Org Divers Evol
DOI 10.1007/s13127-010-0007-1

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

The world's economically most important chelonians represent a diverse species complex (Testudines: Trionychidae: *Pelodiscus*)

Uwe Fritz · Shiping Gong · Markus Auer · Gerald Kuchling · Norbert Schneeweiß · Anna K. Hundsdörfer

Received: 26 February 2009 / Accepted: 2 July 2009

Abstract *Pelodiscus* is one of the most widely distributed genera of softshell turtles, ranging from south-eastern Siberia and Korea over central and southern China to Vietnam. Economically, *Pelodiscus* are the most important chelonians of the world and have been bred and traded in high numbers for centuries, resulting in many populations established outside their native range. Currently, more than 300 million turtles per year are sold in China alone, and the bulk of this figure comprises farmed *Pelodiscus*. Due to easy availability, *Pelodiscus* also constitutes a model organism for physiological and embryological investigations. Yet, diversity and taxonomy of *Pelodiscus* are poorly understood and a comprehensive investigation using molecular tools has never been published. Traditionally, all populations were assigned to the species *P. sinensis* (Wiegmann, 1834); in recent years up to three additional species have been recognized by a few authors, while others have continued to accept only *P. sinensis*. In the present study, we use trade specimens and known-locality samples from Siberia, China, and Vietnam, analyze 2,419 bp of mtDNA and a 565-bp-long fragment of the nuclear C-mos gene to elucidate genetic diversity, and compare our data with sequences available from GenBank. Our findings provide evidence for the existence of at least seven distinct genetic lineages and suggest interbreeding in commercial turtle farms. GenBank sequences assigned to *P. axenaria* (Zhou, Zhang & Fang, 1991) are highly distinct. The validity of *P. maackii* (Brandt, 1857) from the northernmost part of the genus' range is confirmed, whereas it is unclear which names should be applied to several taxa occurring in the central and southern parts of the range. The diversity of *Pelodiscus* calls for caution when such turtles are used as model organisms, because the respective involvement of more than a single taxon could lead to irreproducible and contradictory results. Moreover, our findings reveal the need for a new assessment of the conservation status of *Pelodiscus*. While currently all taxa are subsumed under '*P. sinensis*' and listed as 'vulnerable' by the IUCN Red List of Threatened Species, some could actually be endangered or even critically endangered.

Keywords Asia · Conservation · Diversity · Phylogeography · Phylogeny

U. Fritz (✉) · M. Auer · A. K. Hundsdörfer
Museum of Zoology (Museum für Tierkunde),
Senckenberg Natural History Collections Dresden,
Königsbrücker Landstr. 159,
01109 Dresden, Germany
e-mail: uwe.fritz@senckenberg.de

S. Gong
Guangdong Provincial Public Laboratory for Wild Animal Conservation and Management,
South China Institute of Endangered Animals,
Guangzhou 510260, Peoples' Republic of China

G. Kuchling
School of Animal Biology, The University of Western Australia,
35 Stirling Highway,
Perth, Western Australia 6009, Australia

N. Schneeweiß
Landesumweltamt Brandenburg, Naturschutzstation Rhinluch,
16833 Linum, Germany

Introduction

Softshell turtles (Trionychidae) are an ancient group of chelonians the earliest known record of which is from the Lower Cretaceous of Asia (Meylan and Gaffney 1992;

Published online: 20 March 2010

Nessov 1995). The family comprises 30 extant species in 13 genera and is distributed in Africa, Asia, North America and New Guinea (Ernst et al. 2000; Fritz and Havaš 2007; Praschag et al. 2007). Trionychids are characterized by a highly derived morphology. Their shell is flattened and covered by a leathery skin instead of horny scutes; the bony shell is much reduced. The neck is long and retractile, and the snout usually is a long proboscis. The jaws are concealed by fleshy lips, a unique character among extant chelonians, and limbs are paddle-like, with three strong claws each. All trionychids are highly aquatic and many species are vigorous predators of fish. As a rule, external morphology is difficult to use for taxonomic and phylogenetic purposes in this group; past studies focused on osteological characters (Ernst et al. 2000; Meylan 1987). In recent years, essential new insights in phylogeny and diversity have been gained by molecular methods (Engstrom et al. 2002, 2004; McGaugh et al. 2008; Praschag et al. 2007; Weisrock and Janzen 2000). However, all African and most Asian genera were never studied in detail using molecular tools.

One of the most widely distributed genera of softshell turtles is *Pelodiscus*. It occurs in a large native distribution range from south-eastern Siberia (Amur and Ussuri River basins) and Korea through central and southern China (including Hainan and Taiwan) to Vietnam (Fig. 1; Ernst et al. 2000; Iverson 1992). This area spans about 4,500 km in its maximum north-south extension and about 2,000 km in east-west direction. *Pelodiscus* turtles are the economically most important chelonians of the world (van Dijk et al. 2000; Maran 2003; Nurizan and Ong 1997; Shi et al. 2008; Zhang et al. 2008), constituting a highly appreciated part of many dishes of the East and Southeast Asian cuisine. For centuries, *Pelodiscus* turtles have been bred and traded in high numbers, resulting in many populations established outside their native range (Japan, Guam, Hawaii, Mariana and Bonin Islands, Timor: Ernst et al. 2000; Philippines: Diesmos et al. 2008; Sarawak, Malaysia: Jensen and Das 2008), and, most probably, the admixture of distinct genetic lineages (Ernst et al. 2000; Sato and Ota 1999). In China alone, more than 300 million turtles are sold per year, and the bulk of this figure comprises farmed *Pelodiscus* (Shi et al. 2008). Yet, their diversity and taxonomy are poorly understood. Whereas in the 19th and early 20th centuries many species and subspecies were described (Table 1), merely a single monotypic species, *P. sinensis* (Wiegmann, 1834), was recognized later (Ernst and Barbour 1989; Ernst et al. 2000; Mertens and Wermuth 1955; Wermuth and Mertens 1961, 1977; Zhao and Adler 1993). Only in recent years two new species, *P. axenaria* (Zhou, Zhang & Fang, 1991) and *P. parviformis* Tang, 1997, were described (Tang 1997; Zhou et al. 1991), and another species, *P. maackii* (Brandt, 1857), was suggested to be valid (Chkhikvadze

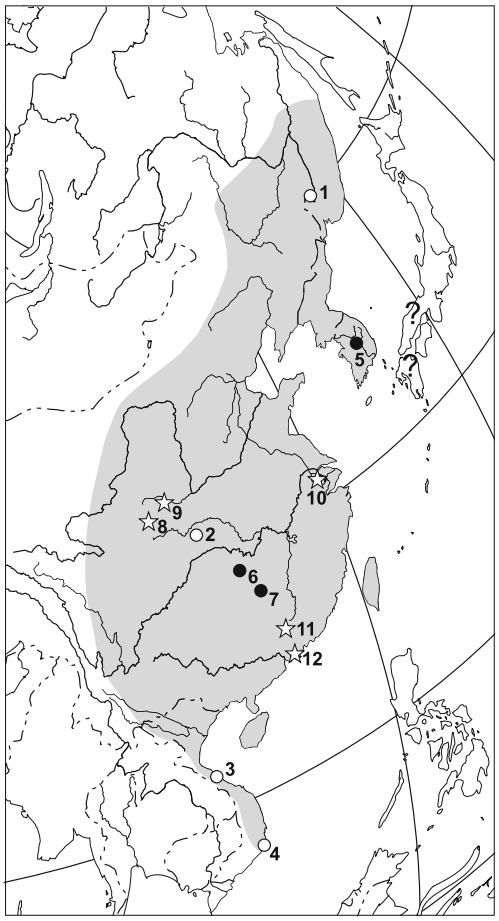

Fig. 1 Distribution range of *Pelodiscus* (*shaded*; after map in Iverson 1992) and origins of samples and GenBank sequences. *Open circles*, wild-caught turtles: (1) Lake Khanka, southern Siberia, Russia; (2) Changling village, Shaanxi, China; (3) Phong Na-Ke Bang Reserve, Quang Binh, Vietnam; (4) Nha Trang, Khánh Hòa, Vietnam. *Closed circles*, known-locality GenBank sequences: (5) Republic of Korea ('*P. sinensis*' sensu Jung et al. 2006); (6) Changde, Hunan, China ('*P. axenaria*' sensu Chen et al. 2006); (7) Binzhou, Anren Xian, Hunan, China ('*P. axenaria*' sensu Chen et al. 2005, 2006). *Stars*, market specimens (all from China): (8) Guangyuan, Sichuan; (9) Hanzhong, Shaanxi; (10) Suzhou, Jiangsu; (11) Shaoguan, Guangdong; (12) Hong Kong

1987; Fritz and Havaš 2007; Fritz and Obst 1999). However, this has not been generally accepted; some authors continue to recognize only *P. sinensis* (van Dijk et al. 2000; Ernst et al. 2000; Zhao and Adler 1993). Two recent studies found mtDNA sequences of *P. axenaria* and *P. sinensis* to be significantly distinct (Chen et al. 2005, 2006), supporting the view that *Pelodiscus* represents a species complex. However, until now no comprehensive molecular genetic or phylogeographic evaluation of *Pelodiscus* was undertaken.

