Genetic evidence for wild-living *Aspideretes nigricans* and a molecular phylogeny of South Asian softshell turtles (Reptilia: Trionychidae: *Aspideretes, Nilssonia*)

P. Praschag, A. K. Hundsdörfer, A. H. M. A. Reza & U. Fritz

Praschag, P., Hundsdörfer, A. K., Reza, A. H. M. A. & Fritz, U. (2007). Genetic evidence for wild-living *Aspideretes nigricans* and a molecular phylogeny of South Asian softshell turtles (Reptilia: Trionychidae: *Aspideretes, Nilssonia*). — *Zoologica Scripta*, ******, *****–*****.

Aspideretes nigricans was long thought to be one of the rarest turtle species of the world, being restricted to a single site in Bangladesh; its specific distinctness was repeatedly doubted. Using mtDNA sequence data of all four *Aspideretes* species, we provide evidence that *A. nigricans* is a distinct species that is sister to *A. hurum*. Furthermore, *A. nigricans* is not endemic to Bangladesh, but also occurs in Assam, India. While all applied phylogenetic analyses (Bayesian Analysis, Maximum Likelihood, Maximum Parsimony, Neighbor Joining) strongly suggest a well-supported clade containing the four *Aspideretes* species and *Nilssonia formosa*, the monophyly of *Aspideretes* is at best weakly supported. We propose to synonymise the genera *Aspideretes* Hay, 1904 and *Nilssonia* Gray, 1872, resulting in an expanded genus *Nilssonia* with the species *N. formosa* (Gray, 1869), *N. gangetica* (Cuvier, 1825), *N. hurum* (Gray, 1831), *N. leithii* (Gray, 1872) and *N. nigricans* (Anderson, 1875). Genetic structure within *N. nigricans* is weak, while we detected two distinct genetic lineages within *N. gangetica*, one occurring in the Brahmaputra River system and the other in the Ganges and Indus River basins.

Peter Praschag, Am Katzelbach 98, A-8054 Graz, Austria. E-mail: peter@praschag.at

Anna K. Hundsdörfer, Uwe Fritz, Museum of Zoology (Museum für Tierkunde), Natural History State Collections Dresden, Königsbrücker Landstr. 159, D-01109 Dresden, Germany. E-mails: anna.hundsdoerfer@snsd.smwk.sachsen.de, uwe.fritz@snsd.smwk.sachsen.de

A. H. M. Ali Reza, Department of Natural Resources Management, Texas Tech University, Lubbock, TX 79401, USA and Department of Zoology, Jahangirnagar University, Savar, Dhaka, Bangladesh. E-mail: ali.reza@ttu.edu

Introduction

Softshell turtles (Trionychidae) are an ancient family of chelonians with a highly derived morphology, dating back at least to the Lower Cretaceous of Asia (Meylan & Gaffney 1992; Nessov 1995). The family comprises 30 extant species in 14 genera and is distributed in Africa, Asia, North America and New Guinea (Fritz & Havaš 2007). The shell of trionychid turtles is flattened and covered by a leathery skin instead of horny scutes; the bony shell is much reduced. Their neck is long and retractile, and limbs are paddle-like, with three strong claws on each. The snout is usually a long proboscis. All softshell turtles are highly aquatic and many species are vigorous predators of fish. External morphology is often difficult to use for taxonomic and phylogenetic purposes and much emphasis has been given to osteological characters (Meylan 1987; Ernst *et al.* 2000; Praschag & Gemel 2002).

The South Asian genus *Aspideretes* comprises four large-sized species (*Aspideretes gangeticus, A. hurum, A. leithii, A. nigricans*) with maximum shell lengths of 60–94 cm (Ernst *et al.* 2000). Until recently, one of these species, the Black Softshell Turtle (*A. nigricans*) was thought to be confined to the artificial pond at the Hazrat Sultan Bayazid Bostami Shrine in Nasirabad near Chittagong, Bangladesh. From this site, *A. nigricans* was described by Anderson (1875), and subsequent authors believed that the species either became extinct in the wild (Pritchard 1979; Groombridge 1982) or that it 'descended from introduced individuals of the more widespread *Trionyx* [now *Aspideretes*] *gangeticus*' (Groombridge 1982; Khan 1987) or of *A. hurum* (Rashid 1990). For a long time, *A. nigricans* was thought to be one of the world's most endangered chelonian species, comprising not more than 150–300 individuals in the semicaptive colony near Chittagong (Groombridge 1982;

Ahsan *et al.* 1991; Hilton-Taylor 2000). Due to its apparent rareness and the tale that the Muslim Saint Bayazid Bostami, when founding the turtle shrine in the year 830, turned the evil spirits of the site into turtles, *A. nigricans* became nationally and internationally renowned as the 'holy' turtle of Bangladesh (e.g., Pritchard 1979; Khan 1980; Groombridge 1982; Obst 1986; Ernst & Barbour 1989; Ernst *et al.* 2000). However, using mainly skull osteology as well as colouration and pattern characters, Praschag & Gemel (2002) suggested that *A. nigricans* occurs not only in the shrine pond near Chittagong, but also in the neighbouring Indian state of Assam.

In the present study we use for the first time mtDNA sequences of all four nominal *Aspideretes* species to address the following questions: (i) is *A. nigricans* a species genetically distinct from its three congeners or merely a morphological variant of *A. gangeticus* or *A. hurum*, and (ii) are samples of Assamese turtles morphologically assigned to *A. nigricans* distinct from Black Softshell Turtles from Chittagong, Bangladesh? Furthermore, we present a complete molecular phylogeny for *Aspideretes* and re-investigate its possible paraphyly with respect to *Nilssonia formosa*, as recently suggested by Engstrom *et al.* (2004).

