaf turtle species *Cyclemys gemeli* (Testudines: Geoemydidae)

PETER PRASCHAG[1], ANNA K. HUNDSDÖRFER[2] & UWE FRITZ[2,3]

[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, A. B. Meyer Building, D-01109 Dresden, Germany. E-mails: anna.hundsdoerfer@snsd.smwk.sachsen.de, uwe.fritz@snsd.smwk.sachsen.de*
[3]*Corresponding author*

Abstract

We describe external morphology and habitat of ten specimens of *Cyclemys gemeli*, a recently discovered leaf turtle species from north-eastern India, previously known only from its incomplete holotype and photos of a live female. Further, we assess the phylogenetic position of *C. gemeli* using sequence data of the mitochondrial cytochrome *b* gene as well as of three nuclear DNA fragments (C-mos, Rag2 genes, intron 1 of R35 gene) and confirm its genetic distinctiveness. Mitochondrial data strongly suggest a sister group relationship of *C. gemeli* and *C. fusca*, another species occurring in Myanmar. According to our new records, the Naga Hills and the Arakan Mts could constitute the geographical divide between *C. gemeli* and *C. fusca*. Morphologically, *C. gemeli* resembles other dark-bellied *Cyclemys* species and determination by external morphology alone is quite difficult.

Key words: India, Myanmar, *Cyclemys fusca*, *Cyclemys gemeli*, molecular phylogenetics

Introduction

The Southeast Asian leaf turtle genus *Cyclemys* Bell, 1834 is a morphologically difficult group comprising cryptic species. A recent investigation using mitochondrial, nuclear genomic and morphological data revealed the existence of seven species, three of which were described as new for science (Fritz *et al.* 2008). The description of one of the new species, *C. gemeli*, was based only on an incomplete bony shell, sequence data of its mitochondrial cytochrome *b* gene (cyt *b*) and photos of another live turtle from Assam, India. No nuclear genomic data were available. In the present paper, we publish photos of the live specimen mentioned in Fritz *et al.* (2008) and data of nine additional live leaf turtles from north-eastern India. Further, we use saliva samples of four of these individuals for sequencing the mitochondrial cyt *b* gene and the three nuclear DNA fragments (C-mos, Rag2 genes, intron 1 of R35 gene) utilized for phylogenetic reconstructions in Fritz *et al.* (2008) in order to assess their taxonomic and phylogenetic position. We also provide some observations on collection sites and distribution range.

Material and methods

Sampling. During field work in the states of Assam, Meghalaya and Nagaland (north-eastern India), the senior author had the opportunity to study 10 juvenile and adult leaf turtles and to obtain salivary samples (oral swabs) of four specimens for later genetic investigation. Two turtles were kept by locals, while the others were field-collected. Of each turtle, photographs were taken and straight-line carapacial lengths were measured using a calliper (Table 1). Unfortunately the exact measurements of three turtles studied in 2007 were lost on journey.

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Laboratory procedures. Saliva samples were kept frozen at -20°C until processing. DNA extraction, amplification and sequencing of the mitochondrial cyt *b* gene followed Praschag *et al.* (2007) and Fritz *et al.* (2008). Nuclear genomic sequences of the C-mos and Rag2 genes were obtained using PCR protocols and primers Cmos1, Cmos3, F2, and R2-1 of Le *et al.* (2006). Sequencing was performed on an ABI 3130 (Applied Biosystems) using the forward primers Cmos1 and F2. The R35 intron was amplified with the primers R35Ex1 and R35Ex2 (Fujita *et al.* 2004); however, direct sequencing was not possible for this fragment. Therefore, PCR products were extracted from agarose gels using the peqGOLD Gel Extraction Kit (peqlab) and cloned (TOPO TA Cloning Kit, Invitrogen). The transformation was successful for three individuals (MTDT 5055, 5057-58; MTDT = Museum of Zoology Dresden, tissue collection). Six colonies per individual were picked and grown in liquid medium. After plasmid isolation using the peqGOLD Plasmid Miniprep Kit (peqlab), the inserts were sequenced in both directions using the standard primers M13 Forward (-20) und M13 Reverse (Invitrogen). The sequences obtained from different colonies of PCR products of the same individual showed singletons at up to eight sites that were coded as ambiguities. Accession numbers of sequences obtained in this study are FM877759-FM877773.

