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Phylogeography of the Asian softshell turtle *Amyda cartilaginea* (Boddaert, 1770): evidence for a species complex

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Abstract

Using up to 2456 bp mtDNA and up to 2716 bp nDNA of fresh samples and short sequences of three mitochondrial genes of historical museum material, we examine the phylogeography of *Amyda cartilaginea*. This data set provides evidence for the existence of deeply divergent genetic lineages which we interpret as three distinct species, two of which are polytypic. On the Great Sunda Islands, the distribution ranges of the two subspecies of *Amyda cartilaginea* (Boddaert, 1770) sensu stricto and of an undescribed species match palaeodrainage systems. *Amyda cartilaginea cartilaginea* occurs in the East Sunda palaeodrainage, with records in eastern Borneo and Java. Also a record from Sulawesi, most probably not representing a native population, refers to *A. c. cartilaginea*. In the North Sunda palaeodrainage (Sumatra, western Borneo) lives *Amyda cartilaginea maculosa* subsp. nov., which is described herein. One sample from the Baram river (Sarawak, Malaysia) is genetically highly distinct and represents a new species. We refrain from naming this taxon until more material becomes available for morphological characterization. For the continental populations, we resurrect the species *Amyda ornata* (Gray, 1861). We identify Asian softshell turtles from the Mekong drainage with the nominotypical subspecies, while the genetically distinct populations from Thailand and Myanmar are assigned to *Amyda ornata phayrei* (Theobald, 1868). Samples from Bangladesh are also genetically distinct and represent an undescribed subspecies and the first country record for *Amyda*.

Key words

Amyda cartilaginea cartilaginea; *Amyda cartilaginea maculosa* subsp. nov.; *Amyda ornata ornata*; *Amyda ornata phayrei*; Great Sunda Islands; Southeast Asia, Subspecies; Taxonomy; Testudines; Trionychidae.

Introduction

Among extant turtles, softshells (Trionychidae) belong to the species which deviate most from the general chelonian body plan. Softshell turtles possess a much reduced bony shell and the shell surface is covered by a leathery skin instead of a horny epidermis. The snout is a proboscis resembling a pig nose, and trionychids are the only turtles having fleshy lips. Softshells are highly aquatic, and their paddle-like feet possess extensive webbing. Each foot is armed with three strong claws (Meylan 1987; Ernst et al. 2000; Delfino et al. 2010) used for killing and dissecting prey. Trionychids belong to the fastest swimming freshwater turtles (Webb 1962; Meylan 1987) with good manoeuvre skills, making many species vigorous predators of fish and crayfish (Delfino et al. 2010). While most softshell turtles are thought to be completely confined to freshwater habitats (Ernst et al. 2000), two species are

known to enter regularly marine habitat (*Pelochelys cantorii*: Das 2008; *Trionyx triunguis*: Shanas et al. 2012). The greatest diversity of extant trionychids is found in Asia, where both subfamilies occur (Cyclanorbinae and Trionychinae, with 3 and 21 species in 1 and 10 genera, respectively). This corresponds to 43% of all seven extant cyclanorbine species and 88% of all 24 extant trionychine species. The remaining extant softshell turtles occur in Africa (2 cyclanorbine genera with 4 species) and North America (1 trionychine genus with 3 species). The eponymous genus *Trionyx* (Trionychinae) occurs mainly in Africa but reaches in the eastern Mediterranean region also the Asian continent (*cf.* Fritz & Havaš 2007; van Dijk et al. 2012; Le et al. 2014).

External morphology of softshell turtles is often only of limited utility for taxonomy and systematics, because drastic ontogenetic changes in coloration and body proportions blur diagnostic characters. Another problem is posed by the fact that many species reach considerable sizes with shell lengths beyond 30–40 cm, so that adult individuals are rarely present in natural history museums. Therefore, much emphasis was given to osteological characters. While this has allowed a pioneering assessment of the phylogenetic relationships (Meylan 1987), the rarity of material of many taxa hampered a detailed understanding of the species diversity. Thus, it is not surprising that several studies using molecular genetic approaches have gained a much refined understanding of trionychid taxonomy (Weisrock & Janzen 2000; Engstrom et al. 2002, 2004; Praschag et al. 2007, 2011; McGaugh et al. 2008; Fritz et al. 2010; Stuckas & Fritz 2011; Liebing et al. 2012; Le et al. 2014) and resulted in the recognition of six additional species (*Chitra chitra*, *C. vandijki*, *Lissemys ceylonensis, Pelodiscus axenaria*, *P. maackii*, *P. parviformis*; Engstrom et al. 2002; McCord & Pritchard 2003; Fritz et al. 2010; Praschag et al. 2011; Stuckas & Fritz 2011) and two additional subspecies (*Chitra chitra javanensis*, *Lissemys punctata vittata*; Engstrom et al. 2002; McCord & Pritchard 2003; Praschag et al. 2011) compared to previous morphology-based assessments (Webb 1982; Meylan 1987). However, molecular studies have not yet been conducted for the majority of trionychid species, so that the existence of further distinct, but currently unrecognized, taxa is most likely. If it is considered that softshells are seriously threatened by massive overexploitation for food and Traditional Chinese Medicine (Auliya 2000; van Dijk & Palasuwan 2000; Kuchling et al. 2004; Chen et al. 2009), such bad taxonomy may even contribute to the loss of species due to lacking legislative protection based on erroneous categories of threat. Thus, a better understanding of the taxonomy of many softshell turtles is of paramount interest for conservation, although current export practices of samples for scientific study are in this context often completely counterproductive.

The present investigation illustrates this situation and may serve as an exemplar study for turtle species classified in low IUCN categories of threat based on bad taxonomy (*cf.* Petzold et al. 2014 for another case study). As currently understood the Asian softshell turtle, *Amyda cartilaginea* (Boddaert, 1770), is a widely distributed monotypic species (Ernst et al. 2000; Fritz & Havaš 2007; van Dijk et al. 2012) and listed in the category "Vulnerable" by the IUCN (2013). Asian softshells are large-sized, with adult shell lengths exceeding 40 cm (van Dijk 1992) and a reported maximum shell length of 83 cm (Ernst et al. 2000). *Amyda cartilaginea* is distributed in northeastern India (Mizoram), Myanmar, Laos, Vietnam, Cambodia and Thailand and ranges through the Malay Peninsula to Sumatra, Java and Borneo (Iverson 1992; van Dijk et al. 2012); populations on the Lesser Sunda Islands and Sulawesi are thought to be introduced (Koch et al. 2008; van Dijk et al. 2012), and this could be also true for Yunnan, China (Kuchling 1995).