Materials and methods
Sampling
Ten specimens of *Aspideretes gangeticus*, seven of *A. hurum*, two of *A. leithii* and 11 of *A. nigricans* were sampled by clipping off a small piece of the shell or extracting muscle tissue from carcasses. Six of our *A. nigricans* samples originated from the shrine pond near Chittagong, Bangladesh (type locality), four from the Kachapukhuri pond on Nilachal Hill, next to the Kamakhya Tantra Temple (near Gauhati), Assam, and one from a wild-caught turtle from Jia Bhoroli River, Assam (Fig. 1). The Jia Bhoroli is a northern tributary of the Brahmaputra River. In addition, one sequence each of *A. gangeticus* and *A. hurum* was downloaded from GenBank (Table 1). 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 ~300 bp), together comprising almost the complete cytochrome *b* gene (cyt *b*) and the adjacent portion of the
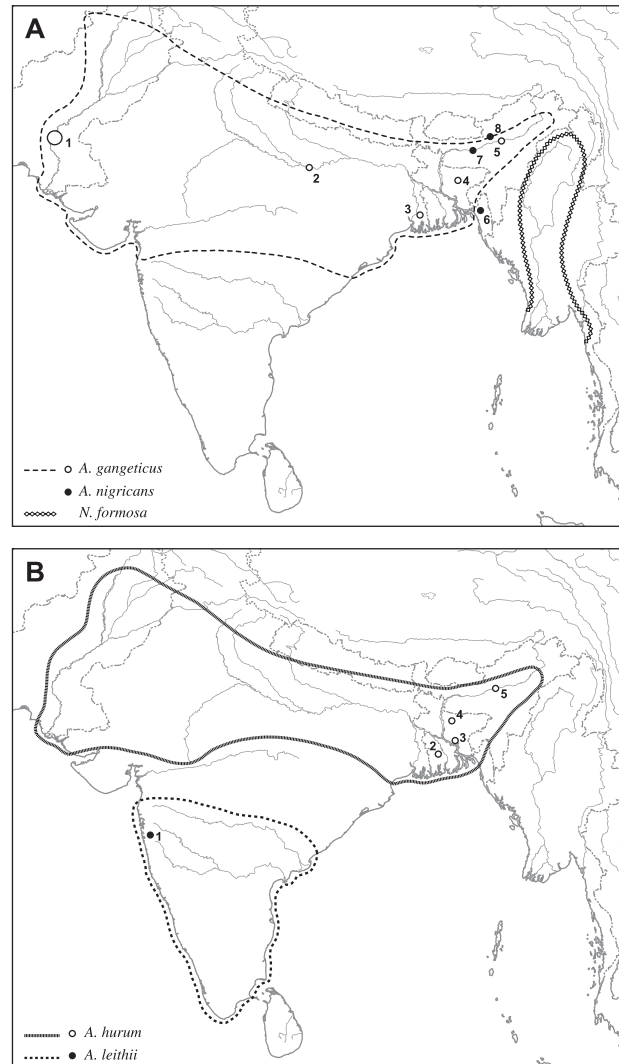
Fig. 1 A, B. Distribution of *Aspideretes* species and *Nilssonia formosa* and sampling sites. —A. *Aspideretes gangeticus*, *A. nigricans* and *Nilssonia formosa*: (1) large symbol indicates imprecise collection site along Indus River system, Pakistan, (2) Chambal, Uttar Pradesh, India, (3) Howrah, West Bengal, India, (4) Mymensingh, Bangladesh, (5) Biswanath, Assam, India, (6) Chittagong, Bangladesh, type locality of *A. nigricans*, (7) Kamakhya near Gauhati, Assam, India, (8) Jia Bhoroli River, Assam, India. —B. *Aspideretes hurum* and *A. leithii*: (1) Pawna River, Maharashtra, India, (2) Khulna, Bangladesh, (3) Sunargon, Bangladesh, (4) Mymensingh, Bangladesh, (5) Biswanath, Assam, India. Range borders of *A. gangeticus* according to Schneider & Djalal (1970), Das (1995, 2001) and Praschag & Gemel (2002); of *A. hurum* according to Iverson (1992) and Khan (2006); of *A. leithii* according to Das (1995, 2001); and of *N. formosa* according to Iverson (1992) and Kuchling *et al.* (2004).

Table 1 Studied *Aspideretes* samples and sequences downloaded from GenBank.

Species	Origin	MTD T	Museum specimen	Accession number
Aspideretes gangeticus (Cuvier, 1825)	Bangladesh: Mymensingh: Old Brahmaputra	3411	—	AM495208
Aspideretes gangeticus (Cuvier, 1825)	Bangladesh: Mymensingh: Old Brahmaputra	3412	—	AM495209
Aspideretes gangeticus (Cuvier, 1825)	Bangladesh: Mymensingh: Old Brahmaputra	3413	—	AM495210
Aspideretes gangeticus (Cuvier, 1825)	India: Assam: Biswanath	3136	—	AM495211
Aspideretes gangeticus (Cuvier, 1825)	India: Uttar Pradesh: Chambal River	3087	—	AM495212
Aspideretes gangeticus (Cuvier, 1825)	India: West Bengal: Howrah Market	3096	—	AM495213
Aspideretes gangeticus (Cuvier, 1825)	India: West Bengal: Howrah Market	3097	—	AM495214
Aspideretes gangeticus (Cuvier, 1825)	Pakistan	3401	NHMW 30265 : 5	AM495215
Aspideretes gangeticus (Cuvier, 1825)	Pakistan	3402	NHMW 30265 : 3	AM495216
Aspideretes gangeticus (Cuvier, 1825)	Pakistan	3403	NHMW 30265 : 2	AM495217
Aspideretes hurum (Gray, 1831)	Bangladesh: Khulna	3421	—	AM495218
Aspideretes hurum (Gray, 1831)	Bangladesh: Khulna	3422	—	AM495219
Aspideretes hurum (Gray, 1831)	Bangladesh: Mymensingh: Old Brahmaputra	3414	—	AM495220
Aspideretes hurum (Gray, 1831)	Bangladesh: Mymensingh: Old Brahmaputra	3429	—	AM495221
Aspideretes hurum (Gray, 1831)	Bangladesh: Sunargon Market	3539	—	AM495222
Aspideretes hurum (Gray, 1831)	Bangladesh: Sunargon Market	3426	—	AM495223
Aspideretes hurum (Gray, 1831)	India: Assam: Biswanath	3428	—	AM495224
Aspideretes leithii (Gray, 1872)	India: Maharashtra: Pawna River	3099	—	AM495225
Aspideretes leithii (Gray, 1872)	India: Maharashtra: Pawna River	3100	—	AM495226
Aspideretes nigricans (Anderson, 1875)	Bangladesh: Chittagong: Shrine Pond	3415	—	AM495227
Aspideretes nigricans (Anderson, 1875)	Bangladesh: Chittagong: Shrine Pond	3416	—	AM495228
Aspideretes nigricans (Anderson, 1875)	Bangladesh: Chittagong: Shrine Pond	3417	—	AM495229
Aspideretes nigricans (Anderson, 1875)	Bangladesh: Chittagong: Shrine Pond	3418	—	AM495230
Aspideretes nigricans (Anderson, 1875)	Bangladesh: Chittagong: Shrine Pond	3419	—	AM495231
Aspideretes nigricans (Anderson, 1875)	Bangladesh: Chittagong: Shrine Pond	3420	—	AM495232
Aspideretes nigricans (Anderson, 1875)	India: Assam: Jia Bhoroli River	3430	—	AM495233
Aspideretes nigricans (Anderson, 1875)	India: Assam: Gauhati: Kamakhya Temple Pond	3427	—	AM495234
Aspideretes nigricans (Anderson, 1875)	India: Assam: Gauhati: Kamakhya Temple Pond	3540	—	AM495235
Aspideretes nigricans (Anderson, 1875)	India: Assam: Gauhati: Kamakhya Temple Pond	3541	NHMW 36996	AM495236
Aspideretes nigricans (Anderson, 1875)	India: Assam: Gauhati: Kamakhya Temple Pond	3551	—	AM495237
GenBank sequences:				
Amyda cartilaginea (Boddaert, 1770)	Thailand	—	—	AY259550
Aspideretes gangeticus (Cuvier, 1825)	Bangladesh: Dhaka Market	—	—	AY259549
Aspideretes hurum (Gray, 1831)	Bangladesh: Dhaka Market	—	—	AY259548
Dogania subplana (Geoffroy Saint-Hilaire, 1809)	Malaysia: Penang	—	—	AY259551
Nilssonia formosa (Gray, 1869)	Myanmar	—	—	AY259547
Palea steindachneri (Siebenrock, 1906)	Chinese-Vietnamese border	—	—	AY259552
Pelodiscus sinensis (Wiegmann, 1834)	China: Shanghai	—	—	AY259553

