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Molecular phylogeny of the softshell turtle genus *Nilssonia* revisited, with first records of *N. formosa* for China and wild-living *N. nigricans* for Bangladesh

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> Abstract

Based on 2354 bp of mitochondrial DNA (12S rRNA, ND4, cyt *b*) and 2573 bp of nuclear DNA (C-mos, ODC, R35), we re-examine the phylogenetic relationships of *Nilssonia* species. Individual and combined analyses of mitochondrial and nuclear DNA using Maximum Likelihood and Bayesian approaches confirm the monophyly of the genus. While mitochondrial data alone could not resolve the phylogenetic position of *N. formosa*, nuclear data support a sister group relationship of *N. formos*a and the remaining *Nilssonia* species. Combined analyses of mitochondrial and nuclear DNA suggest the following branching pattern, with *N. formosa* as the sister taxon of the remaining species: *N. formosa* + ((*N. gangetica* + *N. leithii*) + (*N. hurum* + *N. nigricans*)). Among the samples we studied is the first record of *N. formosa* for Yunnan, China, and the first record of wild-living *N. nigricans* for Bangladesh. In *N. gangetica*, each of the studied major river basins harbours a genetically distinct population, suggesting that at least three distinct management units should be distinguished: (1) Brahmaputra River; (2) Indus and Ganges Rivers plus Ganges Delta; and (3) Mahanadi River.

> Key words

Reptilia, Testudines, Trionychidae, Asia, Bangladesh, China, India, Myanmar, Pakistan.

Introduction

Nilssonia Gray, 1872 is a little known genus of South Asian and Southeast Asian softshell turtles. Until a few years ago *Nilssonia* was thought to be monotypic, with its only species *N. formosa* of Myanmar (Meylan, 1987; Ernst & Barbour, 1989; Ernst *et al.*, 2000). However, based on molecular and morphological evidence Engstrom *et al.* (2004) and Praschag *et al.* (2007) concluded that *N. formosa* is so closely allied to the four species of the South Asian genus *Aspideretes* Hay, 1904 that all species should be

placed in the same taxon. Within the framework of a rank-free phylogenetic nomenclature, Engstrom *et al.* (2004) recommended to abandon the usage of generic names and to treat all five species only as members of the clade Aspideretini. By contrast, Praschag *et al.* (2007) synonymized *Aspideretes* with *Nilssonia*, resulting in a polytypic genus *Nilssonia* with the five species *N. formosa* (Gray, 1869), *N. gangetica* (Cuvier, 1825), *N. hurum* (Gray, 1830), *N. leithii* (Gray, 1872) and *N. nigricans* (Anderson, 1875). All of these species are morphologically similar, large-sized softshell turtles, with maximum shell lengths of 60 to 94 cm. Hatchlings and juveniles are characterized by conspicuous large ocelli on their back (Ernst & Barbour, 1989; Ernst *et al.*, 2000). Yet, Rhodin *et al.* (2010) were reluctant to accept an expanded genus *Nilssonia*, and only recently van Dijk *et al.* (2011) conceded that this classification is now widely accepted in the herpetological community. Nevertheless, especially palaeontologists continue to treat *Aspideretes* as a distinct genus (e.g., Joyce & Lyson, 2010; Vitek, 2012).

The molecular data set of Engstrom *et al.* (2004) consisted of the mitochondrial cyt *b* and ND4 genes plus the intron 1 of the nuclear R35 gene, and these authors combined their molecular data for phylogenetic analyses with morphological evidence from Meylan (1987). However, Engstrom *et al.* (2004) studied only three species (*N. formosa*, *N. gangetica*, *N. hurum*) represented by one individual each, and the only morphological character separating *N. formosa* from the former *Aspideretes* species is the lower number of neural plates in the bony carapace, resulting from the fusion of the first and second neural plate (Meylan, 1987). Using a comprehensive sampling of all *Nilssonia* species and the mitochondrial cyt *b* gene as a marker, Praschag *et al.* (2007) conducted a phylogeographic study. Like Engstrom *et al.* (2004), Praschag *et al.* (2007) found the monophyly of the studied *Nilssonia* species well-supported. However, while the phylogenetic relationships of *N. gangetica*, *N. hurum*, *N. leithii* and *N. nigricans* were well-resolved, the placement of *N. formosa* remained problematic (Praschag *et al.*, 2007).

To re-examine the phylogenetic position of *N. formosa*, we supplement the data set of Praschag *et al.* (2007) with sequence data of the mitochondrial 12S rRNA and ND4 genes (the latter plus adjacent DNA coding for tRNAs), the nuclear C-mos and ODC genes, and the intron 1 of the nuclear R35 gene and analyse this expanded data set using Maximum Likelihood and Bayesian methods. We include in our analyses additional samples of *N. gangetica*, *N. hurum* and *N. nigricans* and replace the GenBank sequence of *N. formosa* used by Praschag *et al.* (2007) by fresh material of two individuals of this species. One of these turtles was caught near Shuangbai, Yunnan, China, and constitutes the first record of *N. formosa* for the northern catchment basin of the Mekong. Among our new material of *N. gangetica* are for the first time samples from the Mahanadi River system, India. Furthermore, we include sequences of two *Nilssonia* specimens of questionable taxonomic identity. One of these softshell turtles is an aberrant pale-coloured *Nilssonia* from Manikchhari near Chittagong, Bangladesh. The other is a large shell of a freshly killed large turtle from Sreemangal (Shreemongal), Sylhet District, Bangladesh.