TABLE 1. Examined leaf turtles from north-eastern India. Asterisks indicate that saliva samples were taken and analyzed genetically. Locality numbers refer to Fig. 1. Guwahati is also frequently spelled as Gauhati.

Age class and sex	Locality	Carapace length (mm)	Date
Adult female	1 – Assam: Nameri National Park: Jia Bhoroli River region, 35 km to Tezpur	232.0	4 Nov 1998
Subadult*	1 – Assam: Nameri National Park: Jia Bhoroli River region, 35 km to Tezpur	152.3	9 Nov 2007
Juvenile	1 – Assam: Nameri National Park: Jia Bhoroli River region, 35 km to Tezpur	106.4	26 Oct 1999
Adult male	2 – Assam: SE Goalpara: Damra village, close to Meghalayan border	approx. 190	19 Nov 2007
Adult female	2 – Assam: SE Goalpara: Damra village, close to Meghalayan border	approx. 210–220	19 Nov 2007
Juvenile	3 – Meghalaya: between Guwahati and Shillong, near Barni Hat	80.4	10 Nov 1999
Juvenile	3 – Meghalaya: between Guwahati and Shillong, near Barni Hat	101.7	10 Nov 1999
Adult male*	4 – Nagaland: Dimapur	approx. 180–190	13 Nov 2007
Juvenile*	4 – Nagaland: Dimapur	95.2	13 Nov 2007
Juvenile*	5 – Nagaland: between Amguri and Mokokchung: Tuli village, close to Assamese border	65.2	15 Nov 2007

Phylogenetic analyses. Sequences of the Indian *Cyclemys* were collapsed manually into haplotypes and identical sequences were excluded from further analysis. Mitochondrial and nuclear gene sequences were analysed separately using the same outgroups as in Fritz *et al.* (2008), *Heosemys spinosa* and *Leucocephalon yuwonoi*. For phylogenetic analyses of the mitochondrial cyt *b* gene (984 bp), we selected from the data set of Fritz *et al.* (2008) two sequences of each other *Cyclemys* species and the sequence of the putative *C. fusca* x *C. oldhamii* hybrid. The concatenated nuclear gene sequences of MTDT 5055, 5057 and 5058 (C-mos: 573 bp; Rag2: 628 bp; R35 intron: 1075 bp) were added to the respective data set of Fritz *et al.* (2008). For MTDT 5056, no R35 sequence was available and its C-mos and Rag2 fragments were identical to at least one of those of MTDT 5055, 5057 or 5058, providing no extra phylogenetic information, which is why we excluded the C-mos and Rag2 sequences of MTDT 5056 from analysis.

Data were analysed in PAUP* 4.0b10 (Swofford 2002) under the optimality criteria Maximum Parsimony (MP; equal weighting; commands: hs add = cl) and Maximum Likelihood (ML), as well as with Bayesian inference of phylogeny (BA) using MrBayes 3.1 (Ronquist & Huelsenbeck 2003; settings: ngen = 10000000 sample = 500 temp = 0.2 savebrlens = yes startingtree = random; burn-in set to sample only the plateau of

most likely trees). The best evolutionary model for each of the four fragments and the concatenated nuclear sequences was established in Modeltest 3.06 (Posada & Crandall 1998) using the Akaike information criterion (Table 2). For BA, concatenated nuclear sequences were analysed using mixed-model settings. For the cyt *b* gene, 720 of 984 aligned sites were constant and 182 characters were variable and parsimony-informative; 82 variable characters were parsimony-uninformative. For the ingroup taxa, 800 characters were constant, 168 variable characters were parsimony-informative, and 16 variable characters were parsimony-uninformative. The alignment of the three nuclear fragments comprised 2276 sites of which 2197 were constant; of 79 variable sites 26 were parsimony-informative. For the ingroup taxa, 2249 characters were constant; 13 variable characters were parsimony-informative and 14 were singletons. Bootstrap support was calculated in PAUP* 4.0b10 (settings nreps = 1000, maxtre = 1000 for MP and nreps = 100, maxtre = 1 for ML).