Even though *A. cartilaginea* is generally accepted as a monotypic species, van Dijk (1992) distinguished three morphologically distinguishable forms which could represent either distinct taxa or, favoured by van Dijk (1992), a single variable species. Here we use fresh samples representing these morphotypes and most of the distribution range of *A. cartilaginea* to generate up to 2456 bp mitochondrial DNA (mtDNA) and up to 2716 bp nuclear DNA (nDNA). Our material also includes samples from three large softshells from the Chittagong region (Bangladesh) which were morphologically identified as *A. cartilaginea*, although the species was not recorded from that country yet (van Dijk et al. 2012). In addition, we supplement our data set with historical DNA sequences from museum specimens (up to 241 bp mtDNA). We analyse these DNA sequences using standard approaches (Maximum Likelihood, Bayesian Inference) and try to correlate the revealed differentiation pattern with nominal taxa currently synonymised with *A. cartilaginea*. In doing so, we provide for the first time a molecular genetic assessment of the phylogeography and taxonomy of *A. cartilaginea*.

Material and methods

Sampling and laboratory procedures

For the present study, 19 fresh blood or tissue samples of *Amyda cartilaginea* plus 14 tissue samples from historical museum specimens were used (Table S1). For fresh material, three mitochondrial and three nuclear DNA fragments were chosen, which have been successfully used in softshell turtles for phylogenetic and phylogeographic purposes (Weisrock & Janzen 2000; Engstrom et al. 2002, 2004; Praschag et al. 2007, 2011; McGaugh et al. 2008; Fritz et al. 2010; Stuckas & Fritz 2011; Liebing et al. 2012; Le et al. 2014), namely, part of the 12S ribosomal RNA gene (12S rRNA or 12S), the complete

cytochrome *b* gene (cyt *b*) plus adjacent DNA coding for tRNA-Thr, part of the 3' half of the NADH dehydrogenase subunit 4 gene (ND4) plus adjacent DNA coding for tRNAs, part of the gene coding for oocyte maturation factor Mos (Cmos), part of the gene coding for ornithine decarboxylase (ODC), and part of intron 1 of the orphan G protein-coupled receptor gene R35 (R35). For PCR and sequencing of the 12S, ND4, Cmos, ODC and R35 fragments of fresh samples, the same primer pairs were applied as in Liebing et al. (2012). However, for the cyt *b* gene, which was amplified and sequenced in two parts overlapping by 383 bp, the primer pairs CytbG + mt-E-Rev2 and mt-c-For2 + mt-f-na (Spinks et al. 2004; Fritz et al. 2006) were used.

Since DNA from historical museum specimens is much degraded and fragmented, only short fragments corresponding to highly variable regions of the three mitochondrial markers 12S, cyt *b* and ND4 were sequenced using new primers (Table S2). These primers were designed using consensus sequences of the fresh *Amyda* samples.

Total genomic DNA of fresh samples was extracted using the innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany). PCR was performed using 1–5 µl of DNA extraction in a 20 µl volume containing 0.5 µM of each primer, 0.5 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 1 unit of *Taq* polymerase (Bioron DFS-Taq, Bioron GmbH, Ludwigshafen, Germany), 2 µl PCR buffer 10× incl. $MgCl_2$, and ultrapure H_2O. For applied PCR programs, see Table S3. PCR products were visualised on a 1% agarose gel and cleaned up using the ExoSAP-IT reagent (USB Europe GmbH, Staufen, Germany; 1:20 dilution, modified protocol: 30 min at 37°C, 15 min at 80°C). If necessary, DNA bands were alternatively excised from a 2% agarose gel and purified using the peqGOLD Gel Extraction Kit (peqlab Biotechnologie GmbH, Erlangen, Germany). For cycle sequencing the same forward and reverse primers were used as for PCR. The total reaction volume of 10 µl contained 2 µl sequencing buffer, 1 µl premix, 0.5 µM of the respective primer, 0.5–6 µl DNA template, and ultrapure H_2O. Using the ABI PRISM Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 25–30 cycles were run at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Reaction products were purified using Sephadex™ G-50 fine (GE Healthcare, München, Germany). Sequencing was performed on an ABI 3130xl Genetic Analyser (Applied Biosystems).

Extraction of historical material was carried out in a clean room, which is physically isolated from all other DNA processing facilities. Prior to this study, no *Amyda* material had been studied there (fresh samples used in the present study were processed in the main laboratory, which is located in another building). All work in the clean room was performed in three different HERAsafe KSP 9 safety cabinets (Thermo Fisher Scientific, Waltham, MA, USA) corresponding to the working steps sample preparation, DNA extraction, and PCR setup. Safety cabinets and clean room were irradiated with UV light for at least 6 h before and after every working step. For DNA extraction, the sbeadex forensic kit (LGC Genomics, Berlin, Germany) was used according to the manufacturer's standard protocol. PCR setup was identical to fresh samples, except that 1 µl of Mg^{2+} and 0.2 µl (20 ng/µl) of Bovine Serum Albumin (Fermentas) were added to each sample. Primers and PCR programs used for historical samples are summarized in Tables S2 and S3. Thermocycling was carried out in the main laboratory, and a positive control (containing DNA of a fresh *Amyda* sample, extracted with standard methods in the normal DNA processing facility) and a negative control (all reagents except the DNA template) were always processed downstream along with the historical samples. Additional working steps were identical as for fresh material. The lengths of the resulting gene fragments (after primers were trimmed) were 63 bp for 12S, 108 bp for cyt *b*, and 70 bp for ND4 (Table S2).

Alignment and phylogenetic analyses, uncorrected *p* distances

Obtained DNA sequences were checked manually for base-calling errors and aligned in bioedit 7.0.9.0 (Hall 1999). For phylogenetic analyses, our newly generated sequences were concatenated and merged with previously published homologous sequences of *Dogania subplana*, *Palea steindachneri*, *Pelodiscus maackii* and all species of *Nilssonia* (Table S1). According to Engstrom et al. (2004), *Nilssonia* sensu lato (see Praschag et al. 2007), *D. subplana*, *Palea steindachneri* and *Pelodiscus* represent the successive sister taxa of *Amyda*. Two data sets were used for calculations. One alignment of 2456 bp length comprised only the concatenated mtDNA sequences. In the second alignment of 5172 bp length, nDNA sequences were added as far as available. For phylogenetic analyses, the alignments were partitioned by gene. However, all DNA coding for tRNAs was lumped together in one partition, so that 394 bp corresponded to 12S, 1140 bp to cyt *b*, 710 bp to ND4, 212 bp to the DNA coding for tRNAs, 590 bp to Cmos, 1081 bp to ODC, and 1045 bp to R35.