Materials and methods

Sampling and gene selection

Fifty-three *Nilssonia* samples were studied, representing the five currently recognized species *Nilssonia formosa*, *N. gangetica*, *N. hurum*, *N. leithii* and *N. nigricans* (see Appendix). Three mitochondrial genes were sequenced that have previously been shown to be useful for assessing the phylogenetic relationships of terminal chelonian taxa (e.g., Le *et al.*, 2006; Fritz *et al.*, 2010, 2012a; Vargas-Ramírez *et al.*, 2010; Wiens *et al.*, 2010; Praschag *et al.*, 2011), viz. the partial 12S ribosomal RNA (12S rRNA) gene, the partial NADH dehydrogenase subunit 4 (ND4) gene, and the cytochrome *b* (cyt *b*) gene. The DNA sequence containing the partial ND4 gene embraced also the flanking DNA coding for tRNA-His, tRNA-Ser and tRNA-Leu. The DNA sequence containing the cyt *b* gene included also approximately 20 bp of the adjacent DNA coding for tRNA-Thr. Twenty-nine of the cyt *b* sequences originated from a previous study using the same samples (Praschag *et al.*, 2007). In addition, up to three nuclear loci were generated, viz. the partial genes coding for the oocyte maturation factor Mos (C-mos) and for ornithine decarboxylase (ODC), and the intron 1 of the RNA fingerprint protein 35 (R35) gene. These loci are increasingly applied for phylogenetic investigations of turtles and tortoises (e.g., Georges *et al.*, 1998; Fujita *et al.*, 2004; Vargas-Ramírez *et al.*, 2010; Wiens *et al.*, 2010; Fritz *et al.*, 2011a, 2012a; Kindler *et al.*, 2012). While all mitochondrial data could be generated for most samples, the nuclear loci could be sequenced only for a subset owing to bad DNA quality or small sample size (see Appendix). Remaining samples and DNA are stored at −80°C in the tissue collection of the Museum of Zoology, Dresden.

Table 1. Primers used for PCR and sequencing.

Primer	Direction	Gene	Primer sequence (5′ to 3′)	Reference
L1091	Forward	12Sr RNA	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	Kocher *et al.* (1989)
H1478	Reverse	12Sr RNA	TGACTGCAGAGGGTGACGGGCGGTGTGT	Kocher *et al.* (1989)
ND4 672	Forward	ND4 + tRNAs	TGACTACCAAAAGCTCATGTAGAAGC	Engstrom *et al.* (2004)
H-Leu	Reverse	ND4 + tRNAs	ATTACTTTTACTTGGATTTGCACCA	Stuart & Parham (2004)
CytbG	Forward	cyt *b*	AACCATCGTTGTWATCAACTAC	Spinks *et al.* (2004)
mt-a-neu3	Forward	cyt *b*	CTCCCAGCCCCATCCAACATCTCHGCHTGATGAAACTTCG	Praschag *et al.* (2007)
mt-c-For2	Forward	cyt *b*	TGAGGVCARATATCATTYTGAG	Fritz *et al.* (2006)
mt-E-Rev2	Reverse	cyt *b*	GCRAATARRAAGTATCATTCTGG	Fritz *et al.* (2006)
mt-f-na3	Reverse	cyt *b*	AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG	Praschag *et al.* (2007)
Cmos1	Forward	C-mos	GCCTGGTGCTCCATCGACTGGGATCA	Le *et al.* (2006)
Cmos3	Reverse	C-mos	GTAGATGTCTGCTTTGGGGGTGA	Le *et al.* (2006)
Nilssonia_Cmos_Seq_F*	Forward	C-mos	CCTGGGCACCATAATCAT	This study
Nilssonia_Cmos_Seq_R*	Reverse	C-mos	TATGCTTAGGGGTTCTCT	This study
Chicken primer 1	Forward	ODC	GACTCCAAAGCAGTTTGTCGTCTCAGTGT	Friesen *et al.* (1999)
Nilssonia_ODC_Seq_F*	Forward	ODC	GAAGCTATGGTCAGTTACGT	This study
Chicken primer 2	Reverse	ODC	TCTTCAGAGCCAGGGAAGCCACCACCAAT	Friesen *et al.* (1999)
R35Ex1	Forward	R35	ACGATTCTCGCTGATTCTTGC	Fujita *et al.* (2004)
R35Ex2	Reverse	R35	GCAGAAAACTGAATGTCTCAAAGG	Fujita *et al.* (2004)

* Newly designed sequencing primer

Laboratory procedures

Total genomic DNA was extracted using either the DTAB method (Gustincich *et al.*, 1991) or the innuPREP DNA Mini Kit (Analytik Jena, Germany).

The partial 12S rRNA gene was amplified using the primers L1091 and H1478; for the DNA fragment comprising the partial ND4 gene plus flanking DNA coding for tRNAs, the primers ND4 672 and H-Leu were used. The cyt *b* gene was routinely amplified using the primer combination CytbG + mt-f-na3; for challenging samples, the primers mt-a-neu3 + mt-f-na3, mt-a-neu3 + mt-E-Rev2, and mt-c-For2 + mt-f-na3 were used. For amplifying the nuclear genes, the following primers were used: Cmos1 + Cmos3 for the C-mos gene, the chicken primers of Friesen *et al.* (1999) for ODC, and the primers R35Ex1 + R35Ex2 for the intron 1 of the R35 gene (Table 1).