TABLE 2. Best-fit substitution models (AIC) and their parameters, established using Modeltest 3.06 (Posada & Crandall 1998).

Fragment	Model	Nst (and TRatio or Rmat)	Rates	Shape	Pinvar
C-mos	TVM	6 (Rmat = 0.2903 1.8900 1.3932 0.000000001 1.8900)	equal	n.a.	0
Rag2	HKY	2 (TRatio = 3.8923)	equal	n.a.	0
R35	TrN+I	6 (Rmat = 1.0000 8.2319 1.0000 1.0000 2.3543)	equal	n.a.	0.8070
all nuclear	TrN+I	6 (Rmat = 1.0000 5.7916 1.0000 1.0000 4.1223)	equal	n.a.	0.7277
cyt *b*	TVM+G	6 (Rmat = 1.2183 12.8010 0.8150 0.00000001 12.8010)	gamma	0.2127	0

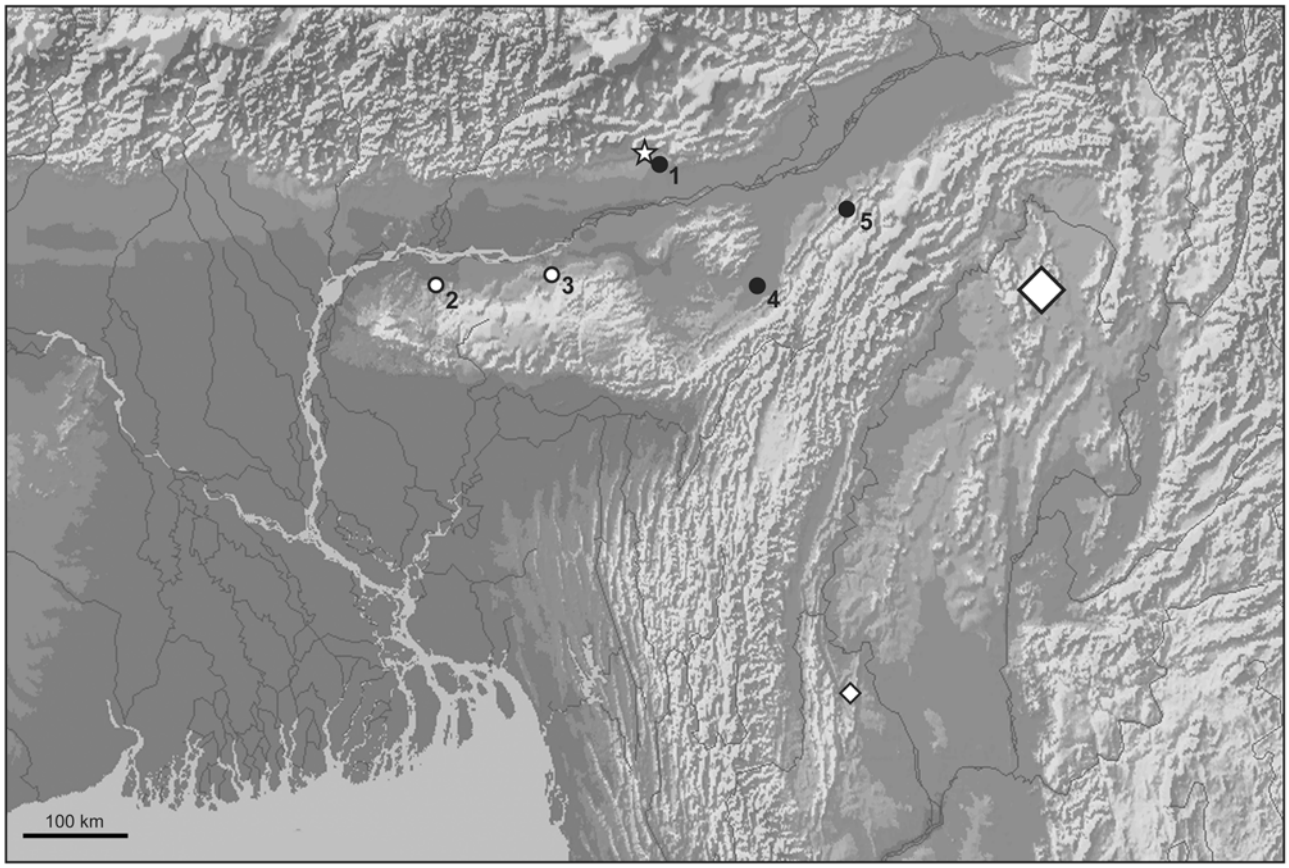

FIGURE 1. Records for *Cyclemys* in north-eastern India and adjacent Myanmar. Star indicates type locality of *Cyclemys gemeli* (Assam: street from Tezpur to Arunachal Pradesh, 5 km to border of Arunachal Pradesh; Fritz *et al.* 2008); open circles, observation sites; closed circles, genetically verified new records of *C. gemeli*. Numbers refer to Table 1. Diamonds represent genetically verified records of *C. fusca* in Myanmar (large symbol corresponds with 'Kachin state'; Fritz *et al.* 2008).

Results and discussion

Species identification and phylogeny. The mitochondrial sequences of our four Indian *Cyclemys* samples are very similar and represent three distinct haplotypes (A–C) differing in two sites only. Haplotype C is identical with the sequence of the holotype of *C. gemeli* (AM931656; Fritz *et al.* 2008), suggesting that the four *Cyclemys* belong to this species. When it is considered that the other six studied leaf turtles originate from the same localities or from localities in close proximity, it seems likely that all ten specimens represent *C. gemeli*. Our new records suggest that the Naga Hills, and to the south the Arakan Mts, constitute the divide between *Cyclemys gemeli* and *C. fusca* and that all *Cyclemys* records west of this mountain chain, including those from Nepal, West Bengal and Bangladesh (Das 1991), represent *C. gemeli* (Fig. 1).