Phylogenetic relationships were then inferred using Bayesian and Maximum Likelihood (ML) approaches. For Bayesian analyses, the best evolutionary model for each partition was established by the Bayesian Information Criterion in jmodeltest 2.3 (Posada 2008), resulting in the TrN+G model for 12S, the TIM2+I model for cyt *b*, the TrN+G model for ND4, the TPM3uf+G model for the merged tRNAs, the K80+I model for Cmos, the F81 model for ODC, and the HKY model for R35. Calculations were performed with mrbayes 3.2.1 (Ronquist et al. 2012) using two parallel runs (each with four chains) and default parameters. The chains ran for 10 million generations with every 100th generation sampled. The calculation parameters were analysed using a burn-in of 2.5 mil-

lion generations to assure that both runs converged. Subsequently, only the plateau of the most likely trees was sampled using the same burn-in, and a 50% majority rule consensus tree was generated. The posterior probability of any individual clade in this consensus tree corresponds to the percentage of all trees containing that clade, and is a measure of clade frequency and credibility.

ML analyses were conducted using RAxML 7.2.8 (Stamatakis 2006) and the default GTR+G model. Five independent ML searches were run using different starting conditions and the fast bootstrap algorithm to explore the robustness of the branching patterns by comparing the best trees. Subsequently, 1000 non-parametric thorough bootstrap replicates were calculated and the values plotted against the best tree.

In addition, average uncorrected *p* distances were obtained for the mitochondrial genes using MEGA 6.06 (Tamura et al. 2013) and the pairwise deletion option. For comparative purposes, all *Nilssonia* sequences from Praschag et al. (2007) and Liebing et al. (2012) were included in these calculations.

Results

Phylogenetic inference

The topologies of the ML and Bayesian trees were for each data set (mtDNA *vs.* mtDNA combined with nDNA) completely identical, and there were only minor differences in the topologies when the mitochondrial trees are compared to the trees based on concatenated mitochondrial and nuclear DNA.

With respect to the phylogenetic relationships of *Amyda*, all analyses found the *Amyda* sequences with high support monophyletic (Fig. 1), and *Nilssonia*, *Dogania subplana* and *Palea steindachneri* constituted, with high support, the successive sister taxa. Parenthetically it may be noted that there is no evidence for a close relationship of *Nilssonia formosa* and *Amyda*, as suggested by van Dijk (1992) who speculated that both taxa could represent the same genus. *Nilssonia formosa* was placed together with the remaining four *Nilssonia* species in a well-supported monophylum. Within *Amyda*, three deeply divergent clades (A, B, C) were revealed, two of which showed further structuring.

A single sample from the Baram river, Sarawak (Borneo), was highly distinct and sister to all remaining samples of *Amyda* (clade 1/A in Fig. 1). The remaining samples grouped in five additional terminal clades in the analyses based on mtDNA alone (clades 2–6 in Fig. 1), which clustered in the two more inclusive clades B and C. One of the terminal clades of clade B (clade 3) was only weakly supported and not found in the calculations using concatenated mtDNA and nDNA. Then, the sequences of the respective samples were placed in clade B in a basal polytomy together with the well-supported clade 2 (trees not shown). In addition, *N. formosa* constituted in the trees based on mtDNA and nDNA, with high support, the sister taxon of the remaining *Nilssonia* species (see also Liebing et al. 2012). Otherwise the trees resulting from the merged mtDNA and nDNA sequences were identical to the trees based on mtDNA alone, and support values showed only negligible differences.

Within clade B, samples from Java, Sulawesi and most samples from Kalimantan (Borneo), plus some samples of unknown geographical provenance, were consistently placed in clade 2. In analyses of mtDNA alone, clade 2 was sister to clade 3 which contained also samples from Borneo (Kalimantan, Sarawak) and one sample from Sumatra. Clade B was sister to the moderately supported clade C comprised of the three well-supported terminal clades 4–6. The first of these terminal clades corresponded to samples from Bangladesh (clade 4), the second to samples from Thailand, China (Yunnan) and Myanmar plus one sample without geographical provenance (clade 5), and the third to samples from Cambodia and Laos (clade 6).

Uncorrected *p* distances

When uncorrected *p* distances of the different clades of *Amyda* are compared to *Nilssonia* it is obvious that some, but not all, of the six terminal clades show pairwise divergences resembling different *Nilssonia* species (Tables 1–3). For *Nilssonia*, the lowest values are found between the sympatric sister species *N. hurum* and *N. nigricans*, with 0.83% sequence divergence for 12S, 4.87% for cyt *b*, and 3.21% for ND4. The divergences of the single softshell from the Baram river (clade 1/A) clearly exceed these values for each gene, while the pairwise divergences for the terminal clades 2–6 are lower. Yet, most values still resemble the species divergences within *Nilssonia*, even though a sometimes contradictory pattern emerges for the individual genes. The divergences between the more inclusive clades B and C generally exceed or, at least, closely resemble the divergence values between *N. hurum* and *N. nigricans*.

Discussion

We discovered considerable phylogeographic structure within what is currently understood as the monotypic species *Amyda cartilaginea*. Using up to 2456 bp mtDNA and 2716 bp nDNA, we found three deeply divergent

Table 1. Uncorrected *p* distances (percentages) for the partial 12S gene (394 bp) of *Amyda*, *Nilssonia* and allied softshell turtles. Data for *Nilssonia* species are from Praschag et al. (2007) and Liebing et al. (2012). Between-group distances below diagonal, within-group distances along diagonal in boldface. Critical divergence value of *Nilssonia hurum* and *N. nigricans* bears asterisk.

	n	1 (A)	2	3	4	5	6	B	C	*for*	*gan*	*hur*	*lei*	*nig*	*sub*	*ste*	*maa*
Amyda – 1 (A)	1	—															
Amyda – 2	11	3.47	**0.38**														
Amyda – 3	4	1.98	3.29	**0.53**													
Amyda – 4	3	3.17	2.39	2.62	**0**												
Amyda – 5	10	4.77	4.23	5.51	0.93	**0**											
Amyda – 6	4	4.76	3.50	4.58	0.53	0.13	**0**										
Amyda – 2+3 (B)	15	3.08	—	—	—	—	—	**1.61**									
Amyda – 4+5+6 (C)	17	4.49	—	—	—	—	—	4.01	**0.29**								
Nilssonia formosa	2	7.94	6.38	8.22	4.63	6.96	4.63	6.87	6.00	**0.26**							
Nilssonia gangetica	18	11.29	6.16	8.14	4.24	7.79	4.51	6.69	6.39	3.23	**0**						
Nilssonia hurum	11	9.52	4.99	7.24	3.74	5.72	3.74	5.59	4.90	2.43	2.10	**0.05**					
Nilssonia leithii	2	9.68	5.28	6.08	4.26	6.98	4.52	5.49	5.92	2.46	2.33	2.31	**0**				
Nilssonia nigricans	18	9.70	5.43	7.68	4.53	6.20	4.53	6.03	5.51	3.24	2.32	0.83*	3.08	**0.05**			
Dogania subplana	1	9.52	10.16	6.24	6.93	10.77	7.47	9.12	9.31	7.40	6.77	6.52	7.03	6.71	—		
Palea steindachneri	1	6.35	6.79	7.39	7.16	9.29	7.69	6.95	8.54	7.36	8.29	8.01	7.51	8.25	8.05	—	
Pelodiscus maackii	1	12.70	13.12	9.78	9.28	13.53	9.81	12.23	11.91	9.95	10.62	9.82	10.08	10.57	10.91	6.98	—