PCR was carried out in a total volume of 25 µl containing 0.2 µl *Taq* polymerase (5 u/µl; Bioron, Ludwigshafen, Germany), 1x buffer as recommended by the supplier, 0.4 µM of each primer, and 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany). Alternatively, for challenging samples a total volume of 20 µl containing 0.2 µl GoTaq® Flexi DNA Polymerase (5 u/µl; Promega, Madison, WI, USA) was used according to the recommendations by the supplier. For cycling protocols, see Table 2. PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Staufen, Germany) and sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Cycle sequencing reactions were purified by ethanol/sodium acetate precipitation or by using Sephadex (GE Healthcare, München, Germany). For sequencing the cyt *b* gene, the internal primers mt-c-For2 and mt-E-Rev2 were used; for all other genes, the same primers as for PCR. However, for sequencing C-mos and ODC of a few challenging samples, newly designed sequencing primers were applied (Table 1). For GenBank accession numbers of newly generated sequences, see Appendix.

Alignment, partitioning and data analyses

DNA sequences were aligned in BIOEDIT 7.0.5.2 (Hall, 1999) with outgroup sequences downloaded from GenBank (*Amyda cartilaginea*, *Dogania subplana*, *Palea steindachneri*, and *Pelodiscus maackii*). These species represent the successive sister taxa of *Nilssonia* (Engstrom *et al.*, 2004). Since not all outgroup sequences were available from GenBank, the missing data were generated as described above using samples from the tissue collection of the Museum of Zoology, Senckenberg Dresden (see Appendix). Furthermore, protein-coding sequences were translated in amino acids and uncorrected *p* distances were calcu-

Table 2. PCR protocols for mitochondrial and nuclear genes.

Gene	Primers	Thermocycling conditions					
		ID	C	D	A	E	FE
12S rRNA	L1091, H1478	94°C, 3 min	30	94°C, 30 s	50°C, 30 s	72°C, 30 s	72°C, 10 min
ND4 + tRNAs	ND4 672, H-Leu	94°C, 5 min	35	94°C, 45 s	53°C, 30 s	72°C, 60 s	72°C, 10 min
cyt *b*	CytbG, mt-f-na3	95°C, 5 min	35	95°C, 45 s	56°C, 30 s	72°C, 60 s	72°C, 8 min
cyt *b*	mt-a-neu3, mt-f-na3	95°C, 5 min	35	95°C, 30 s	56°C, 30 s	72°C, 60 s	72°C, 8 min
cyt *b*	mt-a-neu3, mt-E-Rev2	95°C, 5 min	35	95°C, 30 s	56°C, 30 s	72°C, 60 s	72°C, 8 min
cyt *b*	mt-c-For2, mt-f-na3	95°C, 5 min	35	95°C, 30 s	62°C, 30 s	72°C, 60 s	72°C, 8 min
C-mos	Cmos1, Cmos3	94°C, 5 min	30	94°C, 30 s	58°C, 30 s	72°C, 60 s	72°C, 8 min
ODC	chicken primers of Friesen *et al.* (1999)	94°C, 5 min	35	94°C, 30 s	62°C, 45 s	72°C, 60 s	72°C, 10 min
R35	R35Ex1, R35Ex2	94°C, 5 min	35	94°C, 30 s	62°C, 45 s	72°C, 60 s	72°C, 8 min

Abbreviations: ID = initial denaturing, **C** = number of cycles, **D** = denaturing, **A** = annealing, **E** = extension, **FE** = final extension.

lated for cyt *b* sequences using MEGA 4.0.2 (Tamura *et al.*, 2007).

Aligned sequences of the mitochondrial 12S rRNA gene were of 394 bp length (including gaps), the DNA fragment embracing the partial ND4 gene and adjacent DNA coding for tRNAs was 893 bp long (including gaps), and cyt *b* sequences had 1067 bp. The nuclear C-mos sequences were 590 bp long, and the R35 sequences, 1045 bp (including gaps). The ODC sequences comprised a hardly readable simple-sequence-repeat (SSR) region of 80 bp length, which could not be sequenced for all samples. This region was excluded from further analyses, resulting in a fragment length of 938 bp used for phylogenetic calculations.