Abbreviations: MTD T = Museum of Zoology Dresden, Tissue Collection; NHMW = Natural History Museum Vienna.

tRNA-Thr gene, were amplified using either the primer pair 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 µL volume (50 mM KCl, 1.5 mM MgCl$_2$ 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 58 °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 µL), 1 volume 4 M NH$_4$Ac (30 µL) and 12 volumes EtOH (100%; 360 µL). DNA was pelleted by centrifugation (15 min at 13 000 r.p.m.) and the pellet washed with 70% ethanol. The pellet was dissolved in 20 µL H$_2$O. PCR products were sequenced with the primers mt-c-For2 and mt-E-Rev2 on ABI 3130 or on ABI 3730XL sequencers (Amersham Biosciences). Because no internal stop codons were found and nucleotide frequencies corresponded to those known for coding mtDNA, we conclude that we amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

Sequence divergence, phylogenetic and population genealogy analyses

A sequence of the species *Nilssonia formosa*, representing a monotypic genus closely related with *Aspideretes* (Meylan 1987; Engstrom *et al*. 2004), was downloaded from GenBank and added to the ingroup species. We chose previously published sequences of *Amyda cartilaginea*, *Dogania subplana*, *Palea steindachneri* and *Pelodiscus sinensis* as outgroups (Table 1) because these species represent the closest successive sister taxa of *Aspideretes* and *N. formosa* (Engstrom *et al*. 2004). Sequences were aligned with CLUSTALW using default parameters implemented in MEGA 3.0 (Kumar *et al*. 2004); the alignment comprised 1038 bp of cyt *b*. MEGA 3.0 was also used to calculate genetic distances. For the ingroup species, 854 of 1038 aligned sites were constant, 38 characters were variable but parsimony-uninformative, and 146 variable characters were parsimony-informative.

Data were analysed using Maximum Likelihood (ML), Maximum Parsimony (MP; equal weighting) and Neighbor Joining (NJ; with model corrected ML distances) as implemented in PAUP* 4.0b10 (Swofford 2002), as well as Bayesian inference of phylogeny using 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) to derive the final tree and estimates of the posterior probabilities. 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: GTR + I + G; Posada & Crandall 1998). Under ML, trees were calculated using a NJ starting tree as well as without using a starting tree; resulting ML trees were identical. For ML trees the following parameters were used: Base = (0.3329 0.3272 0.1118), Nst = 6, Rmat = (3.6187 7.9759 3.1144 0.3944 36.0005), Rates = Gamma Shape = 4.1286, Pinvar = 0.5925. Support for resulting ML, MP and NJ topologies was obtained using the bootstrap (Felsenstein 1981) with 100 replicates under ML, 1000 replicates under MP and 1 000 000 under NJ and search parameters matching those for the optimality search.

Intraspecific gene evolution cannot always be represented by dichotomous trees however. Population genealogies are often multifurcated; descendant genes may coexist with persistent ancestors, resulting in reticulate relationships (Posada & Crandall 2001). To find out whether the semicaptive Chittagong population is differentiated from Assamese *A. nigricans* in a manner that is not necessarily depicted by a bifurcating tree, we calculated a parsimony haplotype network as implemented in TCS 1.21 (Clement *et al*. 2000). To compare the extent of variation in *A. nigricans*, we also calculated a haplotype network for *A. gangeticus*. For both species

the same number of sequences was available and our samples of *A. gangeticus* cover the entire range of the species in east–west direction, so that geographically correlated sequence variation is expected. If a similar amount of variation should occur between the Chittagong and Assamese populations of *A. nigricans*, despite their geographical proximity, this would argue for a long-lasting, effective isolation of the turtles in the shrine pond.

Results

Phylogenetic analyses and sequence divergence

Aspideretes nigricans is genetically distinct from the three other *Aspideretes* species. Under all tree-building methods, the sequences of each *Aspideretes* species correspond to a well-supported clade; sequences of Assamese *A. nigricans* group with sequences from the Chittagong shrine (Fig. 2). *Aspideretes hurum* and *A. nigricans* appear with high support values as sister taxa. Within *A. gangeticus* two moderately supported subclades occur. One of these subclades comprises the sequences from the Brahmaputra River system (Mymensingh, Bangladesh; Biswanath, Assam), while the other one contains sequences from more eastern regions of northern India and Pakistan (Ganges and Indus River basins) and one GenBank sequence (AY259549) from a turtle obtained at a market in Dhaka (Dacca), Bangladesh. The clade comprising the sequences of *A. nigricans* is a fork containing two subclades, one corresponding to all four sequences from the Kamakhya Temple Pond, and the other one including five of the six sequences from the Chittagong Shrine Pond. The sixth sequence, as well as the only *A. nigricans* sequence from Jia Bhoroli River, Assam, appears in the polytomy outside of both subclades. Sequence divergence within *A. nigricans* is low and approximately half of the divergence within *A. gangeticus* (Table 2; average sequence divergence within *A. gangeticus*: 0.35%, within *A. nigricans*: 0.16%, uncorrected *p* distances and ML distances in both identical); the two subclades within *A. gangeticus* are differing from one another by an average uncorrected *p* distance of 0.62% (0.58%–0.77%) and the same mean ML distance of 0.62% (0.58%–0.79%).