While *Pelodiscus* turtles are farm-bred in vast numbers for the food trade, the wild populations decline throughout the range (van Dijk et al. 2000). A better understanding of the diversity of *Pelodiscus* is crucial not only for

Table 1 Nominal taxa assigned to *Pelodiscus* according to Fritz and Havaš (2007), in chronological order; for suppressed names, nomina nova and nomina nuda, see Fritz and Havaš (2007)

Taxon	Type locality
Trionyx (Aspidonectes) sinensis Wiegmann, 1834	rice field on a small island, Tiger River, near Macao [Aomen], China
Trionyx stellatus var. *japonica* Temminck & Schlegel, 1835	Japan
Trionyx tuberculatus Cantor, 1842	Chusan [Zhousan Island, Zhejiang Province], China
Tyrse perocellata Gray,1844	Canton [Guangzhou Shi], Guangdong Province, China
Trionyx maackii Brandt, 1857	rivers south of Amur River, specifically Sungari and Ussuri Rivers, and Amur River between Sungari and Ussuri Rivers
Trionyx schlegelii Brandt, 1857	Beijing, China
Landemania irrorata Gray, 1869	Shanghai, China
Ceramopelta latirostris Heude, 1880	vicinity of Ngang-k'ing [Anqing Shi], Anhui Province, China
Cinctisternum bicinctum Heude, 1880	irrigation channel of Ngang-k'ing [Anqing Shi], Anhui Province, China
Coelognathus novemcostatus Heude, 1880	eastern extremity of Tch'ao [Lake Chao], Anhui Province, China
Coptopelta septemcostata Heude, 1880	lakes of Tong-lieou [Dongliu], Anhui Province, China
Gomphopelta officinae Heude, 1880	Houai [Huai River], Ho-nan [Henan Province] and Mé-k'eng Lake, Anhui or Jiangsu Province, China
Psilognathus laevis Heude, 1880	mountain stream south of Ning-kouo fou [Ningguo Co.], Anhui Province, China
Temnognathus mordax Heude, 1880	vicinity of Shanghai, China
Tortisternum novemcostatum Heude, 1880	Tch'ao [Lake Chao], Anhui Province, China
Trionyx cartilagineus var. *newtoni* Bethencourt-Ferreira, 1897	Timor
Amyda schlegelii haseri Pavlov, 1932	Tzu ya ho River [Ziya He] near Sienhien [Xian Co.], central Chili [Hebei Province], China
Amyda schlegelii licenti Pavlov, 1932	Tientsin [Tianjin], China
Amyda schlegelii var. *laoshanica* Pavlov, 1933	Chantong, Laoshan, near Tsingtao [Qingdao Shi], Shandong Province, China
Trionyx axenaria Zhou, Zhang & Fang, 1991	Taoyuan, Pingjiang, Rucheng, Lingling, Shaoyang Counties, Hunan Province, China
Pelodiscus parviformis Tang, 1997	Quanzhou, Xing'an, Guanyang, Ziyuan, Lingchuan Counties of Guangxi Autonomous Region, and Dong'an, Qiyang, Daoxian Counties of Hunan Province, China

Alternate spellings and explanations of type localities in square brackets

conservation and taxonomy, but also for other scientific disciplines. Due to easy availability, *Pelodiscus* constitutes a model organism for physiological and embryological investigations. For instance, a search in the database of the Zoological Record (ISI Web of Knowledge v. 4.4) using the keywords '*Pelodiscus* [or *Trionyx*, the former genus name] *sinensis*', 'embryology', and 'physiology' yielded 378 hits for 1999–2008. If *P. sinensis* represents a species complex, caution is advisable when results of different physiological or embryological investigations are compared. However, the difficulty to obtain samples of wild-caught *Pelodiscus* poses a serious challenge for any effort to assess the taxon's diversity. As a starting point, in the present study we use trade specimens and known-locality samples from Siberia, China, and Vietnam to analyze 2,419 bp of mtDNA and a 565-bp-long fragment of the nuclear C-mos gene. Furthermore, we compare in single-gene analyses our data with sequences available from GenBank.

Materials and methods

Sampling

Eighteen *Pelodiscus* samples were obtained from turtles sold on food markets in Guangdong, Hong Kong, Jiangsu, Shaanxi, and Sichuan (China); additional samples were from one wild-caught individual from Shaanxi (China), two from Vietnam, and eight from the Przewalski Peninsula, southern Siberia (Russia; Table 2). From most individuals, small pieces of the webbing of the feet or skin of the shell were carefully removed and stored in 99% ethanol until processing. Eight individuals were sacrificed and placed in the herpetological collection of the Museum of Zoology, Dresden. From these, thigh muscle tissue was extracted for analysis. Remaining tissue and DNA samples are stored at −80°C in the tissue sample collection of the Museum of Zoology, Dresden. According to their provenance, the

Table 2 *Pelodiscus* samples studied

Sample[a]	Haplotypes		Provenance
	mtDNA	C-mos[b]	
MTD T 4234	A	C-mos4, C-mos4	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4235	A	C-mos4, C-mos4	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4236	A	C-mos4, C-mos5	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4237	A	C-mos4, C-mos4	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4238	A	C-mos4, C-mos4	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4239	A	–	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4240	A	–	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 4241	A	C-mos4, C-mos4	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka, wild-caught
MTD T 5084	B3	C-mos1, C-mos6	China: Guangdong: Shaoguan, market
MTD T 5085	B1	C-mos2, C-mos6	China: Guangdong: Shaoguan, market
MTD D 41570	B3	–	China: Hong Kong, market
MTD D 43357	B3	–	China: Hong Kong, market
MTD D 44189	B3	–	China: Hong Kong, market
MTD D 44190	B1	–	China: Hong Kong, market
MTD D 44287	B3	–	China: Hong Kong, market
MTD D 47035	B3	–	China: Hong Kong, market
MTD T 5555	B3	C-mos6, C-mos6	China: Jiangsu: Suzhou, market
MTD T 5556	B3	C-mos2, C-mos6	China: Jiangsu: Suzhou, market
MTD T 5557	B3	C-mos1, C-mos6	China: Jiangsu: Suzhou, market
MTD T 5558	B3	C-mos1, C-mos6	China: Jiangsu: Suzhou, market
MTD T 5559	B4	C-mos6, C-mos6	China: Jiangsu: Suzhou, market
MTD T 5560	B3	C-mos4, C-mos4	China: Jiangsu: Suzhou, market
MTD T 5561	B3	C-mos2, C-mos6	China: Jiangsu: Suzhou, market
MTD T 5093	B2	C-mos1, C-mos1	China: Shaanxi: Hanzhong, market
MTD T 5097	C	C-mos3, C-mos3	China: Shaanxi: Changling village, wild-caught
MTD T 5091	C	C-mos1, C-mos4	China: Sichuan: Guangyuan, market
MTD T 5092	C	C-mos4, C-mos4	China: Sichuan: Guangyuan, market
MTD D 42534	D2	C-mos6, C-mos6	Vietnam: Khánh Hòa: vicinity of Nha Trang, wild-caught
MTD D 44045	D1	C-mos6, C-mos6	Vietnam: Quang Binh: Phong Nha-Ke Bang Reserve, wild-caught

[a] Sample codes starting with MTD T refer to the tissue sample collection, those starting with MTD D to the herpetological collection of the Museum of Zoology, Dresden

[b] Nuclear C-mos gene could not be sequenced from all samples

Siberian turtles should represent *P. maackii*; the other specimens could correspond to *P. sinensis* or the two other recently described species, *P. axenaria* and *P. parviformis*. Voucher photos of all turtles not collected are kept in the Museum of Zoology, Dresden; a selection of pictures is available from MorphBank (http://www.morphbank.net).

Chosen marker genes, PCR, and sequencing

Three mitochondrial markers (12S rRNA, cyt *b*, ND4) and one nuclear DNA fragment (C-mos) that had been shown to reveal differences and phylogenetic relationships between chelonian terminal taxa (e.g. Fritz and Bininda-Emonds 2007; Fritz et al. 2008a, b; Le et al. 2006; Spinks and Shaffer 2005; Vargas-Ramírez et al. 2008) were chosen. Total genomic DNA was extracted using the DTAB method (Gustincich et al. 1991). The targeted DNA fragments were amplified with the primers given in Table 3 and according to the protocols in the respective references. For a few samples, however, annealing temperatures were lowered and more cycles used. 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 or Fermentas), and 10 pmol of each primer. PCR products were purified by precipitation, using 1 volume PCR product (30 μL), 1 volume 4 M NH_4Ac (30 μL) and 12 volumes EtOH (100%; 360 μL). DNA was pelleted by centrifugation

Table 3 Primers used for amplification and sequencing of mtDNA and nDNA fragments

Fragment	Primer	Sequence (5′–3′)	Reference
mtDNA 1	L1091	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	Kocher et al. (1989)
mtDNA 1	H1478	TGACTGCAGAGGGTGACGGGCGGTGTGT	Kocher et al. (1989)
mtDNA 2	ND4-672	TGACTACCAAAAGCTCATGTAGAAGC	Engstrom et al. (2004)
mtDNA 2	H-Leu	ATTACTTTTACTTGGATTTGCACCA	Stuart and Parham (2004)
mtDNA 3	Cytb-G	AACCATCGTTGTWATCAACTAC	Spinks et al. (2004)
mtDNA 3	mt-f-na	AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG	Fritz et al. (2006)
mtDNA 3	mt-C2	TGAGGACAAATATCATTCTGAGG	Fritz et al. (2006)
mtDNA 3	mt-E-rev2	GCRAATARRAAGTATCATTCTGG	Fritz et al. (2006)
C-mos	Cmos1	GCCTGGTGCTCCATCGACTGGGATCA	Le et al. (2006)
C-mos	Cmos3	GTAGATGTCTGCTTTGGGGGTGA	Le et al. (2006)

Table 4 GenBank sequences of *Pelodiscus* used for comparison. Species allocation according to GenBank. Numbers of nucleotides aligned with our sequences indicated

Accession number	Species	mtDNA			nDNA	Provenance	Reference
		Fragment 1 (391 bp)	Fragment 2 (860 bp)	Fragment 3 (1,168 bp)	C-mos (565 bp)		
AF043413	*sinensis*	391	–	–	–	unknown	Wu et al. (unpubl.)
AY259553	*sinensis*	–	–	1,140	–	trade, China	Engstrom et al. (2004)
AY259603	*sinensis*	–	698	–	–	trade, China	Engstrom et al. (2004)
AY304497	*sinensis*	158	–	–	–	5 specimens bought in a supermarket; 12 farm-bred specimens from Hunan, China	Chen et al. (2005)
AY389697	*axenaria*	158	–	–	–	8 specimens, Yongle River in Binzhou, Anren Xian, Hunan, China	Chen et al. (2005)
AY583692	*sinensis*	–	–	1,140	–	5 specimens bought in a supermarket; 12 farm-bred specimens from Hunan, China	Chen et al. (2006)
AY583693	*axenaria*	–	–	1,140	–	7 specimens from Changde, Hunan; 10 specimens from Yongle River in Binzhou, Anren Xian, Hunan, China	Chen et al. (2006)
AY687385[a]	*sinensis*	391	860	1,168	–	unknown	Nie et al. (unpubl.)
AY743420[b]	*sinensis*	391	–	–	–	unknown	Chen and Zhang (unpubl.)
AY743421[b]	*axenaria*	391	–	–	–	unknown	Chen and Zhang (unpubl.)
AY962573[a]	*sinensis*	391	860	1,168	–	Korea	Jung et al. (2006)
FJ230869	*sinensis*	–	–	–	561	unknown	Naro-Maciel et al. (2008)