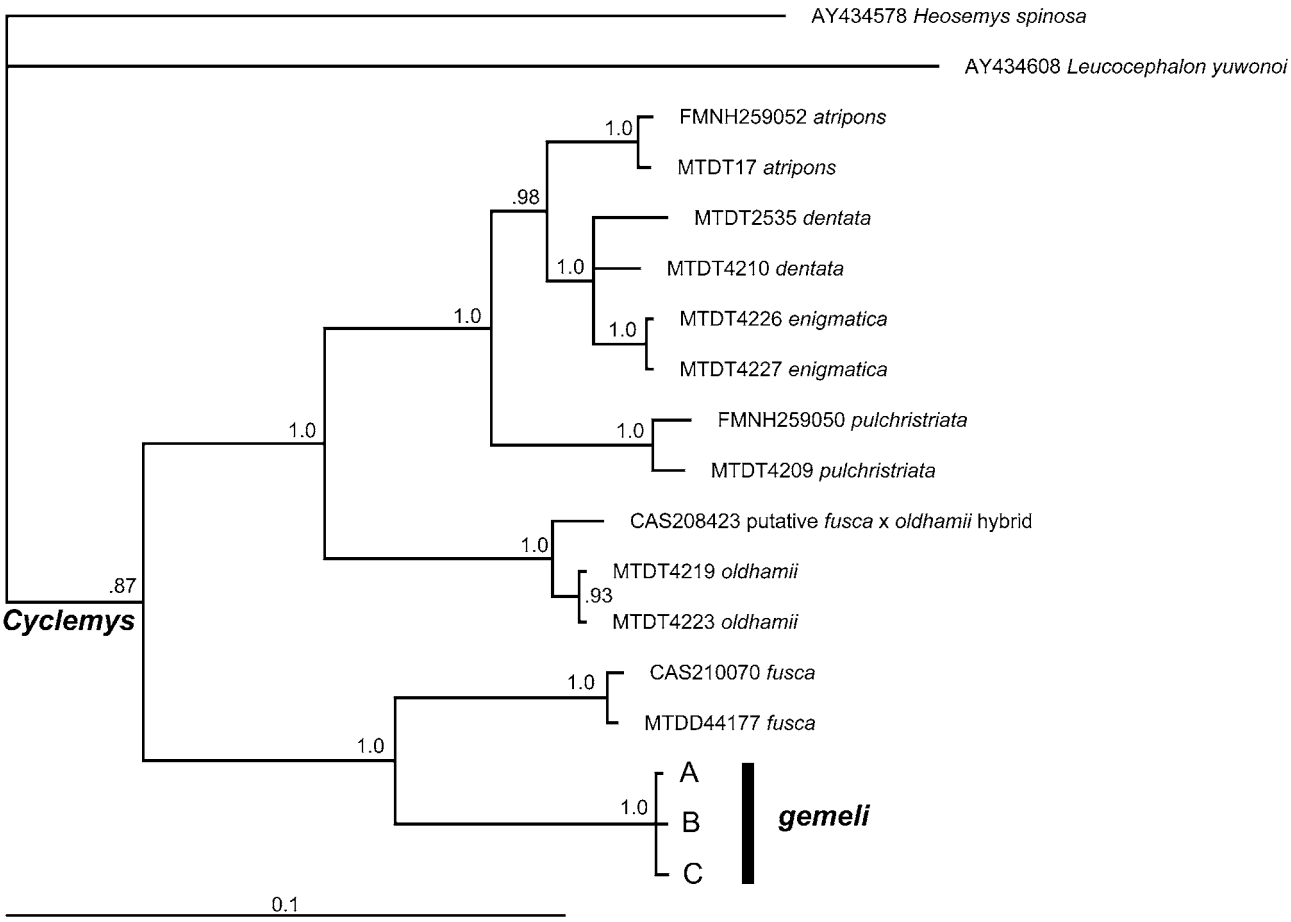

FIGURE 2. Phylogenetic hypothesis for *Cyclemys* species as revealed by Bayesian analysis of 984 bp of the mitochondrial cyt *b* gene. Numbers preceding species names are GenBank accession numbers or voucher numbers (CAS—California Academy of Sciences; FMNH—Field Museum of Natural History; MTDT—Museum of Zoology Dresden, tissue collection). A, B, C denote haplotypes of *C. gemeli*. Haplotype A was identified in two samples (MTDT 5055-5056). The cyt *b* sequence of the type specimen of *C. gemeli* (AM931656; Fritz *et al.* 2008) is identical with haplotype C. Numbers along nodes indicate posterior probabilities. Under ML and MP, bootstrap support of each note is 100%. Note that *C. enigmatica* clusters within *C. dentata*, suggesting introgressive hybridization (for details, see Fritz *et al.* 2008).

For the mitochondrial cyt *b* gene, all tree-building methods revealed the same general topology (Fig. 2), confirming *C. gemeli* as sister species of *C. fusca*. Under parsimony, two equally parsimonious trees were obtained (417 steps; CI = 0.7218, RI = 0.8289), differing only in the branching pattern of the three *C. gemeli* haplotypes. The log likelihood value of the only most likely tree is -ln *L* = 3263.9195.