All phylogenetic analyses provide strong evidence for the monophyly of a clade containing the four *Aspideretes* species as well as *Nilssonia formosa*. Differences between the phylogenetic analyses occur with respect to the branching pattern within the *Aspideretes-Nilssonia* clade. While ML and NJ suggest a weakly supported, monophyletic *Aspideretes* (bootstrap support below 50%) and *N. formosa* being sister of *Aspideretes*, this scenario is not supported under BA and MP that reveal a paraphyly of *Aspideretes* with respect to *Nilssonia*. *Aspideretes gangeticus* and *A. leithii* are sister taxa under BA (posterior probability support 100%), ML (96% bootstrap support) and NJ (69% bootstrap support) and occur under BA in a polytomy together with (*A. hurum* + *A. nigricans*) and *N. formosa*. Under MP, *N. formosa* is placed with *A. gangeticus* and
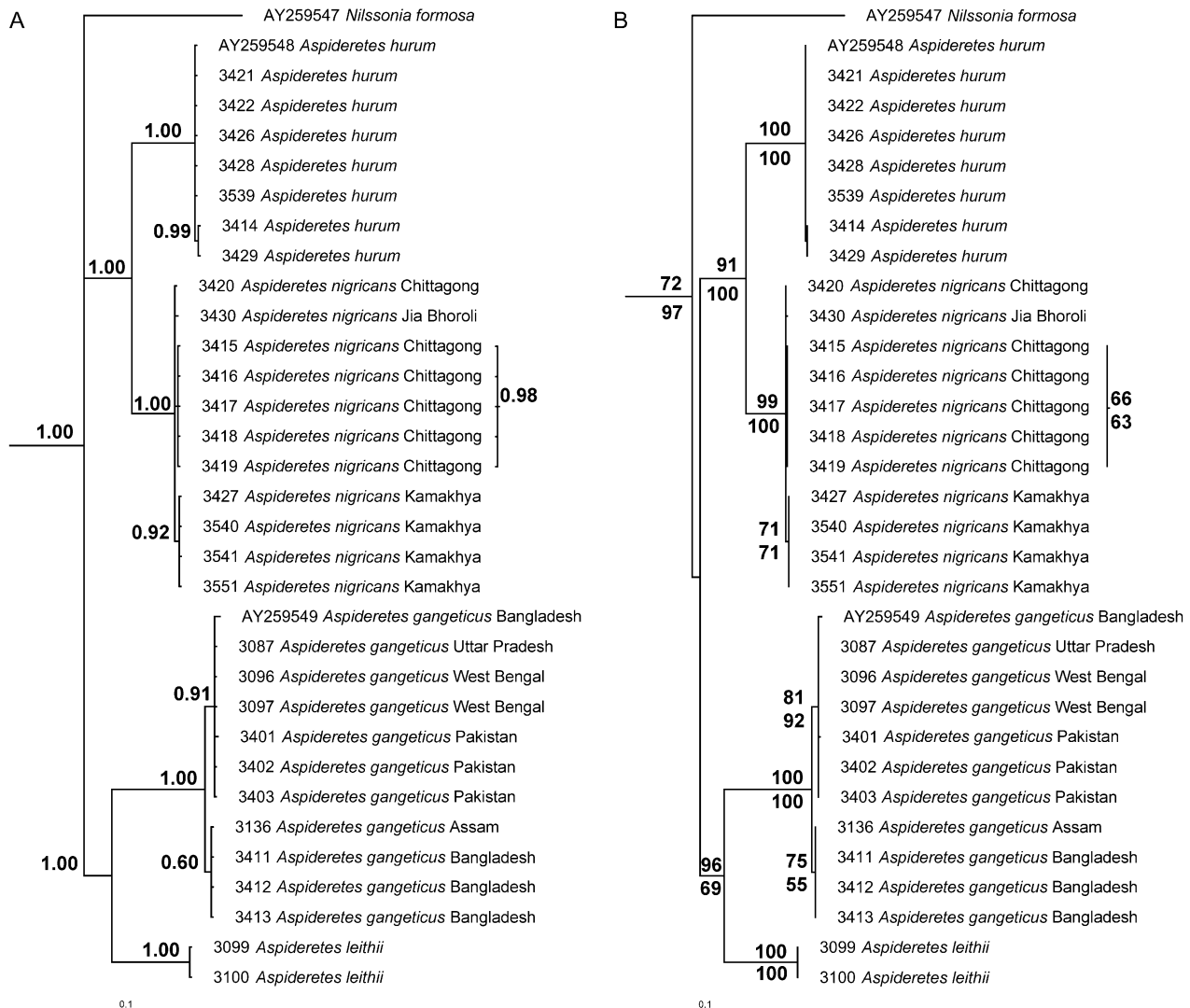
Fig. 2 A–C. Phylogenetic relationships of *Aspideretes* species and *Nilssonia formosa* as revealed by analysis of a 1038 bp long mtDNA fragment (partial cytochrome *b* gene). —A. Bayesian tree. —B. Maximum Likelihood tree. —C. Strict consensus of eight parsimony trees (CI = 0.6416, RI = 0.8595; tree length = 611); this tree is identical to the 50% majority-rule consensus. For all trees, support values are presented above nodes; ML tree includes below nodes also NJ values (BA, posterior probabilities; ML, MP and NJ, bootstrap values greater than 50). Numbers preceding species names are MTD T or accession numbers and refer to Table 1. Branch lengths for the BA and ML trees are proportional to the mean number of substitutions per site; branch lengths for the MP tree, arbitrary. Outgroup taxa (*Amyda cartilaginea*, *Dogania subplana*, *Palea steindachneri*, *Pelodiscus sinensis*) removed for clarity.