[a] Complete mitochondrial genome

[b] Unclear whether sequence used by Chen et al. (2005)

Table 5 Parsimony statistics for the mitochondrial data sets of *Pelodiscus*

Fragment	*n*	Sites			
		Aligned	Conserved	Parsimony-informative	Singletons
mtDNA 1 + 2 + 3	8	2,421	1,880 / 2,333	260 / 29	281 / 59
mtDNA 1	10[a]	393	317 / 364	33 / 11	43 / 18
mtDNA 2	11[a]	860	667 / 810	96 / 23	97 / 27
mtDNA 3	11[a]	1,168	842 / 1,026	177 / 51	149 / 91

Conserved sites, parsimony-informative sites and singletons: Respective first value, alignment including outgroups; second value, ingroups only. *n* Number of *Pelodiscus* haplotypes

[a] Including GenBank sequences

and the pellet washed with 70% ethanol. Subsequently, the pellet was dissolved in 20 μL H_2O and PCR products were sequenced on an ABI 3130 sequencer (Applied Biosystems) using the primers in Table 3. Concerning mtDNA, the 391-bp-long fragment 1 corresponds to the partial 12S rRNA gene, the 860-bp-long fragment 2 to the second half of the NADH dehydrogenase subunit 4 (ND4: 675 bp) plus the adjacent tRNA genes (tRNA-His: 70 bp, tRNA-Ser: 61 bp, partial tRNA-Leu: 54 bp), and the 1,168-bp-long fragment 3 contains 1,143 bp of the cytochrome *b* (cyt *b*) gene and 25 bp of the adjacent tRNA-Thr gene. The nuclear DNA fragment corresponds to 565 bp of the C-mos gene.

Alignment and haplotype determination

Additional sequences of *Pelodiscus* were downloaded from GenBank (Table 4) and aligned with our data in BIOEDIT 7.0.5.2 (Hall 1999); alignments were further inspected in MEGA 4.0.2 (Tamura et al. 2007). Due to gaps, the alignment of the 391-bp-long mtDNA fragment 1 comprised 393 sites. Mitochondrial DNA sequences were manually collapsed into haplotypes. The protein-coding mtDNA fragments contained no internal stop codons, and nucleotide frequencies corresponded to those of coding mtDNA; therefore we conclude that we have amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes. For haplotype determination of the nuclear C-mos gene, the software PHASE 2.1 (Stephens and Donnelly 2003; Stephens et al. 2001) was used. GenBank accession numbers of haplotypes identified in the present study are FM999003–FM999032.

Phylogenetic and haplotype network analyses

Since artificial hybridization is expected due to farm-breeding, only mitochondrial data were used for phylogenetic analyses in order to avoid introducing phylogenetic noise caused by recombinants. For phylogenetic analyses, identical sequences were removed from the alignments. Based on the findings of Engstrom et al. (2004), *Apalone spinifera* (LeSueur, 1827) (accession numbers U81319, AY259607, AY259557), *Palea steindachneri* (Siebenrock, 1906) (AY743419, AY259602, AY259552) and *Rafetus euphraticus* (Daudin, 1801) (FM999033, AY259604, AY259554) served as outgroups; *Apalone* and *Rafetus* constitute the sister group of a clade comprising *Palea* and *Pelodiscus*.

In a first step, a data set was analyzed for *Pelodiscus* haplotypes corresponding to our concatenated three mitochondrial fragments, acknowledging that the complete mitochondrial genome represents one and the same locus. Including gaps in the 12S rRNA fragment, this alignment comprised 2,421 sites. Except for the two complete mitochondrial genomes (AY687385, AY962573), it is unclear whether GenBank sequences were derived from the same individual. Hence, different GenBank sequences were not concatenated in order to avoid creating chimerical sequences. Instead, in a second step the individual GenBank sequences were analyzed together with each of the three separate mitochondrial fragments.

Maximum Likelihood (ML) and Maximum Parsimony (MP) trees were calculated with PAUP* 4.0b10 (Swofford 2002), using the command hs add=cl. Bootstrap support was obtained with 1,000 (ML) and 100,000 replicates (MP), respectively. Parsimony statistics of the data sets are summarized in Table 5; gaps were treated as missing. In addition, each data set was subjected to Bayesian Analysis (BA) in MrBAYES 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003); for the concatenated fragments the mixed-model approach was used. The best evolutionary model was established for each mitochondrial fragment alone, and the three fragments concatenated, using MODELTEST 3.06 (Table 6; Posada and Crandall 1998). Bayesian analysis was performed using the Metropolis-coupled Markov chain Monte Carlo algorithm with two parallel runs, each with four chains. The heating parameter λ was set to 0.1 to obtain convergence. The chains ran for 10 million generations with every 500th generation sam-

Table 6 Best-fit substitution models (AIC) and their parameters, established using MODELTEST 3.06 (Posada and Crandall 1998)

Fragment	Model	Number of substitution types (rate matrix)	Rates	Shape	Pinvar
mtDNA 1 + 2 + 3	GTR+G	6 (3.4216 10.8377 2.9678 0.2815 34.7347)	gamma	0.2464	0
mtDNA 1	TrN+G	6 (1.0000 4.2042 1.0000 1.0000 13.7295)	gamma	0.1898	0
mtDNA 2	GTR+I	6 (3.9203 21.4181 7.4976 0.0000 60.2234)	equal	n.a.	0.6069
mtDNA 3	GTR+G	6 (1.3846 4.2949 0.8612 0.1968 13.3456)	gamma	0.2780	0

pled; the burn-in was set to sample only the plateau of the most likely trees. The remaining trees were used for generating a 50% majority rule consensus tree. The posterior probability of any individual clade in this consensus tree corresponds to the percentage of all trees containing that clade, thus is a measure of clade frequency and credibility.

Within the same species or between closely related species, relationships of haplotypes are likely to be reticulate and ancestral haplotypes may persist, which is why intraspecific gene evolution may be only imperfectly reflected by dichotomous trees (Posada and Crandall 2001). To address this uncertainty, parsimony networks were calculated using TCS 1.21 (Clement et al. 2000). This software is based on statistical parsimony, connects haplotypes via a minimal number of mutational steps, and allows alternative pathways. A further advantage of network analyses is that information about the age of haplotypes may be obtained. Interiorly located haplotypes, having more than one mutational connection, are thought to be ancestral to and older than tip haplotypes (Posada and Crandall 2001). TCS also determines the outgroup probability of each haplotype that is, according to coalescent theory, also correlated with haplotype age (Castelloe and Templeton 1994; Donnelly and Tavaré 1986). Since a conflicting topology of nDNA and mtDNA genealogies is indicative of gene flow, hybridization or incomplete sorting, such a parsimony network was also produced for the haplotypes of the nuclear C-mos gene. Although the TCS algorithm can cope with some ambiguous or missing data when such sequences are placed in the last positions of the alignment, two 12S rRNA sequences from GenBank (AY304497, AY389697) overlapping with our sequences by only 158 bp were excluded from network calculation, due to their short length and possible sequencing errors (see Results).

Results

Our samples contained eight distinct mitochondrial haplotypes (A, B1–B4, C, D1–D2). While all differ in their mtDNA fragment 2 (ND4 + tRNA-His, tRNA-Ser, tRNA-Leu), haplotypes B2–B4 and D1 and D2, respectively, are identical in their fragment 1 (12S rRNA). Moreover, haplotypes B3 and B4 are identical concerning fragment 3 (cyt *b* + tRNA-Thr). Haplotype A was found in all eight samples from the Ussuri Region, southern Siberia. Haplotypes B1–B4 and C were identified in the 18 turtles from Chinese markets; haplotype C occurred also in one sample from a wild-caught individual from Shaanxi, China. Haplotypes D1 and D2 were from the wild-caught Vietnamese turtles (Table 2). For these eight haplotypes, all tree-building methods returned exactly the same branching pattern with robust nodal support (Fig. 2). The log likelihood value of the only ML tree was –ln L=6365.3560. Under MP, a single tree of 714 steps was obtained (CI= 0.8515, RI=0.7330). Haplotype A from Ussuri Region constitutes the sister to all other haplotypes. The haplotypes from the turtles obtained from Chinese markets correspond to two distinct clades (B1–B4 and C), the two haplotypes from Vietnam (D1–D2) to a third clade. The clade comprising haplotypes B1–B4 is the sister group of a clade with the remaining haplotypes C (Shaanxi, China and Sichuan market) and D1 + D2 (Vietnam).