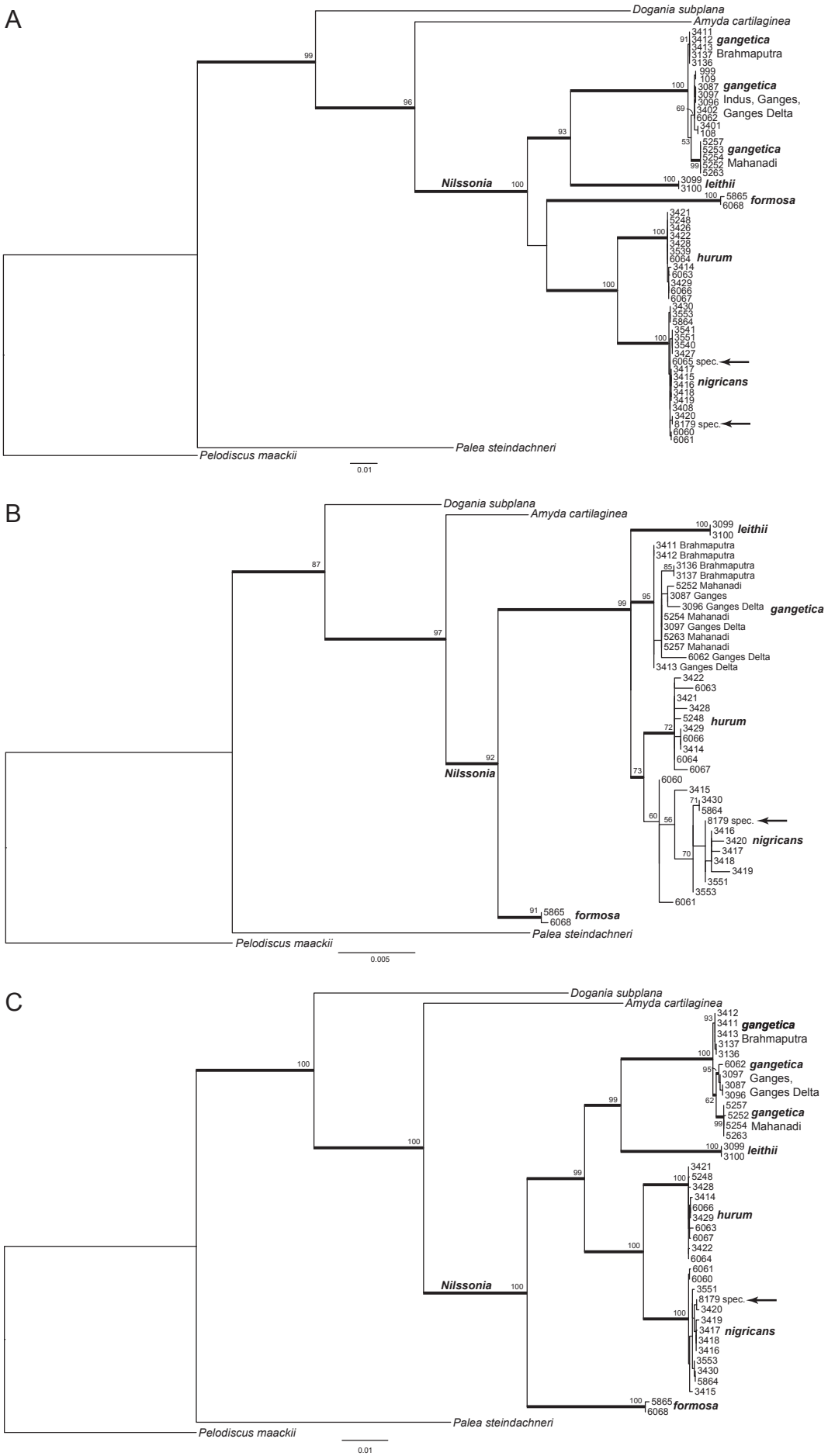
Three data sets were used for inferring phylogenetic relationships: (i) the concatenated mitochondrial sequence data of 53 *Nilssonia* samples, corresponding to an alignment of 2354 bp, including gaps; (ii) the concatenated nuclear sequence data of 40 *Nilssonia* samples, corresponding to an alignment of 2573 bp, including gaps; and (iii) a supermatrix, in which the respective mitochondrial sequence data were merged with the nuclear data of those 40 samples, corresponding to an alignment of 4927 bp, again including gaps.

For each of these data sets, phylogenetic trees were calculated using the Maximum Likelihood approach as implemented in RAxML 7.0.3 (Stamatakis, 2006) and Bayesian Inference of phylogeny as implemented in MrBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). For RAxML analyses, the data sets were partitioned by gene and the GTR+G model was applied across all partitions. Five independent ML calculations were run using different starting conditions and the fast bootstrap algorithm to examine the robustness of the branching patterns by comparing the best-scored trees. Subsequently, 1000 non-parametric thorough bootstrap replicates were computed and plotted against the tree with the highest likelihood value. Analyses with MrBAYES were run using unpartitioned mitochondrial and nuclear data sets; the supermatrix was partitioned in mtDNA and nDNA. The best evolutionary model was established using the Akaike Information Criterion of MrMODELTEST 2.3 (Posada & Crandall, 1998), resulting in the GTR+I+G model for the mtDNA data set and the HKY+G model for the nDNA data set. The chains of MrBAYES run for 10^7 generations, with every 100^{th} generation sampled. For computing the final 50% majority rule consensus tree, a burn-in of 4×10^4 was used.

Results

The phylogenetic trees obtained from the two methods were largely congruent for each data set (Figs 1A–C).

Fig. 1 →. Phylogeny of *Nilssonia* species and allied softshell turtles as inferred by Maximum Likelihood analysis, based on (**A**) an alignment of 2354 bp of mitochondrial DNA, (**B**) an alignment of 2573 bp of nuclear DNA, and (**C**) a supermatrix consisting of the concatenated mitochondrial and nuclear DNA partitions (4927 bp in total). Sample codes at branches are MTD T numbers and refer to the Appendix. Numbers along branches are thorough bootstrap values > 50, except for short terminal branches where support is not shown. Wide branches are supported by posterior probabilities ≥ 0.99 (A, C) or ≥ 0.95 (B) in Bayesian analyses. Note that no nuclear data could be produced for the samples from the Indus River system. Placement of the shell from Sreemangal (Bangladesh, sample 6065) and the morphologically aberrant turtle from Manikchhari (Bangladesh, sample 8179) highlighted by arrows.