Table 2 Uncorrected *p* distances and ML distances (percentages; means and ranges) within and between *Aspideretes* species and *Nilssonia formosa*, based on a 1038 bp long mtDNA fragment (partial cytochrome *b* gene). Uncorrected *p* distances are given below, ML distances above the diagonal. The within-species divergence is given in bold on the diagonal (uncorrected *p* distances/ML distances). The two studied sequences of *A. leithii* were identical.

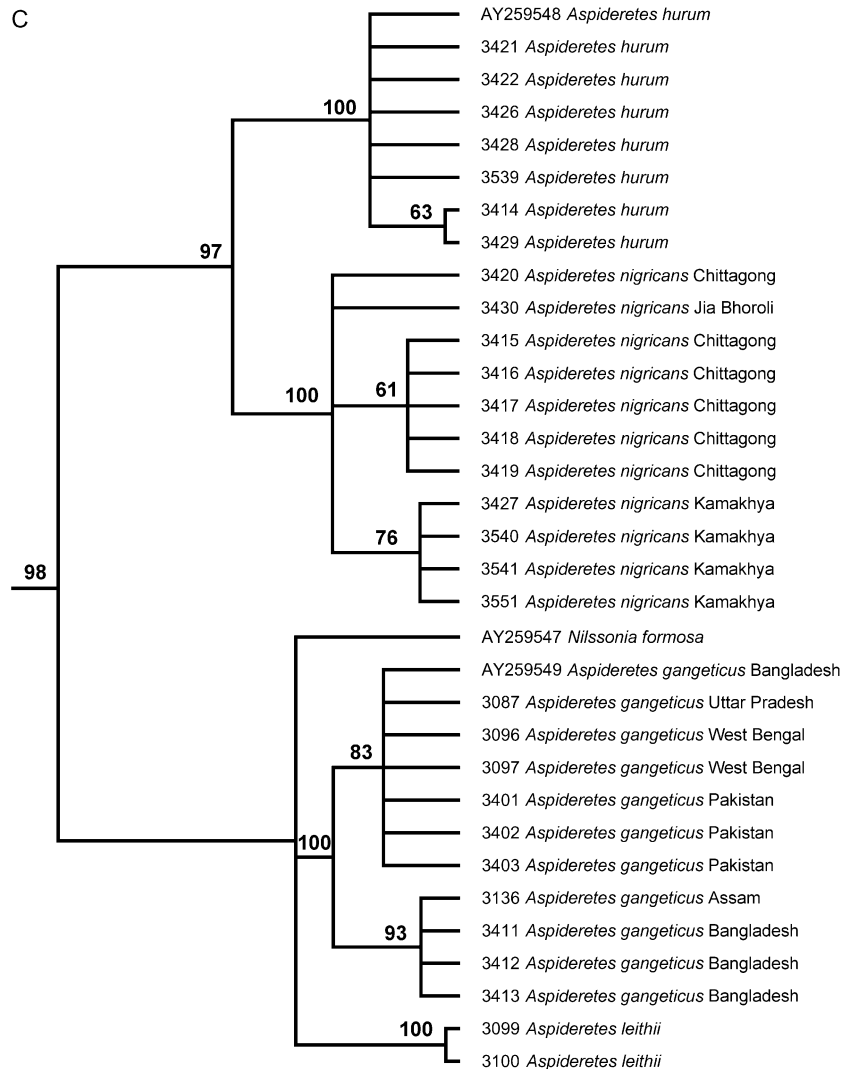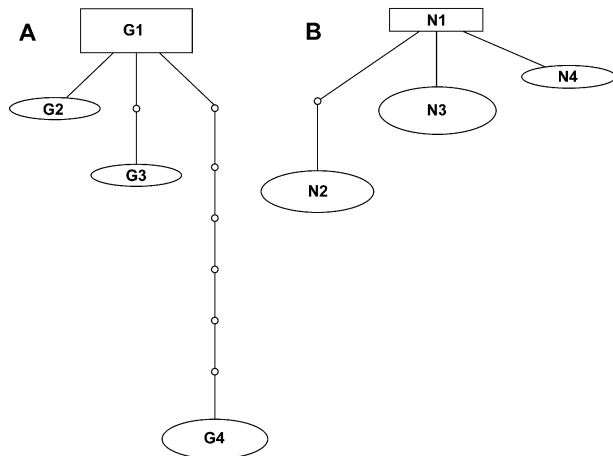|	*Aspideretes gangeticus*	*Aspideretes hurum*	*Aspideretes leithii*	*Aspideretes nigricans*	*Nilssonia formosa*
A. gangeticus	**0.35 (0–0.77)/0.35 (0–0.79)**	13.28 (12.69–14.25)	9.91 (9.68–10.42)	11.79 (11.10–12.58)	15.78 (15.04–16.79)
A. hurum	8.82 (8.58–9.15)	**0.04 (0–0.10)/0.04 (0–0.10)**	12.21 (12.15–12.39)	5.93 (5.81–6.12)	16.17 (16.10–16.38)
A. leithii	7.32 (7.23–7.51)	8.41 (8.38–8.48)	**0/0**	11.34 (11.03–12.23)	14.21 (—)
A. nigricans	8.33 (8.00–8.67)	4.89 (4.82–5.01)	7.96 (7.80–8.37)	**0.16 (0–0.31)/0.16 (0–0.31)**	14.93 (14.63–15.38)
N. formosa	9.63 (9.35–9.92)	10.14 (10.12–10.21)	8.86 (—)	9.58 (9.44–9.85)	—

C

Fig. 2 *Continued.*

A. leithii in a polytomy (bootstrap support below 50%); this (*Nilssonia, A. gangeticus, A. leithii*) clade is sister to (*A. hurum* + *A. nigricans*).

Network analyses of haplotypes of Aspideretes gangeticus and A. nigricans

The 11 sequences of *A. gangeticus* correspond to four haplotypes (G1–G4), differing in one to nine mutation steps (Fig. 3A). The most common haplotype G1 (*n* = 5), found in samples from Uttar Pradesh, West Bengal and Pakistan is under coalescent theory ancestral to the three other haplotypes (outgroup probability of G1: 0.6667). The closely related haplotype G2 from another Pakistani sample differs in one mutation from G1. Haplotype G3 is the GenBank sequence AY259549 from Bangladesh, and is connected over two mutation steps with G1. The most distinct haplotype G4, differing in seven to nine mutation steps from other haplotypes, is represented by the four sequences from the Brahmaputra River system (Mymensingh, Bangladesh; Biswanath, Assam).