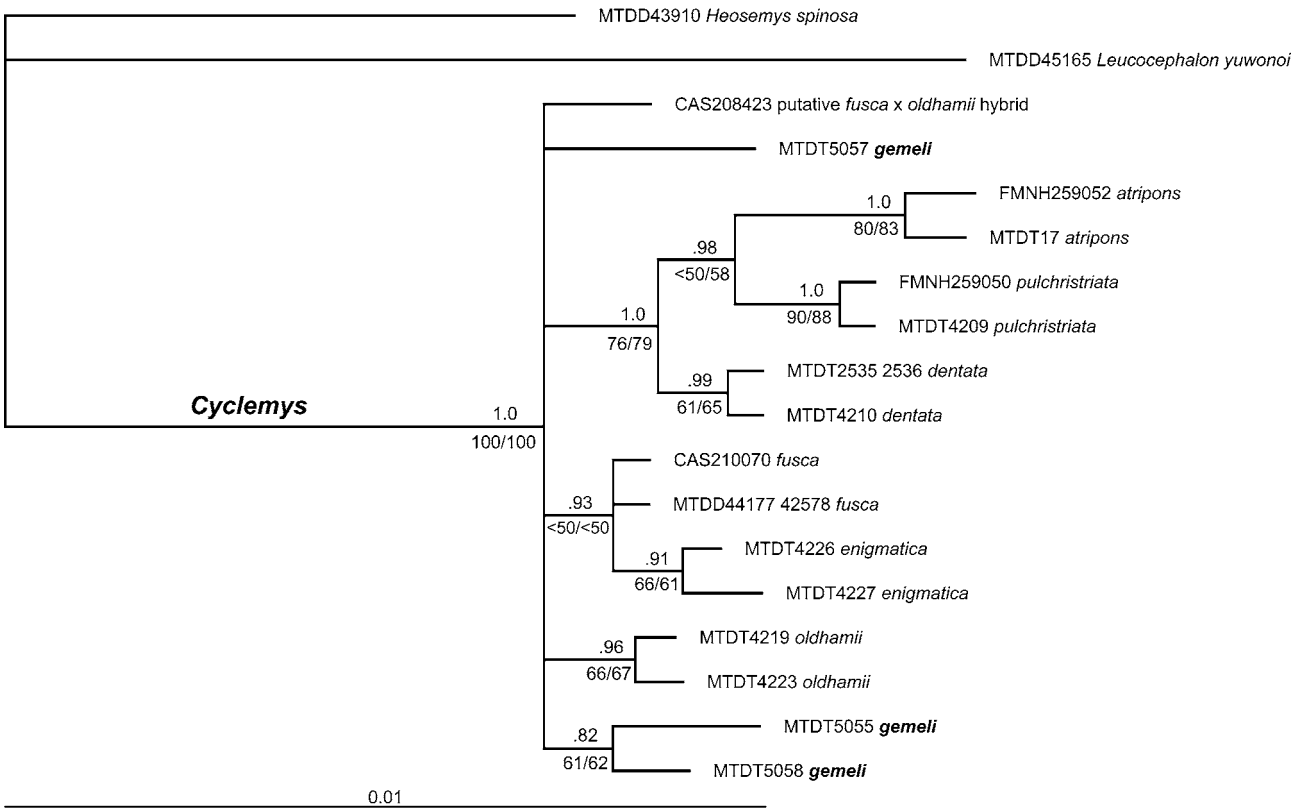

FIGURE 3. Fifty-percent majority rule tree based on mixed-model Bayesian analysis of three nuclear DNA fragments (C-mos: 573 bp; Rag2: 628 bp; R35 intron: 1075 bp) of leaf turtles (*Cyclemys*). Numbers above nodes are posterior probabilities; below nodes, ML and MP bootstrap values. For further explanations, see Fig. 2 and text.

The resolution of the trees obtained from the three nuclear DNA fragments is much worse, reflecting a weak phylogenetic signal with respect to the dark-bellied species. The three yellow-bellied species *C. atripons*, *C. dentata* and *C. pulchristriata* represent a well-supported clade, however, that is located in a basal polytomy with the dark-bellied species and the putative hybrid of the two dark-bellied species *C. fusca* and *C. oldhamii*. Two nDNA sequences of *C. gemeli* are consistently placed in the same clade within the basal multifurcation; the third occurs as distinct basal branch. The topology of the Bayesian tree (Fig. 3) is in perfect agreement with the only most likely tree (-ln *L* = 3738.3693) and the 50% majority rule and strict consensus trees of 163 equally parsimonious trees (83 steps; CI = 0.9639, RI = 0.9211). Only 28 of the 163 equally parsimonious trees (17%) suggest a reciprocal monophyly of all dark-bellied and all yellow-bellied species. Even though the nuclear data set did not result in a well-resolved phylogeny, it provides evidence that *C. gemeli* is distinct at the nuclear genomic level.

Morphology. In adult shell coloration and pattern, *Cyclemys gemeli* resembles the other dark-bellied *Cyclemys* species. Moreover, like in the other dark-bellied species the interfemoral seam is equal to or longer than the length of the interanal seam in adult specimens, implying that species determination by external morphology alone is quite difficult.