A
Dogania subplana
Amyda cartilaginea
3411
3412 gangetica
3413
3137 Brahmaputra
3136
999
109
3087 gangetica
3097
3096 Indus, Ganges,
3402 Ganges Delta
6062
3401
108
5257
5253 gangetica
5254 Mahanadi
5252
5263
Nilssonia
3099 leithii
3100
5865 formosa
6068
3421
5248
3426
3422
3428
3539
6064 hurum
3414
6063
3429
6066
6067
3430
3553
5864
3541
3551
3540
3427
6065 spec.
3417
3415
3416 nigricans
3418
3419
3408
3420
8179 spec.
6060
6061
Palea steindachneri
Pelodiscus maackii
0.01
B
Dogania subplana
Amyda cartilaginea
3099 leithii
3100
3411 Brahmaputra
3412 Brahmaputra
3136 Brahmaputra
3137 Brahmaputra
5252 Mahanadi
3087 Ganges
3096 Ganges Delta gangetica
5254 Mahanadi
3097 Ganges Delta
5263 Mahanadi
5257 Mahanadi
6062 Ganges Delta
3413 Ganges Delta
3422
6063
3421
3428
5248 hurum
3429
6066
3414
6064
6067
Nilssonia
6060
3415
3430
5864
8179 spec.
3416
3420 nigricans
3417
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3419
3551
3553
6061
5865 formosa
6068
Palea steindachneri
Pelodiscus maackii
0.005
C
Dogania subplana
Amyda cartilaginea
3412
3411 gangetica
3413
3137 Brahmaputra
3136
6062 gangetica
3097
3087 Ganges,
3096 Ganges Delta
5257
5252 gangetica
5254 Mahanadi
5263
3099 leithii
3100
3421
5248
3428
3414
6066
3429 hurum
6063
6067
3422
6064
6061
6060
Nilssonia
3551
8179 spec.
3420
3419
3417 nigricans
3418
3416
3553
3430
5864
3415
5865 formosa
6068
Palea steindachneri
Pelodiscus maackii
0.01

Fig. 2. (**A**) *Nilssonia formosa*, juvenile (pet trade, Yangon, Myanmar), photo: P. Praschag; (**B**) *N. gangetica* (Brahmaputra clade), subadult (Biswanath Ghat, Assam, India), photo: P. Praschag; (**C**) *N. gangetica* (Brahmaputra clade), adult (Nagsankar Temple, east of Tezpur, Assam, India), photo: P. Praschag; (**D**) *N. gangetica* (Mahanadi clade), adult (Mahanadi River, Narsinghpur, Odisha, India), photo: P. Praschag; (**E**) *N. hurum*, juvenile (Subarnarekha River, Sibirpur, Odisha, India), photo: P. Praschag; (**F**) *N. leithii*, subadult (Supa River, Karnataka, India), photo: K. Vasudevan; (**G**) *N. nigricans*, juvenile (Jia Bhoroli River, Assam, India), photo: P. Praschag; (**H**) *N. nigricans*, subadult (Biswanath Ghat, Assam, India), photo: P. Praschag; (**I**) *N. nigricans*, adult (Tripura Sundari Temple, Udaipur, Tripura, India), photo: P. Praschag; (**J, K**) *N. nigricans*, unusually pale-coloured subadult (Manikchhari near Chittagong, Bangladesh), photos: S.M.A. Rashid.

Nilssonia constituted always a well-supported monophyletic clade and *Amyda*, *Dogania* and *Palea* were its successive sister taxa. Based on mitochondrial sequences alone and mitochondrial sequences combined with nuclear data, every species within *Nilssonia* corresponded to a well-supported clade. Within *N. gangetica*, three weakly to well-supported clades were revealed. One of these clades comprised sequences of softshell turtles from the Brahmaputra River. Another clade corresponded to sequences from the Indus and Ganges Rivers and the Ganges Delta, and the third clade contained sequences from the Mahanadi River. These clades were not found using nuclear data alone.

Mitochondrial and combined analyses suggested a well-supported sister group relationship of *N. gangetica* + *N. leithii* and of *N. hurum* + *N. nigricans*, re-

spectively. Using nuclear data, the relationships within *Nilssonia* were poorly resolved, except that *N. formosa* constituted with high support the sister taxon of all other species. Also combined analyses of mitochondrial and nuclear sequences supported this placement of *N. formosa*. By contrast, the phylogenetic position of *N. formosa* was poorly resolved by mitochondrial data alone.

Due to small sample size or bad DNA quality, not all genes could be sequenced for all samples (see Appendix). Nevertheless, the phylogenetic analyses allowed an unambiguous taxonomic assignment of all samples. This is of particular interest for the two Bangladeshi samples of questionable taxonomic identity. Sequences of these two samples were consistently embedded among *N. nigricans*.

Using mitochondrial cyt *b* sequences, uncorrected *p* distances between *Nilssonia* species ranged on average from 4.74% to 9.97%; divergences among the three clades within *N. gangetica* ranged from 0.66% to 0.75% (Table 3).

Discussion

Our results based on three mitochondrial genes and three nuclear loci confirm with high support the monophyly of *Nilssonia* sensu lato (cf. Meylan, 1987; Eng-

Table 3. Mean uncorrected *p* distances (percentages) and their standard errors within and between *Nilssonia* species and the three haplotype clades of *N. gangetica*, based on a 1067-bp-long alignment of the mitochondrial cytochrome *b* gene. Distances among groups are given below the diagonal; on the diagonal within-group divergences in boldface. Clade A of *N. gangetica* corresponds to turtles from the Brahmaputra River; clade B, to the Indus and Ganges Rivers and the Ganges Delta; and clade C, to the Mahanadi River.