With respect to mtDNA fragment 1 (12S rRNA), five out of seven *Pelodiscus* sequences from GenBank represent distinct haplotypes. AY389697 and AY743421 are the putative *P. axenaria* sequences from Chen et al. (2005) and Chen and Zhang (unpublished); two other haplotypes correspond to the sequences AY304497 and AY743420 labelled as '*P. sinensis*' by those same authors. AY304497 is one of the two short sequences with only 158 bp aligned nucleotides (Table 4). It is clearly distinct from the second putative *P. sinensis* sequence (AY743420) of Chen et al. (2005) and Chen and Zhang (unpublished), but resembles the corresponding fragment of our haplotypes B1–B4 and C. However, AY304497 differs from our haplotypes in positions 145 (T instead of C) and 154–155 (TC instead of CT). The other short sequence (AY389697), allegedly *P. axenaria*, differs from AY743421, the second putative sequence of this species, only in exactly the same positions and substitutions. Such mutations do not occur in any other *Pelodiscus* sequence, which suggests a sequencing error close to the 3′-end of the fragment. The fifth haplotype is represented by the complete mitochondrial genome of a Korean softshell turtle (AY962573). In the parsimony network excluding the two short sequences (Fig. 3), all

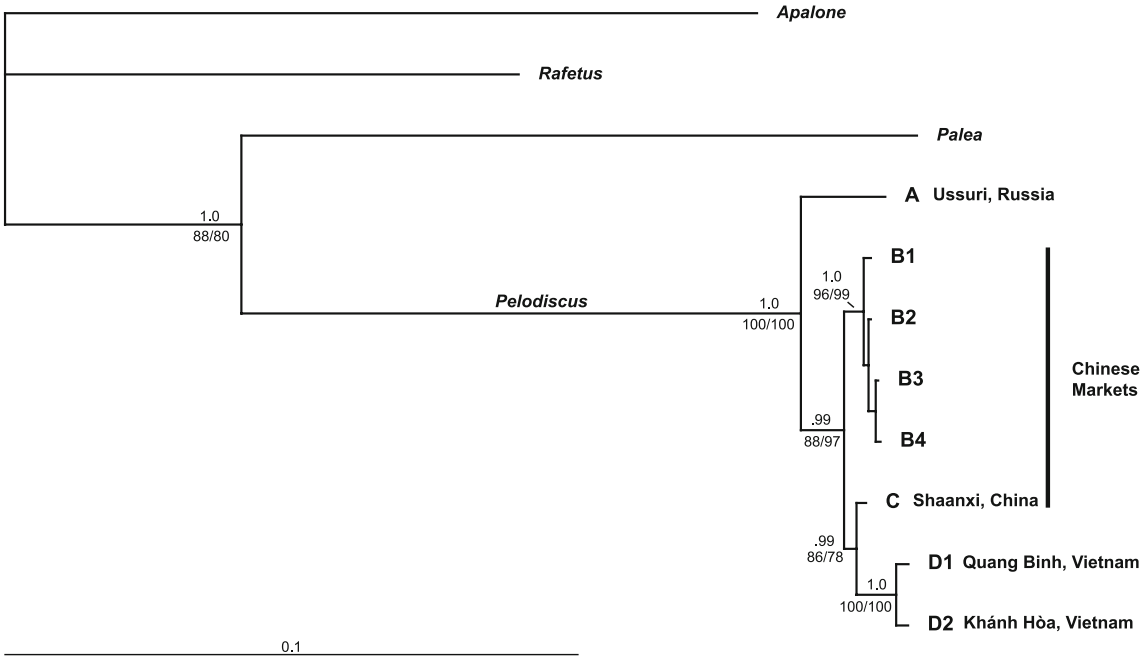

Fig. 2 Bayesian tree for mtDNA haplotypes of *Pelodiscus* based on concatenated sequences of all three mitochondrial fragments from present authors' samples (2,421 sites, mixed-model approach). *Numbers* above branches are Bayesian posterior probabilities; below branches, ML/MP bootstrap values. Support values for clade B3 + B4: 1.0/88/87; for B2 + (B3 + B4): 0.99/65/64. Origins of haplotypes indicated on the *right*. For further explanations, see text

haplotypes are connected under 90–95% probability limits. The most distinct haplotypes correspond to one sequence assigned to *P. sinensis* (AY743420) and another one assigned to *P. axenaria* (AY743421) by Chen et al. (2005) and Chen and Zhang (unpublished). AY743420 and AY743421 are tip haplotypes and differ from haplotypes A and (B2 + B3 + B4) by a minimum of eight and 14 steps, respectively. The sequence AY962573 from Korea is similar to our haplotype A from Ussuri Region and differs from the latter in two positions.

In phylogenetic analyses of haplotypes represented by mtDNA fragment 1, the topology is poorly resolved (Fig. 4) due to weak phylogenetic signal. This is also mirrored by contradictory branching patterns of the ingroup in the four retrieved equally parsimonious trees (98 steps; CI=0.8776, RI=0.7551) and low support values of most nodes within *Pelodiscus*. The log likelihood value of the only returned ML tree for fragment 1 is –ln L=1030.9231. Only the clade comprising our haplotype A and the GenBank haplotypes AY743420 and AY962573 (Korea) obtained somewhat better support values. AY743420 is one of the sequences labelled as *P. sinensis* by Chen et al. (2005) and Chen and Zhang (unpublished) (Table 4). Another one of their '*P. sinensis* sequences' (AY304497) clusters with very weak support with a putative *P. axenaria* sequence (AY389697; Chen et al. 2005), while the second putative *P. axenaria* sequence (AY743421; Chen and Zhang unpublished) is placed in a basal polytomy within *Pelodiscus*. However, the similarity between the only 158-bp-long sequences AY304497 ('*P. sinensis*') and AY389697 ('*P. axenaria*') could be the result of a sequencing error (see above).

For mtDNA fragment 2 (ND4 + tRNA-His, tRNA-Ser, tRNA-Leu), three *P. sinensis* sequences from GenBank differ from our haplotypes. One of these GenBank sequences, AY962573, the complete mitochondrial genome of a Korean *Pelodiscus*, contains an obvious sequencing error at positions 292–293 of the alignment of fragment 2. Here, a double C occurs instead of a single one at 292. Moreover, in the following motif an A is lacking (AAACTACGAC instead of AAACTACGAAC), resulting in mismatches with all other sequences in positions 296–301. When these obvious mistakes are corrected, AY962573 resembles our haplotype A, but is distinct from it in six positions. Fragment 2 haplotypes are assigned by TCS to two unconnected networks and the unconnected haplotype AY259603 when 90–95% probability thresholds are used. When a connection is enforced, these units appear in the network as discrete haplotype clusters (Fig. 5). One of the clusters comprises our haplotype A and GenBank sequence AY962573 from Korea. Both are separated by a minimum of 19 mutational steps from haplotypes of another, diverse cluster comprising our haplotypes B1–B4, C, and D1–D2; GenBank sequence AY687385 occurs in this cluster as well. Within this second cluster, haplotypes are separated by a maximum of 12 steps. GenBank sequence AY259603 represents a highly distinct haplotype,

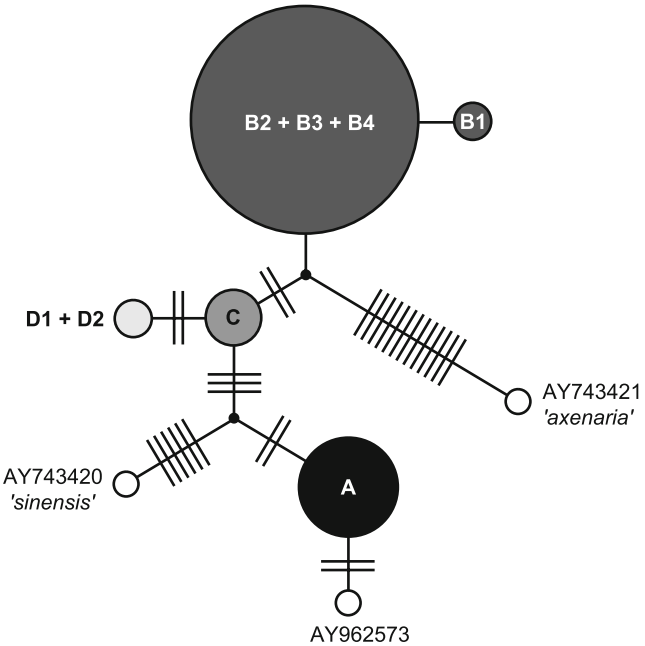

Fig. 3 Parsimony network (spring tree) for fragment 1 haplotypes (mtDNA: 12S rRNA) of *Pelodiscus*. Gaps treated as fifth character state. Connection limit 95%. *Symbol size* corresponds to approximate haplotype frequency; missing node haplotypes, *small solid circles*. Each *uncrossed line* connecting haplotypes indicates one mutational step; where hashmarks across lines are present, each *hashmark* indicates one step. For GenBank sequences representing unique haplotypes (*white*), accession numbers shown; GenBank sequences AF043413 and AY687385 are identical with our fragment 1 haplotype B2 + B3 + B4. Haplotype frequencies: A = 8, B2 + B3 + B4 = 16, C = 3, D1 + D2 = 2, all other haplotypes n=1. Greatest outgroup weight: B2 + B3 + B4 (0.5757). Sequences labelled as *P. axenaria* or *P. sinensis* by Chen et al. (2005) and by Chen and Zhang (unpublished, in GenBank) indicated. Short GenBank sequences AY304497 and AY389697 excluded (see text)

connected by a minimum of 19 steps with haplotypes of the second cluster.

In phylogenetic analyses, two of the three GenBank haplotypes group with haplotypes identified in the present study (AY962573 with haplotype A, AY687385 with B1–B4); the third GenBank haplotype, AY259603, is distinct (Fig. 6). AY259603 is suggested by BA, ML (single ML tree: –ln L=3307.944) and two of the three equally parsimonious trees (263 steps; CI=0.8327, RI=0.7284) as sister to a clade comprising all haplotypes except the basal haplotypes A + AY962573. By contrast, the third MP tree places AY259603 as basal to all other *Pelodiscus* haplotypes.

For mtDNA fragment 3 (cyt *b* + tRNA-Thr), four GenBank sequences differ from our haplotypes; AY687385 is identical with our haplotype B2 (Table 4). In TCS analysis, fragment 3 haplotypes are returned as three discrete networks plus the single unconnected haplotype AY583693 when 90–95% thresholds are used; AY583693 is the putative *P. axenaria* sequence from Chen et al. (2006). When a connection is enforced, each of those units corresponds either to a clearly distinct subnet or to the remote haplotype AY583693 (Fig. 7). The most diverse subnet 1 comprises our haplotypes B1–B4, C, and D1–D2. Subnet 2 contains GenBank sequences AY259553 and AY583692. Subnet 3 corresponds to haplotype A and GenBank sequence AY962573 from Korea. Haplotypes of subnet 1 are separated from haplotypes of subnet 2 by a minimum of 24 steps, and from subnet 3 by a minimum of 29 steps. Haplotypes of subnets 2 and 3 are connected by a minimum of 27 steps. GenBank sequence AY583693 is connected only to subnet 2, but is highly distinct and separated by 87 and 88 steps, respectively, from the haplotypes of subnet 2. Via subnet 2, AY583693 is connected by a minimum of 110 steps with haplotypes of subnet 1, and by 113 steps with subnet 3. Within subnet 1 a maximum of 20 steps occurs, three steps occur within subnet 2, and 14 steps within subnet 3.