All studied adult *C. gemeli* have elongated, relatively flat shells. Compared with the most closely related species, *C. fusca* from Myanmar, the shell outline is somewhat more elongated, with nearly parallel sides, and the carapace is less domed (compare photos of *C. fusca* in Fritz *et al.* 2008). In *C. gemeli* a plastral hinge develops with increasing age, like in the six other *Cyclemys* species. It eventually divides the abdominal scutes, resulting in small triangular extra scutes positioned between the pectoralia and abdominalia. Unlike *C. oldhamii*, the top of the head is uniformly coloured, but not distinctively lighter than the temporal region, as in *C. enigmatica* and *C. fusca*. The temporal region and the neck of adult *C. gemeli* are more or less uniformly

FIGURE 4. *Cyclemys gemeli*: (a) dorsal and (b) ventral aspect of small juvenile (65.2 mm carapace length), Tuli, Nagaland; (c) dorsal and (d) ventral aspect of juvenile (101.7 mm carapace length), between Guwahati and Shillong, near Barni Hat, Meghalaya; (e) head and neck pattern of same turtle; (f) lateral and (g) ventral aspect of adult male (approx. 180–190 mm carapace length), Dimapur, Nagaland; (h) head and neck pattern of same male.

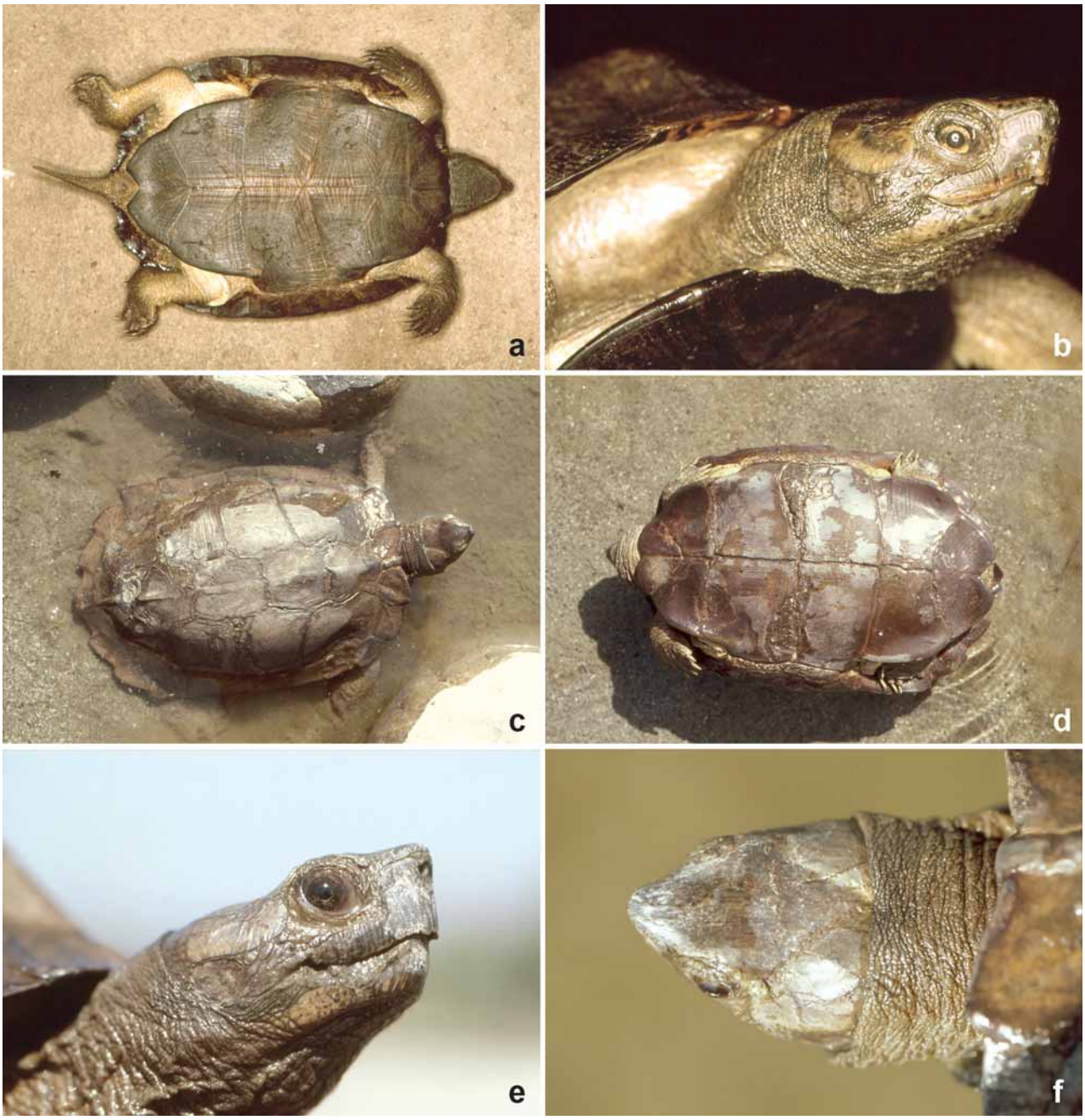

FIGURE 5. *Cyclemys gemeli*: (a) ventral aspect of subadult turtle (152.3 mm carapace length), Jia Bhoroli River region, 35 km to Tezpur, Nameri National Park, Assam; (b) head and neck pattern of same individual; (c) dorsal aspect of adult female (232.0 mm carapace length), Jia Bhoroli River region, 35 km to Tezpur, Nameri National Park, Assam; (d) ventral aspect of same female, note the small triangular extra scutes between pectoralia and abdominalia due to a secondary division of the abdominalia by the plastral hinge; (e) lateral and (f) dorsal head and neck pattern of same female. The turtle depicted in (c)–(e) is the individual mentioned in the original description of *C. gemeli* (Fritz *et al.* 2008).