	formosa	*gangetica* (all)	*gangetica* A	*gangetica* B	*gangetica* C	*hurum*	*leithii*	*nigricans*
formosa	**0.19 ± 0.13**							
***gangetica* (all)**	9.46 ± 0.90	**0.48 ± 0.14**						
***gangetica* A**	9.56 ± 0.91	—	**0**					
***gangetica* B**	9.37 ± 0.88	—	0.70 ± 0.26	**0.07 ± 0.05**				
***gangetica* C**	9.36 ± 0.88	—	0.75 ± 0.26	0.66 ± 0.24	**0**			
hurum	9.97 ± 0.91	8.70 ± 0.82	8.63 ± 0.86	8.76 ± 0.85	8.67 ± 0.84	**0.05 ± 0.05**		
leithii	8.72 ± 0.91	7.44 ± 0.78	7.46 ± 0.84	7.40 ± 0.84	7.50 ± 0.83	8.37 ± 0.85	**0**	
nigricans	9.43 ± 0.92	8.27 ± 0.84	8.14 ± 0.85	8.29 ± 0.86	8.36 ± 0.86	4.74 ± 0.61	7.94 ± 0.82	**0.14 ± 0.07**

strom *et al.*, 2004; Praschag *et al.*, 2007) and the previously suggested sister group relationship of *N. gangetica* + *N. leithii* and *N. hurum* + *N. nigricans*, respectively (Praschag *et al.*, 2007). Earlier studies using morphological (Meylan, 1987; Vitek, 2012) and molecular data (Praschag *et al.*, 2007) or combined analyses of morphological and molecular data (Engstrom *et al.*, 2004) could not resolve the phylogenetic placement of *N. formosa*, even though the monophyly of the five species was unequivocal. Our analyses of nuclear data and the combined analyses of nuclear and mitochondrial data revealed now a well-supported sister group relationship of *N. formosa* and the remaining *Nilssonia* species, so that it could be argued that this supports the original classification by Meylan (1987) placing *N. formosa* into a distinct monotypic genus. However, in contrast to other chelonian species where pronounced morphological or phylogenetic gaps justify the usage of monotypic genera (Fritz *et al.*, 2011b), all five *Nilssonia* species are morphologically highly similar (Praschag *et al.*, 2007) and the degree of genetic distinctness of *N. formosa* resembles the divergences among the remaining four species (Fig. 1C; Table 3).

All *Nilssonia* species are characterized by conspicuous ocelli on their carapace, which disappear with increasing age (Fig. 2), and all species are large-sized, reaching maximum shell lengths of 60 to 94 cm (Ernst & Barbour, 1989; Ernst *et al.*, 2000). Meylan's (1987) assignment of *N. formosa* to a monotypic genus was based on just one osteological character. In the bony carapace of *N. formosa*, a single neural plate is present between the first pair of pleurals, resulting from the fusion of neurals one and two, whereas the remaining four *Nilssonia* species have the two anteriormost neurals unfused. However, as Praschag *et al.* (2007) pointed out, the character state in *N. formosa* should be regarded as an autapomorphy that does not contradict the inclusion of all five species in one and the same genus, and we argue that their well-supported monophyly together with their morphological similarity supports the inclusion of all five species in the same genus.

Previously, *N. formosa* was only known with certainty from Myanmar, with a questionable record for Thailand (Fritz & Havaš, 2007; van Dijk *et al.*, 2011). Our sample from Shuangbai (Yunnan), China, suggests that the species crossed the watershed between the Salween and Mekong Rivers and occurs also in Yunnan, China. Photos of a further specimen of *N. formosa* (filed in the Museum of Zoology, Senckenberg Dresden) caught in the Lancang River (Xishuangbanna, Yunnan), which is downstream called Mekong, support this.

Our data provide clear evidence that wild *N. nigricans* occur in Bangladesh. One of the studied Bangladeshi samples originated from the shell of a slaughtered turtle from Sreemangal (Sylhet District), and the other is from a morphologically aberrant pale turtle caught on a hook near Chittagong (Manikchhari; Figs 2J, K). Sequences generated from these samples clustered in all analyses with high support among *N. nigricans* (Fig. 1). This critically endangered species (van Dijk *et al.*, 2011) was long thought to be extinct in the wild and assumed to survive only in an artificial pond of the Hazrat Sultan Bayazid Bostami Shrine in Nasirabad near Chittagong, Bangladesh (Anderson, 1875; Ernst & Barbour, 1989; Ernst *et al.*, 2000). Only ten years ago Praschag & Gemel (2002) suggested that wild *N. nigricans* occur in Assam (India), and this was confirmed genetically by Praschag *et al.* (2007). However, until now wild *N. nigricans* were not known from Bangladesh, so that our genetically identified samples are the first record for this country. Furthermore, the pale softshell turtle from Manikchhari suggests that coloration of *N. nigricans* is more variable than thought before (cf. Fig. 2).

With respect to *N. gangetica*, we discovered a clear association of distinct mitochondrial haplotypes with