Table 2. Uncorrected *p* distances (percentages) for the cyt *b* gene (1140 bp) of *Amyda*, *Nilssonia* and allied softshell turtles. DNA coding for tRNA-Thr was removed so that only protein-coding DNA was used for distance calculations. For further explanations, see Table 1.

	n	1 (A)	2	3	4	5	6	B	C	*for*	*gan*	*hur*	*lei*	*nig*	*sub*	*ste*	*maa*
Amyda – 1 (A)	1	—															
Amyda – 2	11	8.19	**0.34**														
Amyda – 3	4	5.13	2.87	**0.66**													
Amyda – 4	3	5.56	3.95	3.22	**0**												
Amyda – 5	10	5.58	4.37	3.57	1.96	**0.02**											
Amyda – 6	4	7.18	5.01	4.23	2.74	2.46	**0.39**										
Amyda – 2+3 (B)	15	7.38	—	—	—	—	—	**1.42**									
Amyda – 4+5+6 (C)	17	5.95	—	—	—	—	—	4.24	**1.42**								
Nilssonia formosa	2	12.04	15.82	14.05	12.81	14.00	12.81	15.34	13.51	**0.19**							
Nilssonia gangetica	19	12.96	14.85	13.13	11.72	12.89	12.24	14.39	12.53	9.38	**0.44**						
Nilssonia hurum	12	15.74	16.53	14.62	11.70	14.47	12.56	16.02	13.54	10.06	8.79	**0.05**					
Nilssonia leithii	2	13.89	15.20	14.51	11.66	13.38	12.19	15.02	12.79	8.77	7.31	8.42	**0**				
Nilssonia nigricans	17	15.74	15.71	14.38	11.27	14.16	11.98	15.36	13.14	9.51	8.36	4.87*	7.97	**0.14**			
Dogania subplana	1	12.96	15.67	15.10	13.87	15.38	14.07	15.52	14.81	13.49	14.54	13.29	13.58	12.85	—		
Palea steindachneri	1	14.81	16.57	16.02	13.78	15.04	13.97	16.42	14.56	14.45	14.63	14.39	14.84	13.75	13.97	—	
Pelodiscus maackii	1	12.04	14.42	14.47	15.61	14.79	15.92	14.43	15.20	15.51	16.36	15.45	15.03	15.08	13.87	13.49	—

Table 3. Uncorrected *p* distances (percentages) for the partial ND4 gene (715 bp) of *Amyda*, *Nilssonia* and allied softshell turtles. DNA coding for tRNAs was removed so that only protein-coding DNA was used for distance calculations. For further explanations, see Table 1.

	n	1 (A)	2	3	4	5	6	B	C	*for*	*gan*	*hur*	*lei*	*nig*	*sub*	*ste*	*maa*
Amyda – 1 (A)	1	—															
Amyda – 2	11	3.76	**0.37**														
Amyda – 3	4	4.61	1.15	**1.04**													
Amyda – 4	3	3.45	2.18	2.53	**0**												
Amyda – 5	10	5.78	6.00	5.46	4.53	**0**											
Amyda – 6	4	5.17	5.20	3.21	3.12	4.09	**0.33**										
Amyda – 2+3 (B)	15	3.99	—	—	—	—	—	**0.74**									
Amyda – 4+5+6 (C)	17	5.23	—	—	—	—	—	4.94	**2.49**								
Nilssonia formosa	2	12.07	11.98	7.30	12.40	12.40	12.13	10.73	12.34	**0**							
Nilssonia gangetica	19	6.90	10.55	6.78	12.45	11.66	12.84	9.54	12.07	9.35	**0.30**						
Nilssonia hurum	12	10.34	11.39	10.22	12.59	12.08	12.02	11.08	12.16	7.94	10.15	**0.05**					
Nilssonia leithii	2	12.07	13.24	12.52	13.68	14.53	13.43	13.05	14.12	9.09	8.37	9.08	**0**				
Nilssonia nigricans	17	12.07	12.35	10.40	13.16	12.96	12.39	11.83	12.86	8.14	10.98	3.21*	9.24	**0.07**			
Dogania subplana	1	17.24	19.51	17.86	14.34	14.82	13.85	19.07	14.51	12.59	14.00	13.34	14.13	14.25	—		
Palea steindachneri	1	12.07	15.14	10.54	14.86	14.99	15.00	13.91	14.97	13.01	13.07	13.80	12.87	14.29	14.69	—	
Pelodiscus maackii	1	12.07	13.04	8.25	14.85	12.87	13.97	11.76	13.48	14.71	14.81	14.84	14.56	15.30	14.71	14.26	—