Also in our 11 samples of *A. nigricans* four haplotypes were found (N1–N4). Compared with *A. gangeticus*, the level of differentiation is distinctly lower however. Haplotypes N1–N4 differ in one to three mutation steps only. The sequences from the Chittagong Shrine Pond represent two haplotypes (N1, *n* = 1; N3, *n* = 5). Under coalescent theory, N1 is ancestral to all other haplotypes (outgroup probability: 0.5833). The most distinct haplotype N2, differing in two to three mutations from the other haplotypes, corresponds with the four sequences from Kamakhya Temple Pond in Assam. The only sequence from a wild-living *A. nigricans* from Jia Bhoroli River, Assam, represents the fourth haplotype (N4), like N3 differing in one mutation step from the ancestral haplotype N1 (Fig. 3B).

Fig. 3 A, B. Parsimony networks (TCS 1.21) for mtDNA haplotypes of —A. *Aspideretes gangeticus* and —B. *Aspideretes nigricans*. Haplotypes with biggest outgroup probability shown as rectangles on the top. Symbol size corresponds approximately to haplotype frequency; missing haplotypes, small circles. Each line between symbols and haplotypes indicates one mutation step. Haplotype frequencies, G1: $n = 5$ (3087, 3096–7, 3402–3), G2: $n = 1$ (3401), G3: $n = 1$ (AY259549), G4: $n = 4$ (3411–3, 3136); N1: $n = 1$ (3420), N2: $n = 4$ (3427, 3540–1, 3551), N3: $n = 5$ (3415–9), N4: $n = 1$ (3430). Numbers in brackets are MTD T or accession numbers and refer to Table 1.

Discussion

Our data provide evidence that *Aspideretes nigricans* is a distinct species that occurs, besides in the well-known shrine pond near Chittagong, also in Assam, India. Furthermore, our data strongly suggest the existence of a well-supported clade containing the four *Aspideretes* species and *Nilssonia formosa*. This clade corresponds with the tribe Aspideretini proposed by Meylan (1987). According to our phylogenetic analyses, relationships within Aspideretini are not well-resolved, but there is at best weak evidence for the monophyly of *Aspideretes*, suggesting that the genera *Aspideretes* and *Nilssonia* should be lumped. Likewise, average sequence divergences between *N. formosa* and the *Aspideretes* species (mean uncorrected *p* distances: 8.86%–10.14%, mean ML distances: 14.21%–16.17%) resemble the largest average sequence divergence when the four *Aspideretes* species are compared with one another (mean uncorrected *p* distances: 4.89%–8.82%, mean ML distances: 5.93%–13.28%; Table 2), underlining the close relationship of *N. formosa* and *Aspideretes*. Also morphological evidence for the distinctness of *Nilssonia* is weak. *Nilssonia* differs from *Aspideretes* species merely in the number of neural plates between the first pleural pair of the bony carapace (two neurals in *Aspideretes*; only one in *Nilssonia*, resulting from the fusion of the first and second neural; Meylan 1987). This character constitutes an autapomorphy of *N. formosa* and, thus, does not contradict including

N. formosa in the same genus as the *Aspideretes* species. In addition, external morphology of *N. formosa* and *Aspideretes* species is similar. In contrast to other trionychids, juveniles of all five species possess a carapacial pattern consisting of four conspicuous ocelli; *A. hurum*, *A. nigricans* and *N. formosa* are hardly distinguishable (Fig. 4). Adults of all five species are similar sized (maximum shell length of *Aspideretes* species: 60–94 cm; of *N. formosa*: 65 cm; Ernst *et al.* 2000).

Using a patchy taxon sampling and a combined dataset of morphological and genetic characters, already Engstrom *et al.* (2004) suggested that *Aspideretes* is paraphyletic with respect to *N. formosa*. As advocates of the PhyloCode, these authors proposed abandoning the current generic names, and placing all five species in a higher taxon, Aspideretini, defined as the crown clade arising from the last common ancestor of these five species. We prefer to adhere to the International Code of Zoological Nomenclature (ICZN 1999) and synonymise *Aspideretes* Hay, 1904 with *Nilssonia* Gray, 1872, resulting in an expanded genus *Nilssonia* and rendering the recognition of the taxon Aspideretini superfluous. Only in the case of *A. gangeticus* the ending of the species name needs to be changed to feminine gender when transferred to *Nilssonia* (ICZN 1999: Article 31.2), so that *Nilssonia* sensu lato comprises the species *N. formosa* (Gray, 1869), *N. gangetica* (Cuvier, 1825), *N. hurum* (Gray, 1831), *N. leithii* (Gray, 1872) and *N. nigricans* (Anderson, 1875).

While we found little genetic variation between *N. nigricans* from Chittagong and Assam, we detected previously unrecognised differentiation within *N. gangetica*. Our samples from the Brahmaputra River system are distinct from sequences from the Ganges and Indus River basins in all phylogenetic and network analyses. One sequence downloaded from GenBank (AY259549) clusters with our sequences from the Ganges and Indus. The turtle yielding the GenBank sequence was obtained at a market in Dhaka (Dacca), Bangladesh (Engstrom *et al.* 2004). Dhaka lies near the confluence of the Brahmaputra and Ganges Rivers, suggesting that both genetic lineages meet there.

Conclusions

For more than one century *Nilssonia nigricans* was considered to be restricted to a single man-made pond and being highly endangered. The results of the present study provide compelling evidence that Praschag & Gemel (2002) were right and that the range of *N. nigricans* is distinctly larger than previously thought. The total population size of *N. nigricans* must exceed by far the estimate of 150–300 individuals living in the shrine pond (Groombridge 1982; Ahsan *et al.* 1991). Further field-work is needed to assess its status in Assam and its exact distributional borders.