distinct river basins. While the differentiation between the Indus-Ganges system and the Brahmaputra was already known (Praschag *et al.*, 2007), we included in our present study for the first time samples from the Mahanadi River. Also these softshell turtles correspond to a distinct haplotype clade (Fig. 1). This suggests that each major river basin harbours a genetically distinct population of *N. gangetica*, which should be treated as a distinct management unit. In analogy to the widely used barcoding approach (Hebert *et al.*, 2003), uncorrected *p* distances of the mitochondrial cyt *b* gene have repeatedly been used as a yardstick for assessing the taxonomic status of turtles and tortoises (e.g., Spinks *et al.*, 2004; Vargas-Ramírez *et al.*, 2010; Praschag *et al.*, 2011; Stuckas & Fritz, 2011; Fritz *et al.*, 2012a, b; Kindler *et al.*, 2012). The average divergences among the five *Nilssonia* species (Table 3: 4.74-9.97%) are six to fifteen times larger than the differentiation among the three haplotype clades of *N. gangetica* (0.66-0.75%), and the latter values fall into the range as observed within other trionychid species (Stuckas & Fritz, 2011). This suggests that the genetic differentiation among different river basins represents indeed intraspecific variation within *N. gangetica* and that no cryptic species are involved. Nevertheless, considering that *N. gangetica* is an endangered species (van Dijk *et al.*, 2011), the genetic distinctiveness of the populations in different river basins has to be taken into account when future conservation strategies are designed. In this context, it is of interest that Annandale (1912) described a distinct subspecies from the Mahanadi system, *Trionyx gangeticus mahanaddicus*. It was later synonymized with *N. gangetica* (Smith, 1931). If a taxonomic distinction for the management unit in the Mahanadi River should be desired, the name *Nilssonia gangetica mahanaddica* nov. comb. (Annandale, 1912) were available for this population, whereas the name *Nilssonia gangetica gangetica* nov. comb. (Cuvier, 1825) would have to be used for the population in the Indus and Ganges systems.

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Appendix

Nilssonia samples and outgroups used in the present study. MTD refers to samples from the tissue collection of the Museum of Zoology, Senckenberg Dresden. The DNA fragments labelled as ND4 and cyt *b* contain also adjacent DNA coding for tRNAs. ODC1 corresponds to the DNA fragment preceding the SSR region, ODC2 to the DNA fragment after the SSR region (see Materials and Methods).

MTD	Taxon	Provenance	Genbank accession numbers						
			12S	ND4	cyt b	C-mos	ODC1	ODC2	R35
6068	*Nilssonia formosa*	China: Yunnan: Shuangbai	HE801637	HE801688	HE801740	HE801763	HE801806	HE801844	HE801869
5865	*Nilssonia formosa*	Myanmar: Yangon (local pet trade)	HE801638	HE801689	HE801741	HE801764	HE801807	HE801845	HE801870
3411	*Nilssonia gangetica*	Bangladesh: Mymensingh: Old Brahmaputra	HE801639	HE801690	AM495208	HE801765	HE801808	—	HE801871
3412	*Nilssonia gangetica*	Bangladesh: Mymensingh: Old Brahmaputra	HE801640	HE801691	AM495209	HE801766	HE801809	—	HE801872
3413	*Nilssonia gangetica*	Bangladesh: Mymensingh: Old Brahmaputra	HE801641	HE801692	AM495210	HE801767	—	—	HE801873
6062	*Nilssonia gangetica*	Bangladesh: Patuakhali District	HE801642	HE801693	HE801742	HE801768	HE801810	HE801846	HE801874
3136	*Nilssonia gangetica*	India: Assam: Biswanath Ghat	HE801643	HE801694	AM495211	HE801769	HE801811	HE801847	HE801875
3137	*Nilssonia gangetica*	India: Assam: Biswanath Ghat	HE801644	HE801695	HE801743	HE801770	HE801812	HE801848	HE801876
5257	*Nilssonia gangetica*	India: Odisha: Devi River (20 km inland)	HE801645	HE801696	HE801744	HE801771	HE801813	HE801849	HE801877
5252	*Nilssonia gangetica*	India: Odisha: Narsinghpur: Mahanadi River	HE801646	HE801697	HE801745	HE801772	HE801814	HE801850	HE801878
5253	*Nilssonia gangetica*	India: Odisha: Narsinghpur: Mahanadi River	HE801647	HE801698	HE801746	—	—	—	—
5254	*Nilssonia gangetica*	India: Odisha: Narsinghpur: Mahanadi River	HE801648	HE801699	HE801747	HE801773	HE801815	HE801851	HE801879
5263	*Nilssonia gangetica*	India: Odisha: Narsinghpur: Mahanadi River	HE801649	HE801700	HE801748	HE801774	HE801816	HE801852	HE801880
3087	*Nilssonia gangetica*	India: Uttar Pradesh: Chambal River	HE801650	HE801701	AM495212	HE801775	—	—	HE801881
3096	*Nilssonia gangetica*	India: West Bengal: Howrah (Haora) Market	HE801651	HE801702	AM495213	HE801776	HE801817	HE801853	HE801882
3097	*Nilssonia gangetica*	India: West Bengal: Howrah (Haora) Market	HE801652	HE801703	AM495214	HE801777	HE801818	HE801854	HE801883
108	*Nilssonia gangetica*	Pakistan	HE801653	HE801704	HE801749	—	—	—	—
109	*Nilssonia gangetica*	Pakistan	HE801654	HE801705	HE801750	—	—	—	—
999	*Nilssonia gangetica*	Pakistan	HE801655	HE801706	HE801751	—	—	—	—
3401	*Nilssonia gangetica*	Pakistan	HE801656	HE801707	AM495215	—	—	—	—
3402	*Nilssonia gangetica*	Pakistan	—	HE801708	AM495216	—	—	—	—
3421	*Nilssonia hurum*	Bangladesh: Khulna	HE801657	HE801709	AM495218	HE801778	HE801819	HE801855	HE801884
3422	*Nilssonia hurum*	Bangladesh: Khulna	HE801658	HE801710	AM495219	HE801779	HE801820	HE801856	HE801885
3414	*Nilssonia hurum*	Bangladesh: Mymensingh: Old Brahmaputra	HE801659	HE801711	AM495220	HE801780	HE801821	HE801857	HE801886
6063	*Nilssonia hurum*	Bangladesh: Patuakhali District	HE801660	HE801712	HE801752	HE801781	HE801822	HE801858	HE801887
6064	*Nilssonia hurum*	Bangladesh: Patuakhali District	HE801661	HE801713	HE801753	HE801782	HE801823	HE801859	HE801888
3426	*Nilssonia hurum*	Bangladesh: 20 km E Dhaka: Sonargaon Market	HE801662	HE801714	AM495223	—	—	—	—

Appendix continued.