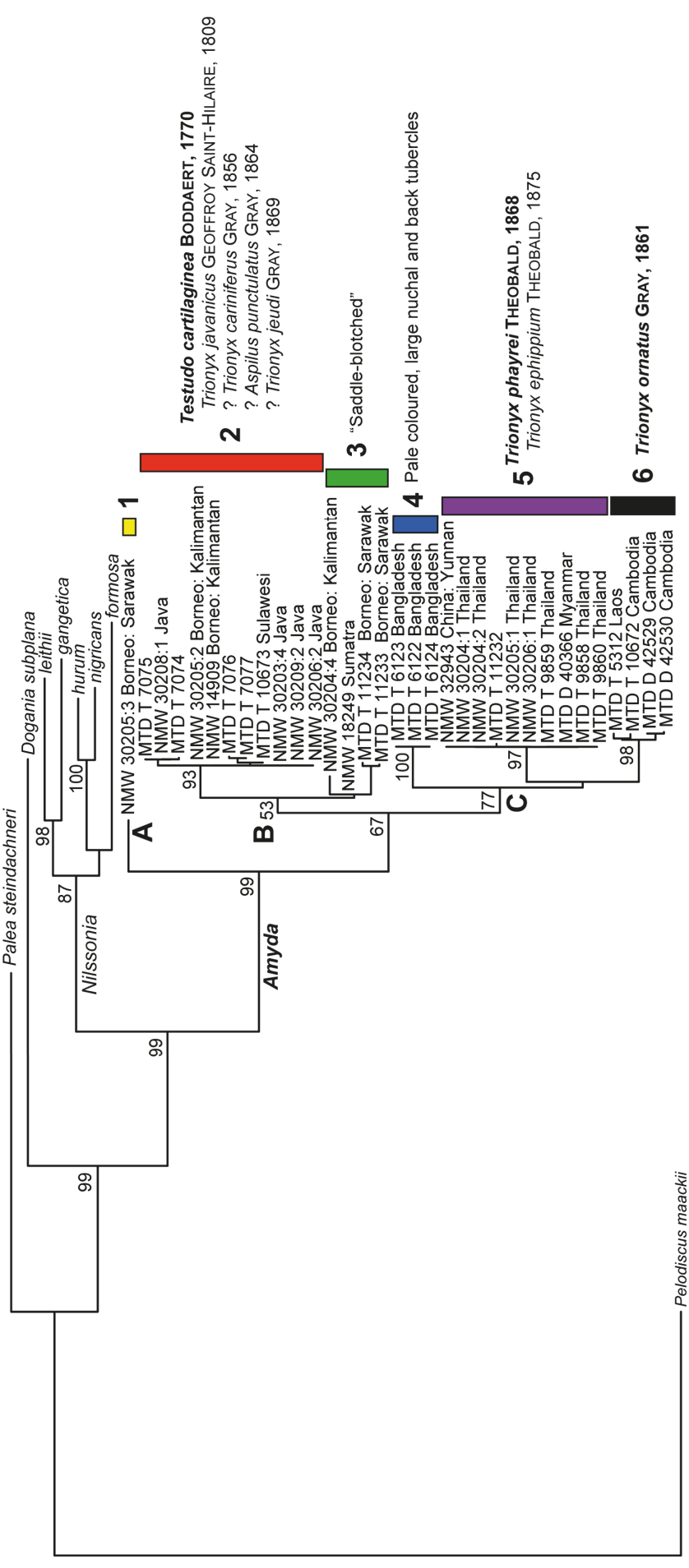

Fig. 1. Maximum Likelihood tree for *Amyda* and allied softshell turtles based on up to 2456 bp mtDNA (12S, cyt *b* and ND4 plus adjacent DNA coding for tRNAs). Values at nodes are thorough bootstrap values greater than 50 (not shown for some terminal clades with short branch lengths). Clade numbers and letters refer to the text; on the right are available names or morphological characters indicated. Oldest available names in bold. The topology of the Bayesian 50% consensus tree was completely identical. In the Bayesian tree, all nodes for which bootstrap values are shown received 100% support, except for the node with 53% bootstrap support. It received a posterior probability of 0.85. When up to 2716 bp nDNA were added for calculations, the topologies remained for *Amyda* unchanged, except for clade 3 which was no longer found monophyletic. Then, the respective sequences were placed in a basal polytomy together with the well-supported clade 2. This, as well as the weak support for the monophyly of clade 3 using mtDNA alone, results from the short sequences of the historical museum specimens NMW 18249 and NMW 30204:4.