In phylogenetic analyses, BA, ML (single ML tree: –ln L=3700.2212) and one of the two equally parsimonious trees (479 steps; CI=0.7871, RI=0.6973) yielded the same branching pattern (Fig. 8). The Korean GenBank sequence AY962573 is sister to our haplotype A, with 100% support. The well-supported clade formed by GenBank sequences AY259553 and AY583692 is weakly supported as the sister group of haplotype A + AY962573. In contrast, AY259553 and AY583692 together are proposed as sister to the clade comprising haplotypes B1–B4, C, and D1 + D2 by the second parsimony tree. In all obtained trees, the putative *P. axenaria* sequence AY583693 is highly distinct and sister to all other *Pelodiscus* haplotypes.

With respect to the nuclear genomic C-mos gene, our samples yielded six haplotypes (Table 2) that differ in up to six mutational steps; when the distinct GenBank haplotype FJ230869 is considered, the maximum number of steps is eight (Fig. 9). The distribution of C-mos haplotypes does not correspond perfectly to mitochondrial haplotypes, and particularly in turtles from Chinese markets two distinct alleles were detected. The frequent haplotype C-mos4 and the rare C-mos5 (identified only once) are the only nuclear haplotypes occurring in the samples from the northernmost part of the species' range (Ussuri Region, Russia; mtDNA haplotype A). However, C-mos4 was also found in samples from Chinese markets that yielded mtDNA haplotypes B3 or C. In one turtle obtained at a market in Guangyuan, Sichuan (mtDNA haplotype C), C-mos4 occurred together with C-mos1. In samples harboring mtDNA haplotypes of clade B, all from market turtles, all C-mos haplotypes other than the rare haplotypes C-mos3 and C-mos5 were found. The distinct GenBank haplotype C-mos7 most likely also originates from a trade turtle. The only wild-caught Chinese turtle (Changling, Shaanxi), mtDNA haplotype C, yielded the rare nuclear haplotype C-mos3 that was not found in any other specimen. The other two samples representing

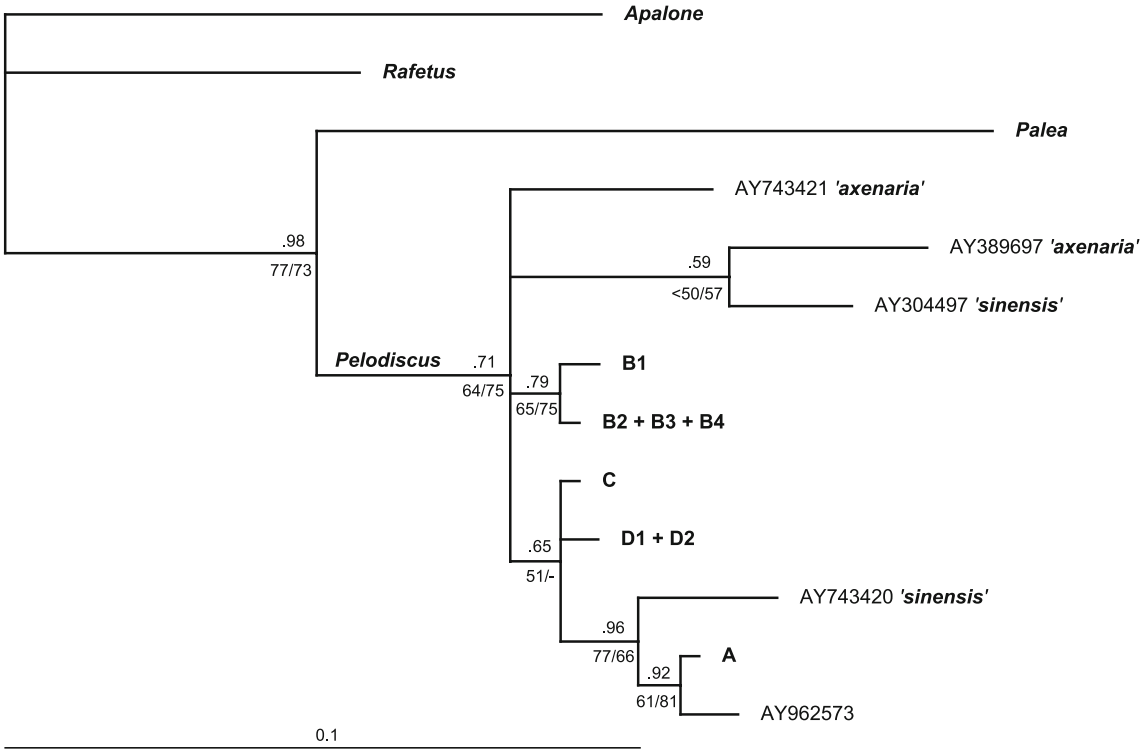

Fig. 4 Bayesian tree for fragment 1 haplotypes (mtDNA: 12S rRNA) of *Pelodiscus.* For GenBank haplotypes, accession numbers shown. *Dash* indicates this branch not found by MP analysis. Sequences labelled as *P. axenaria* or *P. sinensis* by Chen et al. (2005) and by Chen and Zhang (unpublished, in GenBank) indicated. AY389697 ('*P. axenaria*') and AY304497 ('*P. sinensis*') are short sequences of only 158 bp length that share three possible sequencing errors. For further explanations, see Fig. 2 and text

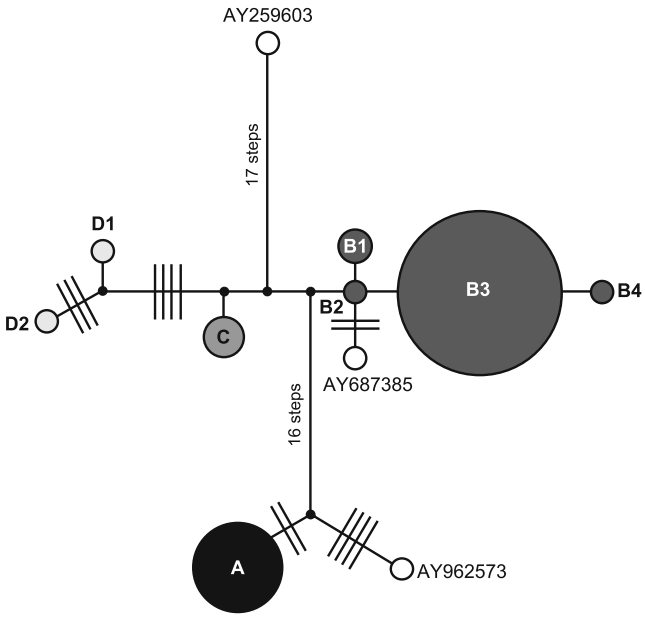

Fig. 5 Parsimony network (spring tree) for fragment 2 haplotypes (mtDNA: ND4 + tRNA-His, tRNA-Ser, tRNA-Leu) of *Pelodiscus*. Connection enforced. Haplotype frequencies: A = 8, B1 = 2, B3 = 12, C = 3, all other haplotypes n=1. Greatest outgroup weight: B2 (0.4). For further explanations, see Fig. 3 and text

mtDNA haplotype C, from market turtles, had the nuclear haplotypes C-mos1 and C-mos4. The two Vietnamese samples (mtDNA haplotypes D1, D2), from the southernmost part of the range, contained haplotype C-mos6, also found in Chinese market specimens.

Discussion

Our study provides evidence for complex genetic variation in *Pelodiscus*. Phylogenetic analyses of mitochondrial sequences identified four clades of haplotypes (A–D) corresponding to distinct genetic lineages. Some of these are so distinct that subnets in TCS analyses are not connected under the 90–95% connection probability thresholds. Lineage B haplotypes were discovered only in market turtles that were most likely farm-bred, while the other lineages were found also or exclusively in wild-caught individuals. Haplotype A, corresponding to a distinct lineage, was identified only from wild-caught turtles from the northernmost part of the range. Haplotype C, representing another lineage, was identified in a wild-caught turtle

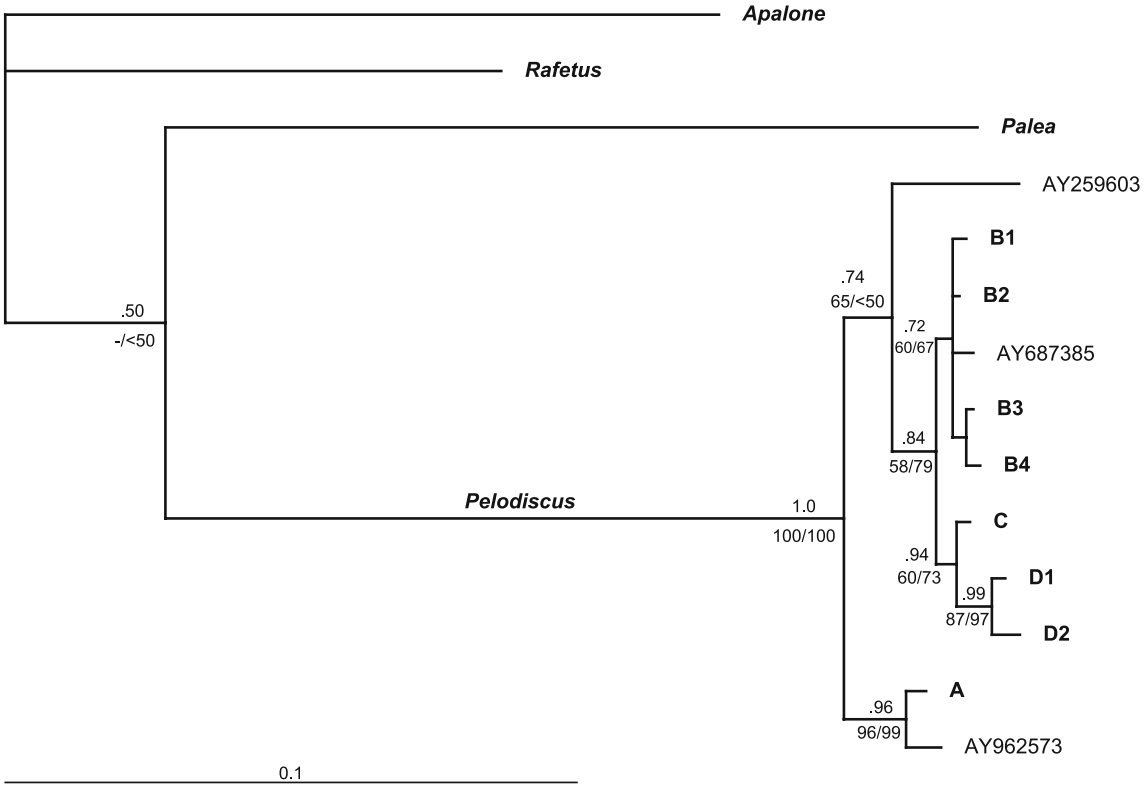

Fig. 6 Bayesian tree for fragment 2 haplotypes (mtDNA: ND4 + tRNA-His, tRNA-Ser, tRNA-Leu) of *Pelodiscus*. For GenBank haplotypes, accession numbers shown. Support values for clade B3 + B4: 0.84/60/63. *Dash* indicates this branch not found by ML analysis. For further explanations, see Figs. 2, 4 and text