dark brown. A striped pattern is present in the juveniles, however, suggesting that it is lost during growth. The smallest juvenile (65.2 mm carapace length; Tuli, Nagaland) has a uniformly reddish brown carapace. On its plastron, diffuse large spots are present. It seems likely that hatchlings have a similar coloration and pattern. In the larger juveniles, carapace and plastron bear a black radiating pattern that obviously gets denser and more extensive with increasing body size, eventually resulting in the uniformly black or blackish brown adult plastral coloration (Figs 4–5).

Notes on habitat. Eight of the studied *Cyclemys gemeli* were hand-collected by the senior author and released after taking measurements, photographs and, in part, salivary samples (Table 1). All turtles were found in the water.

The three specimens from the Jia Bhoroli River (Assam), a tributary of the Brahmaputra, were caught in the river itself and in one of its oxbows. The subadult turtle originates from a site where *Pangshura sylhetensis* and *Nilssonia nigricans* occur as well (Fig. 6). It was captured by diving in approximately 1.5 m depth. At the peak of the dry season, the water is here so clear that the structure of the river bottom is still visible in this depth. Due to fast water flow, high oxygen contents, and low water temperature, the fish fauna differs much from that of the Brahmaputra, as indicated by the occurrence of cyprinids such as *Tor tor*, *T. progeneius*, *T. putitora*, and *Neolissochilus* sp.. The river bottom consists of fine sand and round stones of various sizes. This gravel bed heats up strongly during the day with the effect that daily water temperature can fluctuate within a 3.5°C margin. The water level rises by several metres in the rainy season.

The two juveniles from Meghalaya and one of the juveniles from Nagaland (Tuli) were found in fast flowing creeks, while the other two Nagaland turtles were discovered during a flood in vicinity of Dimapur. According to the locals keeping the couple of adult leaf turtles from Damra village (Assam), these turtles were found basking along a small creek.

FIGURE 6. Jia Bhoroli River, Nameri National Park, Assam. Habitat of *Cyclemys gemeli*, *Pangshura sylhetensis* (Geoemydidae), and *Nilssonia nigricans* (Trionychidae).

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