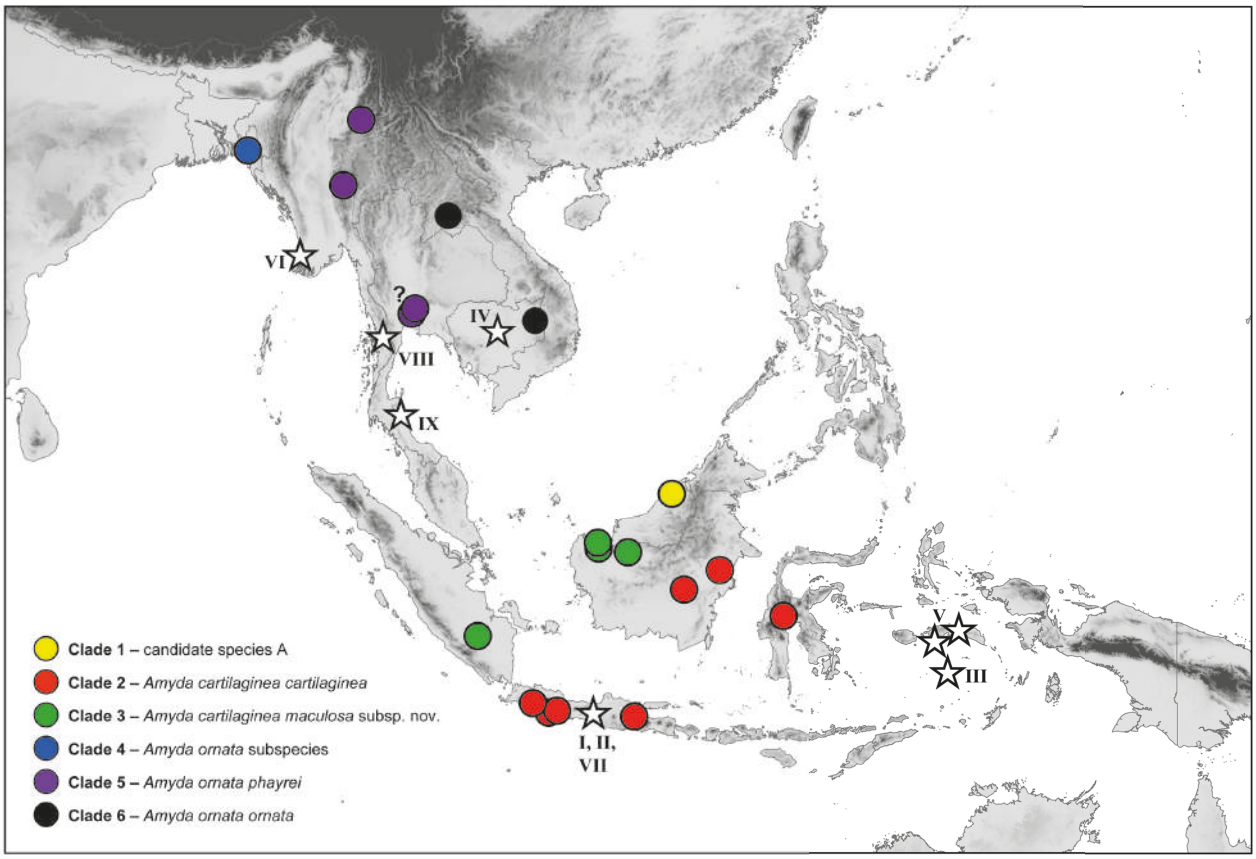

Fig. 2. Geographical distribution of the six terminal clades of *Amyda* and type localities of taxa currently identified with *Amyda cartilaginea* (Boddaert, 1770). Recommended nomenclature indicated. Site colours correspond to Fig. 1. Question mark denotes doubtful localities in the Bangkok region (possibly translocated turtles). Type localities (Fritz & Havaš 2007): (I) Java: *Testudo cartilaginea* Boddaert, 1770; (II) Java and neighbouring islands: *Trionyx javanicus* Geoffroy Saint-Hilaire, 1809; (III) Moluccas: *Trionyx cariniferus* Gray, 1856; (IV) Cambodia: *Trionyx ornatus* Gray, 1861; (V) Amboina or Ceram: *Aspilus punctulatus* Gray, 1864; (VI) Arakan Hills, Bassein District, western Pegu, Burma: *Trionyx phayrei* Theobald, 1868; (VII) Java (?): *Trionyx jeudi* Gray, 1869; (VIII) Tenasserim: *Trionyx ephippium* Theobald, 1875; (IX) Nakorn Sri Thammaraj Province, Thailand: *Trionyx nakornsrithammarajensis* Nutaphand, 1979.

clades, two of which show further substructure. In total, we identified six terminal clades (Fig. 1). One of these six clades (clade 1) is only known from the Baram river, Borneo (Sarawak, Malaysia), another one (clade 2) has a wide distribution and was recorded in eastern Borneo (Kalimantan, Indonesia) and Java. Also a softshell from Sulawesi, where *A. cartilaginea* is thought to be introduced (Koch et al. 2008; van Dijk et al. 2012), belongs to clade 2. The third clade (clade 3) was recorded from Sumatra and western Borneo (Sarawak, Malaysia; Kalimantan, Indonesia), and the remaining three clades 4–6 were found in Mainland Southeast Asia. Clade 4 was identified from samples from the Chittagong region of Bangladesh and represents the first record of *Amyda* for this country. Previously, *Amyda* was only known from the Indian state of Mizoram (van Dijk et al. 2012), which borders Bangladesh. Clade 5 seems to have a wide distribution in Myanmar and Thailand, and was also found in a sample from southwestern Yunnan (China). Clade 6 is represented by samples from Laos and Cambodia (Fig. 2).

The distribution pattern of the different *Amyda* clades on the Great Sunda Islands matches the distribution ranges of other freshwater organisms, for instance halfbeak fishes (Zenarchopteridae: *Dermogenys*, *Hemirhamphodon*, *Nomorhamphus*; de Bruyn et al. 2013). Their ranges largely correspond to Quaternary palaeodrainage patterns in the region (Voris 2000; de Bruyn et al. 2013). During the Quaternary, the Great Sunda Islands and southern Mainland Southeast Asia formed a major landmass, with shared drainage systems of what are now different islands and the mainland (Fig. S1). This explains the occurrence of clade 2 in Java and eastern Borneo (East Sunda palaeodrainage system; Voris 2000) and of clade 3 in western Borneo and Sumatra (North Sunda palaeodrainage system; Voris 2000). Endemic halfbeak species, paralleling the occurrence of clade 1 in the Baram river, are also known (de Bruyn et al. 2013).

Like on the Great Sunda Islands, the distribution of the three *Amyda* clades from Mainland Southeast Asia (Fig. 2) could be associated with different drainage systems (clade 4: Karnaphuli river and clade 6: Mekong drainage). Our records of clade 5, though, seem to contradict this hypothesis because they correspond to two distinct major drainages, the Salween (Thanlwin) and Chao Phraya systems. However, our sampled voucher

specimens from the lower course of the Chao Phraya originate from Bangkok and vicinity (Table S1), which is why their locality data should be treated with care. It cannot be excluded that these turtles were collected in the Salween system and transported for sale to the Bangkok region.

While our data provide firm evidence that *A. cartilaginea* as currently understood consists of distinct genetic lineages, their taxonomic assessment remains a challenge. Many recent studies have used uncorrected *p* distances of mtDNA sequences as a tool for species delimitation in turtles and tortoises (e.g. Engstrom et al. 2002; Fritz et al. 2008, 2012a, b; Kindler et al. 2012; Martin et al. 2013; Petzold et al. 2014; Todd et al. 2014), in analogy to the well-known barcoding approach (Hebert et al. 2003). However, Shen et al. (2013) have pointed out that no universal threshold can serve to identify species-level variation in different taxonomic groups of chelonians. Therefore, thresholds need to be adapted using the most closely related species as a yardstick (Fritz et al. 2012a, b; Kindler et al. 2012; Petzold et al. 2014). For assessing the species status of allopatric or parapatric taxa, divergences of closely related sympatric species are particularly valuable (Fritz et al. 2012b; Kindler et al. 2012).

If this approach is applied to *Amyda*, its sister genus *Nilssonia*, with five distinct and in part sympatric species (Praschag et al. 2007), offers an ideal model (Tables 1–3). It is obvious that the divergences between the three more inclusive clades of *Amyda* resemble or exceed for all three mtDNA fragments the uncorrected *p* distances between *Nilssonia hurum* and *N. nigricans*, two sympatric sister species. By contrast, the divergences between some of the six terminal clades of *Amyda* are below the critical values between *N. hurum* and *N. nigricans*. On the other hand, the divergences for the cyt *b* gene of the terminal clades of *Amyda* (minimum of 1.96%; Table 2) clearly exceed the divergences between populations of *N. gangetica* in different drainage systems (0.66%–0.75%; Liebing et al. 2012).

Thus, the genetic structure of *Amyda* reflects two differentiation levels, which we interpret as corresponding to three species, two of which are polytypic. Accordingly, we identify the three deeply divergent clades A, B and C with distinct species, and the substructure within clades B and C with subspecific variation.

Besides a better understanding of the taxonomy and diversity of *Amyda*, our genetic data provide a powerful tool for tracing back the regions of origin of traded or confiscated softshells and softshell products.

Taxonomy and nomenclature

There are several nominal species which are traditionally treated as synonyms of *Amyda cartilaginea* (Boddaert, 1770) (Boulenger 1889; Smith 1931; Wermuth & Mertens 1961, 1977; Fritz & Havaš 2007; van Dijk et al. 2012). When nomina nuda and replacement names are disregarded, nine names need to be discussed. According to their type localities (Fig. 2), some of these nominal species are easy to identify with a genetic lineage, while the assignment of other names remains speculative.