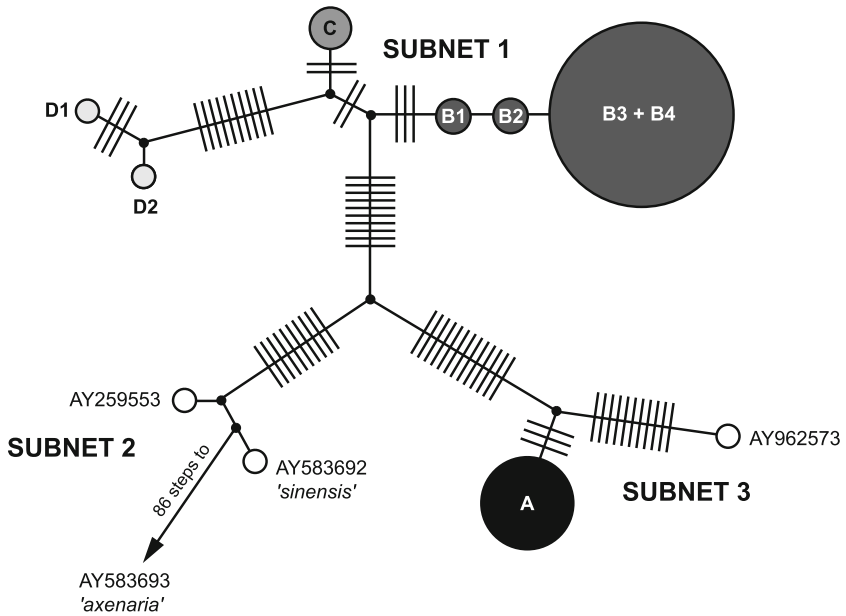

Fig. 7 Parsimony network (spring tree) for fragment 3 haplotypes (mtDNA: cyt *b* + tRNA-Thr) of *Pelodiscus*. Connection enforced. GenBank sequence AY687385 is identical with our haplotype B2. Haplotype frequencies: A = 8, B1 = 2, B2 = 2, B3 + B4 = 13, C = 3, all other haplotypes $n=1$. Greatest outgroup weight: B2 (0.5714). Sequences labelled as *P. axenaria* or *P. sinensis* by Chen et al. (2006) indicated. For further explanations, see Fig. 3 and text

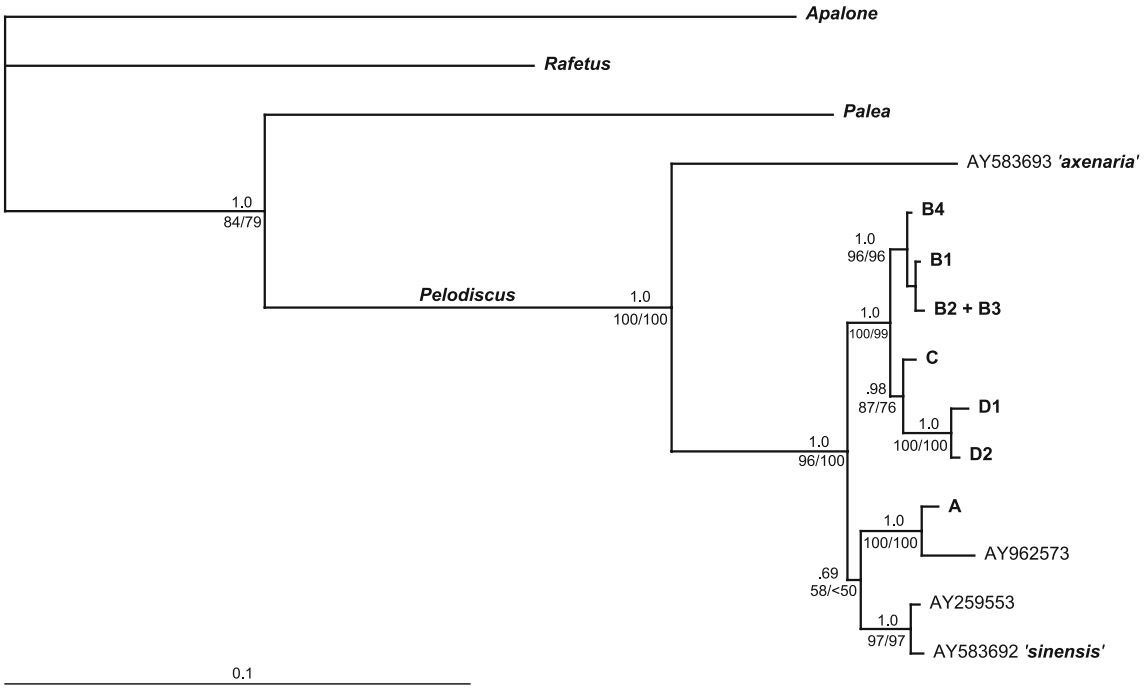

Fig. 8 Bayesian tree for fragment 3 haplotypes (mtDNA: cyt *b* + tRNA-Thr) of *Pelodiscus*. For GenBank haplotypes, accession numbers shown. Sequences labelled as *P. axenaria* or *P. sinensis* by Chen et al. (2006) indicated. Support values for clade B1 + (B2 + B3): 1.0/62/63. For further explanations, see Fig. 2 and text

from Shaanxi, China, and two market specimens. Clade D haplotypes were found only in animals from the southernmost part of the range (Vietnam).

The differentiation pattern of nuclear genomic haplotypes does not completely match the mitochondrial haplotypes. For obvious reasons, this is partially due to the strictly maternal inheritance of the mitochondrial genome and its smaller effective population size compared to the nuclear genome, resulting in accelerated lineage sorting. Nevertheless, there is a conspicuous geographical correlation among both data sets in that the nuclear genomic haplotypes C-mos4 and C-mos5 are the only ones occurring in turtles from the northernmost part of the range, and C-mos6 is the only haplotype identified from the southernmost part of the range, resembling the distribution of mtDNA lineages A and D. This similarity is suggestive of advanced lineage sorting also in the slowly evolving nuclear locus. Mismatches between mitochondrial and nuclear genomic patterns occur mainly in turtles from Chinese food markets. There could be two explanations: (1) this reflects a natural pattern of incomplete sorting or hybridization/introgression, or (2) the market specimens are the result of mixing distinct genetic lineages in turtle farms.

It is impossible to decide which of these two options is more likely, due to the small sample size and, in particular, the difficulty to obtain a range-wide sampling of wild-caught *Pelodiscus*. However, it is obvious that in market turtles, which are most likely farm-bred, distinct alleles of C-mos frequently occur together (Table 2). Only one out of nine genotyped wild-caught *Pelodiscus* was heterozygous (11.1%), but seven out of twelve genotyped market turtles (58.3%), which suggests captive hybridization.

While a few mitochondrial sequences from GenBank are identical or very similar to haplotypes identified by us, highly distinct GenBank sequences demonstrate that we did not sample all genetic lineages of *Pelodiscus* (Table 7). This is also underlined by the distinct nuclear genomic GenBank sequence FJ230869 (C-mos).

Our mtDNA haplotype A consistently clusters with GenBank sequence AY962573, the latter derived from a Korean softshell turtle. Its geographical origin and genetic similarity to our haplotype A suggest that both represent the same taxon. From the northernmost part of the range of *Pelodiscus*, the species *P. maackii* was described. Based on osteology and external morphology, some recent authors have considered it as valid (Chkhikvadze 1987; Fritz and Obst 1999); our genetic data support this view. Morphologically, adult specimens of *P. maackii* are easily recognized by the distinctive light stippling of shell and extremities (Fritz and Obst 1999; Fig. 10). Moreover, according to our observations *P. maackii* may reach up to 32.5 cm in shell length, while adult *Pelodiscus* from the central and southern parts of the range attain only 15–20 cm, the recently described *P. parviformis* from Guangxi

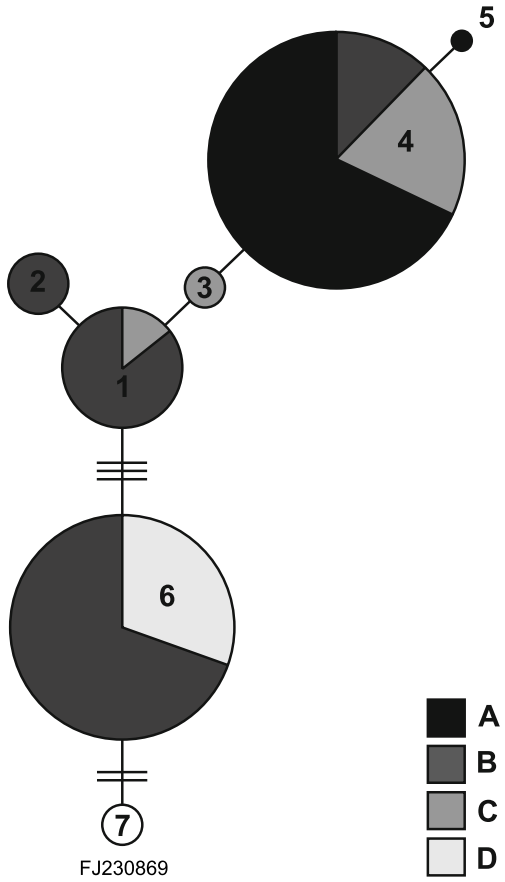

Fig. 9 Parsimony network (spring tree) for nuclear genomic C-mos haplotypes (numbered) of *Pelodiscus*. *Shading of slices* indicates mtDNA haplotypes (A–D). Frequencies of C-mos haplotypes: C-mos1 = 6; C-mos2 = 3; C-mos3 = 2; C-mos4 = 16; C-mos5 = 1; C-mos6 = 14; C-mos7 = 2. Greatest outgroup weight: C-mos3 (0.381). For further explanations, see Fig. 3, Table 2 and text

and Hunan even only 10–12 cm (Tang 1997). However, one aged market specimen harboring haplotype C displayed a color pattern resembling *P. maackii* (Fig. 10) and was of 24 cm carapace length, implying either that turtles of mitochondrial lineage C resemble *P. maackii* and exceed others in size or that this putative farm turtle shows some genetic input from *P. maackii*. The latter possibility is supported by the presence of the nuclear genomic allele C-mos4, typical for *P. maackii* (Table 2).