Also our finding of geographically correlated genetic differentiation within *N. gangetica* calls for further research.
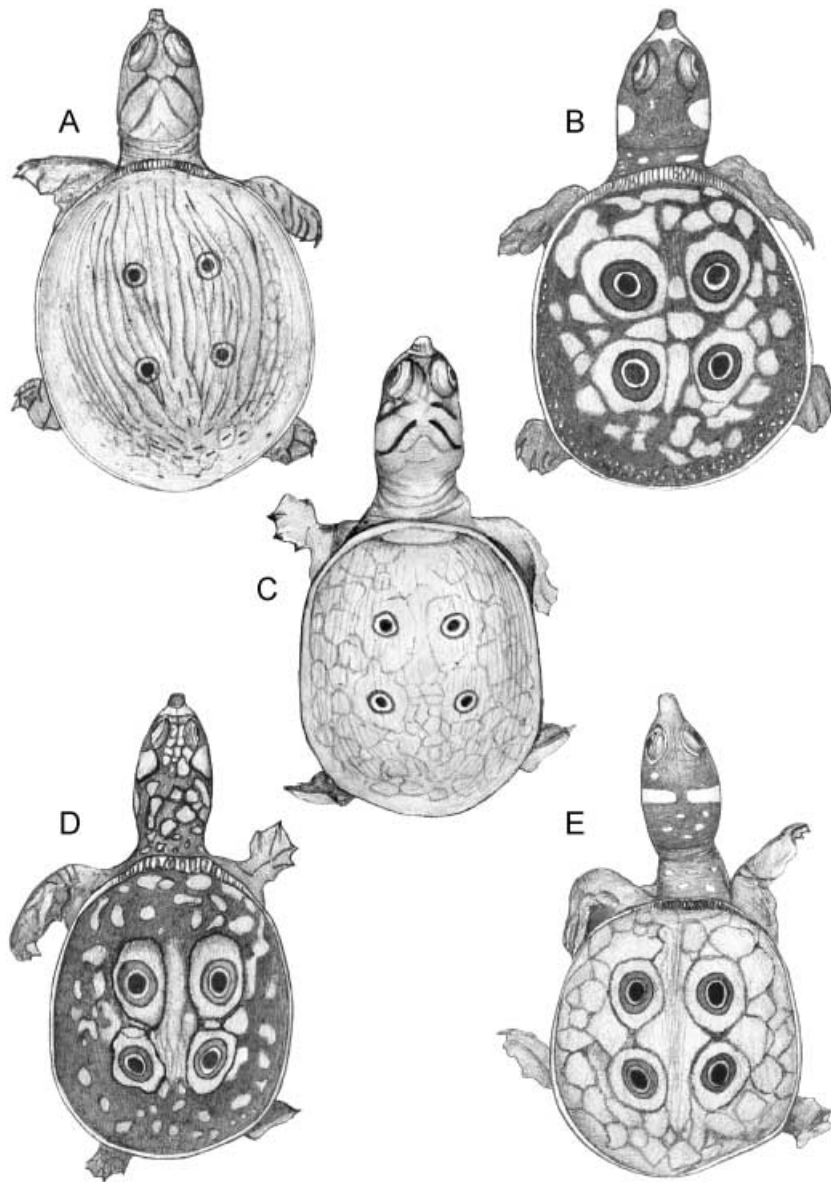
Fig. 4 A–E. Dorsal aspects of hatchlings of South Asian softshell turtles. —A. *Aspideretes gangeticus* (Sarnath near Varanasi, Uttar Pradesh, India; live turtle). —B. *Aspideretes hurum* (Royal Chitwan National Park, Nepal; live turtle). —C. *Aspideretes leithii* ('India'; The Natural History Museum, London, BMNH 1884.3.25.1). —D. *Aspideretes nigricans* (Shrine Pond near Chittagong, Bangladesh; specimen in the collection of the University of Chittagong). —E. *Nilssonia formosa* (Pegu, Myanmar; The Natural History Museum, London, BMNH 1946.1.22.11, holotype of *Trionyx formosus* Gray, 1869). Drawings: P. Praschag.

In view of reintroduction programmes being conducted in this species (Basu 1987; Choudhury *et al.* 2000), a rangewide understanding of its genetic variation is in urgent need to avoid the loss of biodiversity by mixing different stocks.

While the mitochondrial cyt *b* gene was shown to contain too much homoplastic information for revealing deep nodes in softshell turtle phylogeny (Engstrom *et al.* 2004), our results demonstrate that this gene is an ideally suited marker for species-level taxonomy of trionychids and yields a good phylogenetic resolution on the generic level. This is in line with other investigations using cyt *b* for phylogeographical purposes, as well as species- and genus-level taxonomy of softshell turtles (*Apalone*, Weisrock & Janzen 2000) and other chelonian families (Emydidae: Lamb *et al.* 1994; Lenk *et al.* 1999; Fritz *et al.* 2005a; Geoemydidae: Barth *et al.* 2004; Fritz *et al.* 2006a; Testudinidae: Palkovacs *et al.* 2002; Austin *et al.* 2003; Fritz *et al.* 2005b, 2006b, 2007) and suggests its application in further studies.

Acknowledgements

References

Ahsan, M. F., Haque, M. N. & Fugler, C. M. (1991). Observations on *Aspideretes nigricans*, a semi-domesticated endemic turtle from eastern Bangladesh. *Amphibia-Reptilia*, *12*, 131–136.

Anderson, J. (1875). Description of some new Asiatic Mammals and Chelonia. *Annals and Magazine of Natural History (4)*, *16*, 284–285.

Austin, J. J., Arnold, E. N. & Bour, R. (2003). Was there a second adaptive radiation of giant tortoises in the Indian Ocean? Using mitochondrial DNA to investigate speciation and biogeography of *Aldabrachelys*. *Molecular Ecology*, *12*, 1415–1424.

Barth, D., Bernhard, D., Fritzsch, G. & Fritz, U. (2004). The freshwater turtle genus *Mauremys* (Testudines: Geoemydidae) — a textbook example of an east–west disjunction or a taxonomic misconcept? *Zoologica Scripta*, *33*, 213–221.

Basu, D. (1987). Project for rehabilitation of freshwater turtles in India. *Hamadryad*, *12*, 13–14.

Choudhury, B. C., Bhupathy, S. & Hanfee, F. (2000). Status information on the tortoises and freshwater turtles of India. *Chelonian Research Monographs*, *2*, 86–94.

Clement, M., Posada, D. & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, *9*, 1657–1660.

Das, I. (1995). *Turtles and Tortoises of India*. Bombay, Delhi, Calcutta and Madras: WWF India and Oxford University Press.

Das, I. (2001). *Die Schildkröten des Indischen Subkontinents*. Frankfurt am Main: Chimaira.

Engstrom, T. E., Shaffer, H. B. & McCord, W. P. (2004). Multiple data sets, high homoplasy, and the phylogeny of softshell turtles (Testudines: Trionychidae). *Systematic Biology*, *53*, 693–710.