No name is available for the taxon from the Baram river, Sarawak (Malaysia). This species corresponds to clade A (identical with terminal clade 1; Fig. 1). However, we abstain from naming this species. There is too little material available for a morphological characterization of this taxon, and instead we treat it as an unconfirmed candidate species (sensu Padial et al. 2010). Besides the museum specimen which yielded the DNA sequences used in this study (NMW 30205:3, coll. Charles Hose 1901), there is just another specimen with the same data we refer to this species (NMW 30205:4). Both turtles are slightly discoloured small juveniles having very small carapacial nuchal tubercles and well-developed longitudinal carapacial ridges. On the carapace, a slightly darker saddle-shaped blotch is weakly discernible, resembling the pattern of the subspecies of *A. cartilaginea* occurring in Sumatra and Borneo (see below). The soft parts are mainly uniformly dark coloured and only few large light spots are present on the cheeks and on the chin. It is likely that a picture from a softshell from the Loagan Bunut National Park, Sarawak, provided by Indraneil Das, shows the same species (Fig. 3:A). This turtle possesses, in addition to the saddle-shaped pattern, a dark median carapacial stripe and some additional dark blotches. Morphologically, it resembles individuals of clade 3 (see below).

The situation for clade B, with terminal clades 2 and 3 corresponding to two subspecies, is different (Figs 1 and 2). There is no doubt that *Testudo cartilaginea* Boddaert, 1770 and *Trionyx javanicus* Geoffroy Saint-Hilaire, 1809, each with type locality of Java, represent our terminal clade 2. We also refer *Trionyx jeudi* Gray, 1869, which is most likely based on material from Java, to this clade. In addition, it is possible that the two nominal species *Trionyx cariniferus* Gray, 1856 and *Aspilus punctulatus* Gray, 1864, described from Amboina or Ceram and the Moluccas (from where *Amyda* is unknown), represent clade 2. The oldest name for the subspecies correspond-

→ **Fig. 3.** Live Asian softshell turtles from the Great Sunda Islands. Note in (A), (D) and (E) the saddle-shaped dark mark on the carapace. (A) *Amyda* species (candidate species A?), juvenile, Loagan Bunut National Park, Sarawak, Malaysia (Borneo). Photo: Indraneil Das. (B) *Amyda cartilaginea cartilaginea* (terminal clade 2), West Java, Indonesia (trade specimen). Yellow-spotted form of van Dijk (1992). Photo: Mark Auliya. (C) *Amyda* species (not studied genetically). Rantauprapat, Sumatera Utara, Indonesia (northern Sumatra). Yellow-spotted form of van Dijk (1992). Photo: Maren Gaulke. (D) *Amyda cartilaginea maculosa* subsp. nov. (terminal clade 3), Balai Ringin, near Serian, Sarawak, Malaysia (Borneo). Photo: Indraneil Das. (E) *Amyda cartilaginea maculosa* subsp. nov. (terminal clade 3), juvenile, Tanjung Lasa, Kapuas Hulu, West Kalimantan, Indonesia (Borneo). Note the different facial pattern compared to (A). Photo: Mark Auliya.


A
B
C
D
E

ing to clade 2 is *Testudo cartilaginea* Boddaert, 1770. Consequently, the subspecies has to be named *Amyda cartilaginea cartilaginea* (Boddaert, 1770).

Morphologically, *A. c. cartilaginea* matches the typical 'yellow-spotted form' of *Amyda* (Fig. 3:B), characterized by van Dijk (1992) as having 'an abundance of yellow spots over the whole body, yellow-rimmed black ocelli on the carapace, black reticulation on a yellow-olive-brown ground colour at the crown of the head', and often strongly developed nuchal tubercles. Similarly coloured turtles also occur in northern Sumatra (Fig. 3:C) in the Malacca Straits palaeodrainage (Voris 2000; Fig. S1), from where we could not study any samples genetically. It can only be speculated whether these turtles represent a genetically distinct taxon or *A. c. cartilaginea*. In freshwater fishes, this palaeodrainage system harbours endemic taxa (de Bruyn et al. 2013), so that genetic differentiation in *Amyda* should be expected as well.

No name is available for terminal clade 3, which we describe below as a new subspecies of *A. cartilaginea* (Figs 3:D and 3:E). Turtles of clade 3 correspond to the saddle-blotched colouration type of *Amyda*. This morphotype bears a conspicuous saddle-shaped mark on the carapace. Smith (1931), van Dijk (1992) and Auliya (2000) pointed out that this form is common in Sumatra and Borneo, and van Dijk (1992) believed that it could represent a distinct species. However, also in Myanmar, where another clade occurs, a few similarly patterned turtles have been recorded (Theobald 1868; Smith 1931), and all turtles we identify with clade A show a very similar dark carapacial mark.

With respect to clade C, comprised of terminal clades 4, 5 and 6 (Figs 1 and 2), the identification of *Trionyx ornatus* Gray, 1861 (type locality: Cambodia) with clade 6 is unambiguous. The type locality of *Trionyx phayrei* Theobald, 1868 near Pathein (Bassein), Myanmar, belongs to the delta region of the Irrawaddy (Ayeyarwady), which most probably harbours the same taxon as the neighbouring Salween (Thanlwin) river. Some of our samples of clade 5 originate in the Salween basin, and their morphology matches the turtles studied by Kuchling et al. (2004) in the upper Chindwin, the largest tributary of the Irrawaddy. Consequently, we refer the name *T. phayrei* to clade 5. Furthermore, we tentatively identify *Trionyx ephippium* Theobald, 1875 (type locality: Tenasserim) with our clade 5. Thus, as we treat clades 5 and 6 together with clade 4 (for which no name is available) as conspecific, clade 6 has to be named *Amyda ornata ornata* (Gray, 1861), and clade 5, *Amyda ornata phayrei* (Theobald, 1868).

Our assessment of these two subspecies (Figs 4A–C) is completely in line with the conclusions by van Dijk (1992) who had, however, no material from Bangladesh or India available. *Amyda ornata* corresponds to his 'arrow-headed form' from western Thailand and Cambodia. According to van Dijk (1992), this 'arrow-headed form' differs from what is now *A. c. cartilaginea* by a lighter base colour and a more diffuse yellow spotting, which is 'usually restricted to the cheeks, there are no ocelli but black dots may be present on the carapace, the nuchal tubercles are always weakly developed and the animals always show three (or rarely two) converging black lines on the crown of the head'. However, we wish to point out that some specimens of *A. o. ornata*, including genetically verified ones, closely resemble yellow-spotted *A. c. cartilaginea*, which could be the explanation why van Dijk (1992) identified specimens from central Thailand with his 'yellow-spotted form'.