The cyt *b* sequences AY259553 and AY583692 from turtles of unknown origin (Table 4) constitute a well-supported clade that is suggested as sister to *P. maackii* (haplotype A + AY962573 from Korea; Fig. 8); in parsimony network analysis AY259553 and AY583692 appear as a clearly distinct subnet (Fig. 7). This suggests that a further taxon exists that is allied to *P. maackii* (Table 7: distinct lineage I). It seems possible that the distinct 12S rRNA sequence AY743420 (Chen and Zhang unpublished), originating from the same team as AY583692 (Chen et al. 2006), represents the same lineage.

With respect to mtDNA fragment 2 (ND4, tRNA-His, tRNA-Ser, tRNA-Leu), there is another GenBank sequence (AY259603) that cannot be identified with any of the other distinct GenBank sequences or mitochondrial lineages characterized in this study (Table 7: distinct lineage II). According to most phylogenetic analyses, AY259603 is basal to all available sequences of this mtDNA fragment except *P. maackii* (haplotype A + AY962573) (Fig. 6). This is also supported by network analysis (Fig. 5) in that the sequence differs by a minimum of 19 steps from haplotype C and the cluster comprising haplotypes B1–B4, by 23 steps from D1 + D2, but by 36 steps from *P. maackii*.

Finally, GenBank sequences of 12S rRNA and cyt *b* labelled as '*P. axenaria*' (Chen et al. 2005, 2006; Chen and Zhang unpublished) differ significantly from all other sequences and provide evidence for the existence of a highly distinct taxon that seems to be sister to all other *Pelodiscus* lineages. When the many taxa described from the central part of the range of *Pelodiscus* are considered (Table 1), however, it cannot be ruled out that a name older than *Trionyx axenaria* Zhou, Zhang & Fang, 1991 is available.

Conclusions

Our study provides evidence that '*Pelodiscus sinensis*' represents a diverse species complex. Mitochondrial DNA sequences from the northernmost part of the range are clearly distinct, and their nuclear genomic C-mos haplotypes indicate an advanced state of lineage sorting, supportive of the view that the morphologically distinctive *P. maackii* (Brandt, 1857) is a valid species. Data from the present study and GenBank sequences suggest that several taxa occur in the central and southern parts of the range. Our data indicate the occurrence of three further mitochondrial lineages and interbreeding in turtle farms. GenBank sequences assigned to *P. axenaria* are highly distinct and suggest that this taxon is sister to all other *Pelodiscus*. Further GenBank sequences attributed to '*P. sinensis*' correspond to two further mitochondrial lineages of unknown geographical provenance. One of these seems to be closely allied to *P. maackii*, while the second is basal to a clade comprising the three lineages from the central and southern parts of the range identified in our study. In summary, the combined data suggest that *Pelodiscus* consists of at least seven taxa (Table 7). With the exception of *P. maackii*, it is unclear which of the available names should be applied to these taxa.

In the light of the present study, great caution is warranted when *Pelodiscus* is used as model organism,

Table 7 Tentative allocation of mitochondrial GenBank sequences of *Pelodiscus*

Accession number	mtDNA fragment	Remarks	Mitochondrial lineage/taxon
AF043413	1	identical with corresponding fragment of haplotypes B2, B3, B4	lineage B
AY259553	3	not identifiable with any haplotype represented by other mtDNA fragments; differing by 3 steps from AY583692; both sequences highly distinct and constituting the sister group of *P. maackii* (haplotype A + AY962573)	distinct lineage I
AY259603	2	not identifiable with any haplotype represented by other mtDNA fragments; highly distinct; differing by a minimum of 19 steps from other haplotypes; in phylogenetic analyses basal to a clade comprising lineages A, B, and C	distinct lineage II
AY304497	1	distinct; differing by 3 steps each from (B2 + B3 + B4) and C (sequencing error?)	lineage B or C
AY389697	1	distinct; differing by 3 steps from AY743421 (sequencing error?)	'*P. axenaria*'[a]
AY583692	3	not identifiable with any haplotype represented by other mtDNA fragments; differing by 3 steps from AY259553; both sequences highly distinct and constituting the sister group of *P. maackii* (haplotype A + AY962573)	distinct lineage I
AY583693	3	extremely distinct; differing by a minimum of 87 steps from other haplotypes; sister to all other fragment 3 sequences	'*P. axenaria*'[a]
AY687385	1, 2, 3	distinct, but closely resembling haplotype B2; fragment 1 identical with B2, B3, B4; fragment 2 differing by 2 steps from B2; fragment 3 identical with B2	lineage B
AY743420	1	highly distinct; differing by a minimum of 8 steps from other haplotypes; sister to *P. maackii* (haplotype A + AY962573)	distinct lineage I?
AY743421	1	highly distinct; differing by a minimum of 14 steps from other haplotypes	'*P. axenaria*'[a]
AY962573	1,2,3	distinct, but resembling haplotype A with which it clusters with strong support in phylogenetic analyses; fragment 1 differing by 2 steps from A; fragment 2 differing by 6 steps from A; fragment 3 differing by 14 steps from A	*P. maackii* (lineage A)

Lineages distinct from *P. maackii* (lineage A), *Pelodiscus* spp. (lineages B, C, or D), and the putative *P. axenaria* bear Roman numerals. For further data, see Table 4

[a] For this taxon, an older name may be available

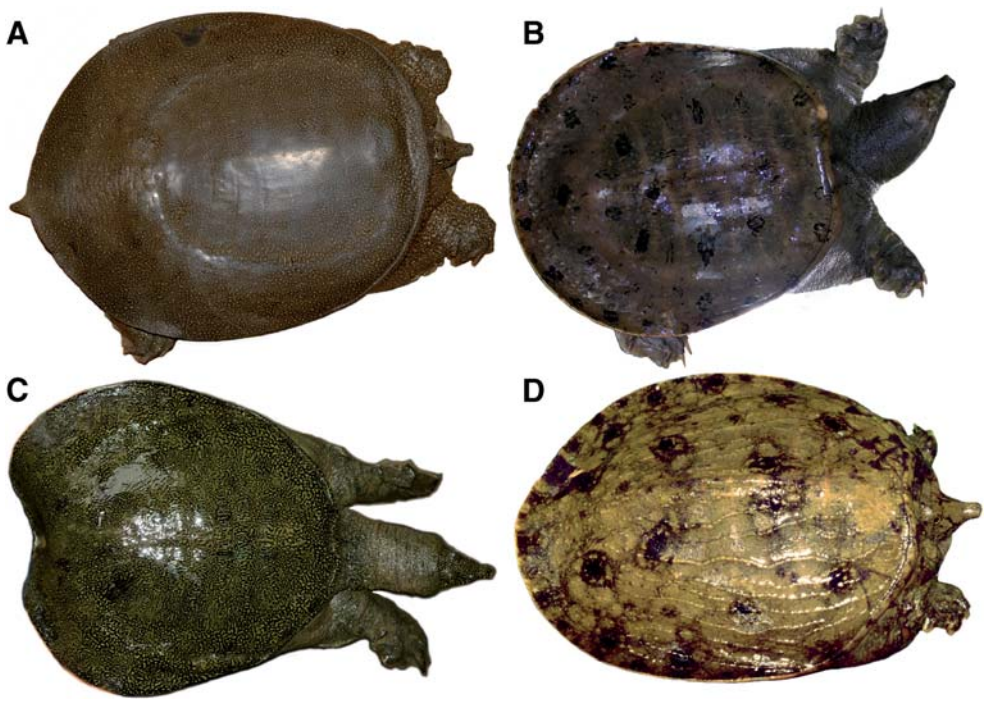

Fig. 10 Adult representatives of distinct mitochondrial lineages of *Pelodiscus*. **A** *Pelodiscus maackii* from Lake Khanka, southern Siberia, Russia. Note distinctive light stippling of shell and extremities. Photo: N. Schneeweiß. **B** *Pelodiscus* sp., market specimen from Suzhou, Jiangsu, China. Photo: G. Kuchling. **C** *Pelodiscus* sp., market specimen from Guangyuan, Sichuan, China. Note color pattern resembling *P. maackii* (see text). Photo: M. Auer. **D** *Pelodiscus* sp., Phong Nha-Ke Bang Reserve, Quang Binh, Vietnam. Coloration and pattern match description of *P. parviformis* Tang, 1997. Photo: T. Ziegler

because unrecognized involvement of distinct taxa could lead to irreproducible results. For instance, contradictory findings with respect to the mode of sex determination and the presence or absence of sex chromosomes in '*P. sinensis*' (Choo and Chou 1992; Ji et al. 2003; Kawai et al. 2007; Nie et al. 2001; Ran and Yuan 2004; Zhu and Sun 2000) are now easily explained, because it is most likely that more than a single taxon was used. Moreover, the results of our study call for a new assessment of the conservation status of *Pelodiscus*. While currently all taxa are subsumed under '*P. sinensis*' and listed as 'vulnerable' by the IUCN Red List of Threatened Species (IUCN 2009), some could actually be endangered or even critically endangered.

Acknowledgements Lab work was done by Anke Müller. Markus Auer's work in China benefited from a grant of the EAZA Shellshock Campaign. Bing He, Zunliang Li, Guofang Zhong, and Jianping Zou helped to collect samples in Guangdong. Christian Schmidt (Dresden) translated Chinese papers for us. Thomas Ziegler (Köln) provided photos of Vietnamese *Pelodiscus*.