MTD	Taxon	Provenance	Genbank accession numbers						
			12S	ND4	cyt b	C-mos	ODC1	ODC2	R35
3539	*Nilssonia hurum*	Bangladesh: 20 km E Dhaka: Sonargaon Market	—	HE801715	AM495222	—	—	—	—
6066	*Nilssonia hurum*	Bangladesh: Sylhet District: Sreemangal	HE801663	HE801716	HE801754	HE801783	HE801824	HE801860	HE801889
6067	*Nilssonia hurum*	Bangladesh: Sylhet District: Sreemangal	HE801664	HE801717	HE801755	HE801784	—	—	HE801890
5248	*Nilssonia hurum*	India: Odisha: Sibirpur: Subarnarekha River	HE801665	HE801718	HE801756	HE801785	HE801825	HE801861	HE801891
3428	*Nilssonia hurum*	India: Assam: Biswanath Ghat	HE801666	HE801719	AM495224	HE801786	HE801826	HE801862	HE801892
3429	*Nilssonia hurum*	India: Assam: Biswanath Ghat	HE801667	HE801720	AM495221	HE801787	HE801827	HE801863	HE801893
3099	*Nilssonia leithii*	India: Maharashtra: Pawna River	HE801668	HE801721	AM495225	HE801788	HE801828	HE801864	HE801894
3100	*Nilssonia leithii*	India: Maharashtra: Pawna River	HE801669	HE801722	AM495226	HE801789	HE801829	HE801865	HE801895
3415	*Nilssonia nigricans*	Bangladesh: Chittagong: Nasirabad: Shrine Pond	HE801670	HE801723	AM495227	HE801790	HE801830	HE801830	HE801896
3416	*Nilssonia nigricans*	Bangladesh: Chittagong: Nasirabad: Shrine Pond	HE801671	HE801724	AM495228	HE801791	HE801831	HE801831	HE801897
3417	*Nilssonia nigricans*	Bangladesh: Chittagong: Nasirabad: Shrine Pond	HE801672	HE801725	AM495229	HE801792	HE801832	—	HE801898
3418	*Nilssonia nigricans*	Bangladesh: Chittagong: Nasirabad: Shrine Pond	HE801673	HE801726	AM495230	HE801793	HE801833	HE801833	—
3419	*Nilssonia nigricans*	Bangladesh: Chittagong: Nasirabad: Shrine Pond	HE801674	HE801727	AM495231	HE801794	HE801834	HE801834	HE801899
3420	*Nilssonia nigricans*	Bangladesh: Chittagong: Nasirabad: Shrine Pond	HE801675	HE801728	AM495232	HE801795	HE801835	HE801835	HE801900
3408	*Nilssonia nigricans*	India: Assam: Guwahati: Kamakhya Temple Pond	HE801676	HE801729	—	—	—	—	—
3427	*Nilssonia nigricans*	India: Assam: Guwahati: Kamakhya Temple Pond	HE801677	HE801730	AM495234	—	—	—	—
3540	*Nilssonia nigricans*	India: Assam: Guwahati: Kamakhya Temple Pond	HE801678	HE801731	AM495235	—	—	—	—
3541	*Nilssonia nigricans*	India: Assam: Guwahati: Kamakhya Temple Pond	HE801679	HE801732	AM495236	—	—	—	—
3551	*Nilssonia nigricans*	India: Assam: Guwahati: Kamakhya Temple Pond	HE801680	HE801733	AM495237	HE801796	HE801836	HE801866	HE801901
3430	*Nilssonia nigricans*	India: Assam: Jia Bhoroli River	HE801681	HE801734	AM495233	HE801797	HE801837	—	HE801902
3553	*Nilssonia nigricans*	India: Assam: Jia Bhoroli River	HE801682	HE801735	HE801757	HE801798	HE801838	—	HE801903
5864	*Nilssonia nigricans*	India: Assam: Jia Bhoroli River	HE801683	HE801736	HE801758	HE801799	HE801839	HE801867	HE801904
6060	*Nilssonia nigricans*	India: West Bengal: Jalpaiguri District: Alipurduar: Swaneswar Temple	HE801684	HE801737	HE801759	HE801800	HE801840	—	HE801905
6061	*Nilssonia nigricans*	India: West Bengal: Jalpaiguri District: Alipurduar: Swaneswar Temple	HE801685	HE801738	HE801760	HE801801	—	—	HE801906
8179	*Nilssonia* spec.	Bangladesh: Manikchhari near Chittagong	HE801686	HE801739	HE801761	HE801802	—	—	HE801907
6065	*Nilssonia* spec.	Bangladesh: Sylhet District: Sreemangal	HE801687	—	HE801762	—	—	—	—
	Amyda cartilaginea		—	AY259600	AY259550	HE801803	HE801841	—	HE801908
	Dogania subplana		AF366350	AF366350	AF366350	—	HE801842	—	HE801909
	Palea steindachneri		FJ541030	FJ541030	FJ541030	HE801804	HE801843	HE801868	HE801910
4235, 4236	*Pelodiscus maackii*	Russia: Primorsky Territory: Lake Khanka	FM999003	FM999019	FM999011	HE801805	—	—	HE801911