Our three samples from Bangladesh represent the distinct terminal clade 4, and we identify them with an undescribed subspecies of *A. ornata*. The samples were taken from three live adults which are morphologically characterized by a rather uniform pale colouration and very large tubercles in the nuchal and back region of the carapace (Figs 4:D and 4:E). The lack of museum material for turtles of clade 4 prevents us from describing this taxon.

Finally, the taxonomic allocation of *Trionyx nakornsrithammarajensis* Nutaphand, 1979 remains unclear. Its type locality (Nakorn Sri Thammaraj Province, Thailand; Fig. 2) belongs to the Siam palaeodrainage system (Fig. S1), which harbours endemic freshwater fishes (de Bruyn et al. 2013). Even though we have studied some samples from the vicinity of Bangkok which could originate in the Chao Phraya system, part of the Siam palaeodrainage system (Voris 2000), we regard their locality data as unreliable (see above). Therefore, we are reluctant to identify the morphologically distinctive *T. nakornsrithammarajensis* (Nutaphand 1979; van Dijk 1992) with our terminal clade 5 (*A. o. phayrei*).

Conclusions

The genus *Amyda* is comprised of three genetically deeply divergent species, two of which are polytypic. An undescribed species of *Amyda* seems to be confined to Sarawak, Malaysia (northern Borneo) and is only known from the Baram river.

Amyda cartilaginea cartilaginea (Boddaert, 1770) occurs in the East Sunda palaeodrainage system, with records in eastern Borneo and Java. In the North Sunda palaeodrainage system (Sumatra, western Borneo) lives a new subspecies of *A. cartilaginea*, which is diagnosed below. A sample from Sulawesi, most probably not from a native population, belongs to the nominotypical subspecies of *A. cartilaginea*.

Samples from Mainland Southeast Asia correspond to the reinstated species *Amyda ornata* (Gray, 1861), consisting of three genetically well-differentiated subspecies. The distribution range of the nominotypical subspecies of *A. ornata* is associated with the Mekong drainage system, with genetically verified records from Laos and Cambodia. Samples from Thailand, Myanmar and Yunnan (if

Fig. 4. Live Asian softshell turtles from Mainland Southeast Asia. (A) *Amyda ornata ornata* (terminal clade 6), southern Vietnam or Cambodia. Arrow-headed form of VAN DIJK (1992). Note the smooth rear carapace. Photo: TIMOTHY MCCORMACK. (B, C) *Amyda ornata phayrei* (terminal clade 5), Thailand. Note the different head colouration compared to *A. o. ornata* and the pronounced shell tubercles. Photos: PETER PRASCHAG. (D, E) *Amyda ornata* subspecies (terminal clade 4), Chittagong Hills, Bangladesh. Note the pale shell colouration, the indistinct head pattern and the pronounced shell tubercles. Photos: PETER PRASCHAG.

native there) are genetically distinct and identified with the subspecies *Amyda ornata phayrei* (THEOBALD, 1868). Three turtles from Bangladesh are also genetically distinct and represent an undescribed subspecies and the first country record for *Amyda*. We cannot exclude that further distinct taxa exist in unstudied drainage systems.

Amyda cartilaginea maculosa subsp. nov.

Diagnosis. *Amyda cartilaginea maculosa* differs from the nominotypical subspecies by a more massive head with a relatively short and blunt proboscis, a lighter base colouration (olive to brown instead of dark brown to blackish), the lack of contrasting yellow spotting and less pronounced nuchal tubercles. Juveniles and young adults bear on their back a characteristic saddle-shaped dark mark. In addition, *A. c. maculosa* is genetically well-differentiated from all other taxa of *Amyda*.

Holotype. Natural History Museum Vienna, NMW 30204:3, Nanga Badau, Kalimantan, Indonesia ("Nanga Bandang, Borneo"). Franz Steindachner leg. 1874; Fig. 5.

Description of the holotype. Juvenile specimen, carapacial length approximately 60 mm, carapacial width approximately 51 mm; discus length 45 mm (all straight line). Head and neck dorsally dark with diffuse small light spots; occiput with two light triangles with tips meeting in the midline. Carapace olive brownish, with diffuse lighter mottling and a saddle-shaped dark mark in its anterior half; posterior half with dark central line. Nuchal tubercles small, several longitudinal ridges present which dissolve posteriorly in tubercles. Shell ventrally uniform beige.

Paratypes. Natural History Museum Vienna, NMW 18249, subadult, Sumatra, F. Schubert-Soldern leg. 22 June 1942; NMW 30204:4, juvenile, same data as holotype; NMW 30210:1, juvenile, Deli, Sumatra, C. Maschmeyer leg. July 1903.

Derivatio nominis. The subspecies name *maculosa* ('blotched') is a Latin adjective in feminine gender, and refers to the characteristic dark carapacial mark of younger individuals.

Distribution. *Amyda cartilaginea maculosa* seems to be endemic to the North Sunda palaeodrainage system (*cf.* Voris 2000) in eastern Sumatra and western Borneo; but see below.

Remarks. *Amyda cartilaginea maculosa* corresponds to our terminal clade 3 (Fig. 1). The dark carapacial mark gradually fades with age and size and, as shown by the genetically studied paratype NMW 18249, subadult individuals may have a more or less uniform pale olive colouration. The paratype NMW 30210:1, a morphologically typical juvenile of 126 mm carapacial length, from "Deli, Sumatra" suggests that *A. c. maculosa* could also occur beyond the North Sunda palaeodrainage. However, for historical museum specimens often the dispatch location, and not the collection site, has been recorded, so that the locality data of NMW 30210:1 should be treated with care. Nevertheless, it should be noted that at least some *Amyda* from the Malacca Straits palaeodrainage of Sumatra are morphologically highly distinct and resemble *A. c. cartilaginea* (Fig. 3C). Unfortunately no samples of this population were available for genetic investigation. However, if *A. c. maculosa* should occur sympatrically with the spotted *Amyda* from the Malacca Straits system, this would argue for species status of both taxa.

Fig. 5. Dorsal aspect of the holotype of *Amyda cartilaginea maculosa* subsp. nov. (Natural History Museum Vienna, NMW 30204:3, Nanga Badau, Kalimantan, Indonesia). Photo: Peter Praschag.

Supporting Information

The Supporting Information is available electronically at www.vertebrate-zoology.de (Back Volumes).
Fig. S1. Quaternary palaeodrainage systems and shorelines in Southeast Asia according to Voris (2000).
Table S1. Used samples, GenBank sequences and their accession numbers.
Table S2. Primer sequences and lengths of PCR products for historical samples.
Table S3. PCR conditions for historical and fresh samples.

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