References

Castelloe, J., & Templeton, A. R. (1994). Root probabilities for intraspecific gene trees under neutral coalescent theory. *Molecular Phylogenetics and Evolution, 3*, 102–113.

Chen, H.-G., Liu, W.-B., & Zhang, X.-J. (2005). Comparative analysis of mitochondrial DNA 12S rRNA region between *Pelodiscus sinensis* and *Pelodiscus axenaria* and their molecular marker for identification. *Journal of Fisheries of China, 29*, 318–322 [in Chinese, with English abstract].

Chen, H.-G., Liu, W.-B., Li, J.-Z., & Zhang, X.-J. (2006). Comparative analysis of mitochondrial DNA *cytb* gene and their molecular identification markers in three species of soft-turtles. *Acta Hydrobiologica Sinica, 30*, 380–385 [in Chinese, with English abstract].

Chkhikvadze, V. M. (1987). O sistematicheskom polozhenii dal'nevostochnogo trioniksa. *Bulletin of the Academy of Sciences of the Georgian SSR, 128*, 609–611.

Choo, B. L., & Chou, L. M. (1992). Does incubation temperature influence the sex of embryos in *Trionyx sinensis*? *Journal of Herpetology, 26*, 341–342.

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

Diesmos, A. C., Brown, R. M., Alcala, A. C., & Sison, R. V. (2008). Status and distribution of nonmarine turtles of the Philippines. *Chelonian Conservation and Biology, 7*, 157–177.

Donnelly, P., & Tavaré, S. (1986). The ages of alleles and a coalescent. *Advances in Applied Probability, 18*, 1–19.

Engstrom, T. N., Shaffer, H. B., & McCord, W. P. (2002). Phylogenetic diversity of endangered and critically endangered Asian softshell turtles (Trionychidae: *Chitra*). *Biological Conservation, 104*, 173–179.

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

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

Ernst, C. H., Altenburg, R. G. M., & Barbour, R. W. (2000). *Turtles of the world, ver. 1.2. CD-ROM*. Amsterdam: ETI BioInformatics.

Fritz, U., & Bininda-Emonds, O. R. P. (2007). When genes meet nomenclature: tortoise phylogeny and the shifting generic concepts of *Testudo* and *Geochelone*. *Zoology, 110*, 298–307.

Fritz, U., & Havaš, P. (2007). Checklist of chelonians of the world. *Vertebrate Zoology, 57*, 149–368.

Fritz, U., & Obst, F. J. (1999). Neue Schildkröten aus Südostasien. Teil II. Bataguridae (*Cyclemys*, *Heosemys*, *Mauremys*, *Ocadia*, *Pyxidea*, *Sacalia*) und Trionychidae. *Sauria, 21*, 11–26.

Fritz, U., Auer, M., Bertolero, A., Cheylan, M., Fattizzo, T., Hundsdörfer, A. K., et al. (2006). A rangewide phylogeography of Hermann's tortoise, *Testudo hermanni* (Reptilia: Testudines: Testudinidae): implications for taxonomy. *Zoologica Scripta, 35*, 531–543.

Fritz, U., Ayaz, D., Buschbom, J., Kami, H. G., Mazanaeva, L. F., Aloufi, A. A., et al. (2008a). Go east: phylogeographies of *Mauremys caspica* and *M. rivulata*—discordance of morphology, mitochondrial and nuclear genomic markers and rare hybridization. *Journal of Evolutionary Biology, 21*, 527–540.

Fritz, U., Guicking, D., Auer, M., Sommer, R. S., Wink, M., & Hundsdörfer, A. K. (2008b). Diversity of the Southeast Asian leaf turtle genus *Cyclemys*: how many leaves on its tree of life? *Zoologica Scripta, 37*, 367–390.

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. *BioTechniques, 11*, 298–302.

Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Huelsenbeck, J. P., & Ronquist, F. (2001). MrBAYES. Bayesian inference of phylogenetic trees. *Bioinformatics, 17*, 754–755.

IUCN = International Union for the Conservation of Nature and Natural Resources. (2009). IUCN red list of threatened species. Version 2009.1. http://www.iucnredlist.org. Accessed 23 May 2009.

Iverson, J. B. (1992). *A revised checklist with distribution maps of the turtles of the world*. Richmond: Privately printed.

Jensen, K. A., & Das, I. (2008). Cultural exploitation of freshwater turtles in Sarawak, Malaysian Borneo. *Chelonian Conservation and Biology, 7*, 281–285.

Ji, X., Chen, F., Du, W.-G., & Chen, H.-L. (2003). Incubation temperature affects hatchling growth but not sexual phenotype in the Chinese soft-shelled turtle, *Pelodiscus sinensis* (Trionychidae). *Journal of Zoology, 261*, 409–416.

Jung, S.-O., Lee, Y.-M., Kartavtsev, Y., Park, I.-S., Kim, D. S., & Lee, J.-S. (2006). The complete mitochondrial genome of the Korean soft-shelled turtle *Pelodiscus sinensis*. *DNA Sequence, 17*, 471–483.

Kawai, A., Nishida-Umehara, C., Ishijima, J., Tsuda, Y., Ota, H., & Matsuda, Y. (2007). Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenetic and Genome Research, 117*, 92–102.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., et al. (1989). Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 6196–6200.

Le, M., Raxworthy, C. J., McCord, W. P., & Mertz, L. (2006). A molecular phylogeny of tortoises (Testudines: Testudinidae) based on mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution, 40*, 517–531.

Maran, J. (2003). Visite d'une ferme à tortues au Vietnam. *Manouria, 6*, 8–12.

McGaugh, S. E., Eckerman, C. M., & Janzen, F. J. (2008). Molecular phylogeography of *Apalone spinifera*. *Zoologica Scripta, 37*, 289–304.

Mertens, R., & Wermuth, H. (1955). Die rezenten Schildkröten, Krokodile und Brückenechsen. *Zoologische Jahrbücher / Abteilung für Systematik, Ökologie und Geographie der Tiere, 83*, 323–440.

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.

Naro-Maciel, E., Le, M., FitzSimmons, N. N., & Amato, G. (2008). Evolutionary relationships of marine turtles: a molecular phylogeny based on nuclear and mitochondrial genes. *Molecular Phylogenetics and Evolution, 49*, 659–662.

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.

Nie, L.-W., Guo, C.-W., & Wang, Q. (2001). Sex determination mechanism of *Trionyx sinensis*. *Chinese Journal of Applied and Environmental Biology, 7*, 258–261.

Nurizan, A., & Ong, B. L. (1997). Some problems of cultured softshell turtle (*Pelodiscus sinensis*) in Peninsular Malaysia. *Jurnal Veterinar Malaysia, 9*, 27–28.

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. *TREE, 16*, 37–45.

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, 36*, 301–310.

Ran, C.-X., & Yuan, C.-G. (2004). The incubation temperature and the sex determination of *Trionyx sinensis*. *Journal of Fujian Fisheries, 25*, 51–53.

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

Sato, H., & Ota, H. (1999). False biogeographical pattern derived from artificial animal transportations: a case of the soft-shelled turtle, *Pelodiscus sinensis*, from the Ryukyu Archipelago, Japan. *Developments in Animal and Veterinary Sciences, 29*, 317–334.

Shi, H. T., Parham, J. F., Fan, Z. Y., Hong, M. L., & Yin, F. (2008). Evidence for the massive scale of turtle farming in China. *Oryx, 42*, 147–150.

Spinks, P. Q., & Shaffer, H. B. (2005). Range-wide molecular analysis of the western pond turtle (*Emys marmorata*): cryptic variation, isolation by distance, and their conservation implications. *Molecular Ecology, 14*, 2047–2064.

Spinks, P. Q., Shaffer, H. B., Iverson, J. B., & McCord, W. P. (2004). Phylogenetic hypotheses for the turtle family Geoemydidae. *Molecular Phylogenetics and Evolution, 32*, 164–182.

Stephens, M., & Donnelly, P. (2003). A comparison of Bayesian methods for haplotype reconstruction. *American Journal of Human Genetics, 73*, 1162–1169.

Stephens, M., Smith, N. J., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics, 68*, 978–989.

Stuart, B. L., & Parham, J. F. (2004). Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). *Molecular Phylogenetics and Evolution, 31*, 164–177.

Swofford, D. L. (2002). *PAUP*. Phylogenetic analysis using parsimony (*and other methods), ver. 4.0b10*. Sunderland: Sinauer.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*, 1596–1599.

Tang, Y. (1997). Research on a new species of *Pelodiscus*, Trionychidae in China. *Zoological Research / Kunming Institute of Zoology, 18*, 13–17 [in Chinese, with English abstract].

van Dijk, P. P., Stuart, B. L., & Rhodin, A. G. J. (Eds.) (2000). Asian turtle trade. Proceedings of a workshop on conservation and trade of freshwater turtles and tortoises in Asia. Phnom Penh, Cambodia, 1–4 December 1999. *Chelonian Research Monographs*, 2, 1–164.

Vargas-Ramírez, M., Castaño-Mora, O. V., & Fritz, U. (2008). Molecular phylogeny and divergence times of ancient South American and Malagasy river turtles (Testudines: Pleurodira: Podocnemididae). *Organisms Diversity and Evolution, 8*, 388–398.

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.

Wermuth, H., & Mertens, R. (1961). *Schildkröten, Krokodile, Brückenechsen*. Jena: Fischer.

Wermuth, H., & Mertens, R. (1977). Testudines, Crocodylia, Rhynchocephalia. *Das Tierreich*, 100, i–xxvii + 1–174.

Zhang, L., Hua, N., & Sun, S. (2008). Wildlife trade, consumption and conservation awareness in southwest China. *Biodiversity and Conservation, 17*, 1493–1516.

Zhao, E.-M., & Adler, K. (1993). *Herpetology of China*. Oxford: Society for the Study of Amphibians and Reptiles.

Zhou, G., Zhang, X., & Fang, Z. (1991). Bulletin of a new species *Trionyx*. *Acta Scientiarum Naturalium Universitatis Normalis Hunanensis, 14*, 379–382 [in Chinese, with English abstract].

Zhu, D.-Y., & Sun, X.-Z. (2000). Sex determination in *Trionyx sinensis*. *Chinese Journal of Zoology, 35*, 37–38.