Ernst, C. H., Altenburg, R. G. M. & Barbour, R. W. (2000). *Turtles of the World. World Biodiversity Database, CD-ROM Series, Windows*, Version 1.2. Amsterdam: Biodiversity Center of ETI.

Ernst, C. H. & Barbour, R. W. (1989). *Turtles of the World*. Washington D.C.: Smithsonian Institution Press.

Felsenstein, J. (1981). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*, 783–791.

Fritz, U., Fattizzo, T., Guicking, D., Tripepi, S., Pennisi, M. G., Lenk, P., Joger, U. & Wink, M. (2005a). A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys*. *Zoologica Scripta*, *34*, 351–371.

Fritz, U., Široký, P., Kami, H. & Wink, M. (2005b). Environmentally caused dwarfism or a valid species — is *Testudo weissingeri* Bour, 1996 a distinct evolutionary lineage? New evidence from mitochondrial and nuclear genomic markers. *Molecular Phylogenetics and Evolution*, *37*, 389–401.

Fritz, U., Barata, M., Busack, S. D., Fritzsch, G. & Castilho, R. (2006a). Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa*. *Zoologica Scripta*, *35*, 97–108.

Fritz, U., Auer, M., Bertolero, A., Cheylan, M., Fattizzo, T., Hundsdörfer, A. K., Martín Sampayo, M., Pretus, J. L., Široký, P. & Wink, M. (2006b). A rangewide phylogeography of Hermann's tortoise, *Testudo hermanni* (Reptilia: Testudines: Testudinidae): implications for taxonomy. *Zoologica Scripta*, *35*, 531–543.

Fritz, U. & Havaš, P. (2007). *Checklist of Chelonians of the World*. Bonn and Dresden: German Federal Ministry of Environment, Nature Conservation and Nuclear Safety and Museum of Zoology Dresden.

Fritz, U., Hundsdörfer, A. K., Široký, P., Auer, M., Kami, H., Lehmann, J., Mazanaeva, L. F., Türkozan, O. & Wink, M. (2007). Phenotypic plasticity leads to incongruence between morphology-based taxonomy and genetic differentiation in western Palaearctic tortoises (*Testudo graeca* complex; Testudines, Testudinidae). *Amphibia-Reptilia*, *28*, 97–121.

Groombridge, B. (1982). *The IUCN Amphibia–Reptilia Red Data Book. Part 1. Testudines, Crocodylia, Rhynchocephalia*. Gland: IUCN.

Gustincich, S., Manfioletti, G., del Sal, G., Schneider, C. & Carninci, C. (1991). A fast method for high-quality genomic DNA extraction from whole human blood. *Bio Techniques*, *11*, 298–302.

Hilton-Taylor, C. (2000). *IUCN Red List of Threatened Species*. Gland and Cambridge: IUCN.

ICZN [International Commission of Zoological Nomenclature] (1999). *International Code of Zoological Nomenclature*, 4th edn. London: International Trust for Zoological Nomenclature.

Iverson, J. B. (1992). *A Revised Checklist with Distribution Maps of the Turtles of the World*. Richmond, Indiana: Privately Printed.

Khan, M. A. R. (1980). A 'holy' turtle of Bangladesh. *Hornbill*, *4*, 7–11.

Khan, M. A. R. (1987). *Bangladesher bonnyoprani*, Vol. I. Dhaka: Bangla Academy.

Khan, M. S. (2006). *Amphibians and Reptiles of Pakistan*. Malabar, Florida: Krieger Publishing Co.

Kuchling, G., Ko, W. K., Lwin, T., Min, S. A., Myo, K. M., Khaing, T. T., Mar, W. W. & Khaing, T. T. (2004). The softshell turtles and their exploitation at the upper Chindwin River, Myanmar: range extensions for *Amyda cartilaginea*, *Chitra vandijki*, and *Nilssonia formosa*. *Salamandra*, *40*, 281–296.

Kumar, S., Tamura, K. & Nei, N. (2004). MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, *5*, 150–163.

Lamb, T., Lydeard, C., Walker, R. B. & Gibbons, J. W. (1994). Molecular systematics of map turtles (*Graptemys*): a comparison of mitochondrial restriction site versus sequence data. *Systematic Biology*, *43*, 543–559.

Lenk, P., Fritz, U., Joger, U. & Wink, M. (1999). Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). *Molecular Ecology*, *8*, 1911–1922.

Meylan, P. A. (1987). The phylogenetic relationships of soft-shelled turtles (family: Trionychidae). *Bulletin of the American Museum of Natural History*, *186*, 1–101.

Meylan, P. A. & Gaffney, E. S. (1992). *Sinaspideretes* is not the oldest trionychid turtle. *Journal of Vertebrate Paleontology*, *12*, 257–259.

Nessov, L. A. (1995). On some Mesozoic turtles of the Fergana Depression (Kyrgyzstan) and Dzhungar Alatau Ridge (Kazakhstan). *Russian Journal of Herpetology*, *2*, 134–141.

Obst, F. J. (1986). *Turtles, Tortoises and Terrapins*. Leipzig: Edition Leipzig.

Palkovacs, E. P., Gerlach, J. & Caccone, A. (2002). The evolutionary origin of Indian Ocean tortoises (*Dipsochelys*). *Molecular Phylogenetics and Evolution*, *24*, 216–227.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics*, *14*, 817–818.

Posada, D. & Crandall, K. A. (2001). Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, *16*, 37–45.

Praschag, P. & Gemel, R. (2002). Identity of the black soft-shell turtle *Aspideretes nigricans* (Anderson, 1875), with remarks on related species. *Faunistische Abhandlungen*, *23*, 87–116.

Pritchard, P. C. H. (1979). *Encyclopedia of Turtles*. Neptune, New Jersey: T. F. H. Publications, Inc.

Rashid, S. M. A. (1990). The *Aspideretes nigricans* mystery. *British Herpetological Society Bulletin*, *34*, 42–43.

Ronquist, F. & Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*, 1572–1574.

Schneider, P. & Djalal, A. S. (1970). Erstnachweis einer Weichschildkröte, *Trionyx gangeticus* Cuvier, 1825, in Afghanistan. *Bonner zoologische Beiträge*, *21*, 269–273.

Swofford, D. L. (2002). PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Weisrock, D. W. & Janzen, F. J. (2000). Comparative molecular phylogeography of North American softshell turtles (*Apalone*): implications for regional and wide-scale historical evolutionary forces. *Molecular Phylogenetics and Evolution*, *14*, 152–164.