Mitochondrial DNA sequences suggest a revised taxonomy of Asian flapshell turtles (*Lissemys* Smith, 1931) and the validity of previously unrecognized taxa (Testudines: Trionychidae)

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> Abstract

We investigated relationships among Asian flapshell turtles by using 2286 bp of mitochondrial DNA for phylogenetic reconstructions and relaxed molecular clock calculations. Currently three taxa are recognized, the unspotted species *Lissemys scutata* and *L. punctata*, with the unspotted subspecies *L. p. punctata* and the spotted subspecies *L. p. andersoni*. However, we found five deeply divergent clades, two of which correspond to *L. scutata* (Myanmar; perhaps also adjacent Thailand and Yunnan, China) and *L. p. andersoni* (Indus, Ganges and Brahmaputra drainages; western Myanmar), respectively. Within *L. p. punctata* from peninsular India and Sri Lanka three distinct clades were identified, two from peninsular India and one from Sri Lanka. The two clades from peninsular India are more closely related to *L. p. andersoni* than to flapshell turtles from Sri Lanka. Due to a genetic divergence resembling *L. scutata*, we propose to separate Sri Lankan populations as the distinct species *L. ceylonensis* (Gray, 1856) from *L. punctata*. Furthermore, we suggest to restrict the name *L. p. punctata* (Lacepède, 1788) = *L. p. punctata* (Bonnaterre, 1789) to populations from southern peninsular India, whereas the name *L. p. vittata* (Peters, 1854) should be applied to unspotted flapshell turtles from northern peninsular India. We classify all three taxa from the Indian subcontinent as subspecies because (1) there is morphological and genetic evidence that *L. p. andersoni* intergrades with *L. p. vittata*, and (2) the genetic divergence among *L. p. punctata*, *L. p. andersoni* and *L. p. vittata* resembles the degree of differentiation as observed between the latter two subspecies, whereas the differences between *L. ceylonensis* and *L. scutata* and among these species and the subspecies of *L. punctata* are about twice the values as observed among the subspecies of *L. punctata*. The formation of the subspecies of *L. punctata* was dated to have occurred between the uppermost Miocene and the Early Pleistocene (mean split ages of approx. 4.5 and 4.2 million years); the origin of *L. ceylonensis* and *L. scutata*, to a range between the Early Miocene and the Lower Pliocene (mean split ages of approx. 8 and 11 million years, respectively).

> Key words

Cryptic taxa, *Lissemys ceylonensis* nov. comb., *Lissemys punctata andersoni*, *Lissemys punctata punctata*, *Lissemys punctata vittata* nov. comb., *Lissemys scutata*, phylogeography, revision, systematics.

Introduction

Softshell turtles (Trionychidae) are an ancient group of chelonians known since the Lower Cretaceous of Asia (Meylan & Gaffney, 1992; Nessov, 1995). Trionychids possess a whole array of distinctive morphological characters. In contrast to other extant turtles and tortoises, the bony shell is much reduced, and its flat bone elements have a unique surface sculpturing and a sandwich-like structure, with an internal and external compact layer framing an inner cancellous core. The shell surface is covered by leathery skin

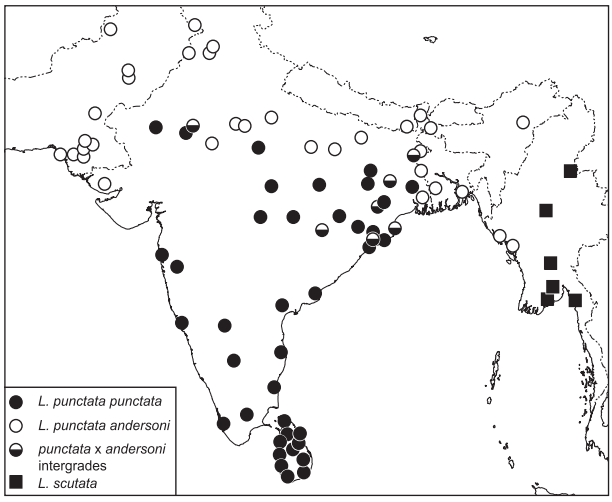

Fig. 1. Distribution of *Lissemys* taxa according to morphological evidence (redrawn from WEBB, 1982). Note the intergradation zone with morphologically intermediate flapshell turtles in northern peninsular India.

instead of horny scutes. The neck is long and retractile, and the snout runs out in a pronounced proboscis. Furthermore, the jaws are concealed by fleshy lips, a unique character among extant chelonians, and the limbs are paddle-like, with three strong claws each. All softshell turtles are highly aquatic, leaving the water only for basking and egg-laying, and mainly or exclusively carnivorous (ERNST & BARBOUR, 1989; ERNST *et al*., 2000; SCHEYER *et al*., 2007).

The family comprises some 35 extant species in 13 genera and two subfamilies. The majority of the species belongs to the Trionychinae, distributed in Africa, Asia, North America and New Guinea. Their sister group, the Cyclanorbinae, are represented by only three genera from Africa and Asia (MEYLAN, 1987; ERNST & BARBOUR, 1989; ERNST *et al*., 2000; FRITZ & HAVAŠ, 2007; PRASCHAG *et al*., 2007; FRITZ *et al*., 2010; RHODIN *et al*., 2010). *Cyclanorbis* and *Cycloderma*, each with two species, are distributed in sub-Saharan Africa, while the genus *Lissemys* is confined to South Asia and western Southeast Asia, at first glance suggestive of an ancient Gondwanan disjunction. However, the oldest known cyclanorbines date only back to the Early Miocene of Kenya and the Sultanate of Oman (18 million years = myr ago; DE LAPPARENT DE BROIN, 2000), and recently it was advocated that the ancestors of the Cyclanorbinae originated in North America (JOYCE & LYSON, 2010). From there, the group is thought to have spread to Asia and only in the Miocene to the Indian subcontinent and Africa.

Compared to the Trionychinae, cyclanorbines are characterized by a stronger ossified, more solid shell with more extensively developed plastral callosities. Moreover, unlike other softshell turtles, all cyclanorbines have well-developed plastra with large fleshy femoral flaps over the hind limb sockets (MEYLAN, 1987; ERNST & BARBOUR, 1989; ERNST *et al*., 2000), being eponymous for their common name 'flapshell turtles'. These femoral flaps and the movable plastral forelobe conceal head and limbs when withdrawn. Molecular and morphological evidence suggests that the African *Cyclanorbis* and *Cycloderma* together constitute the sister group of *Lissemys* (MEYLAN, 1987; ENGSTROM *et al*., 2004; but see JOYCE & LYSON, 2010).

Lissemys is unique among all trionychid turtles in that peripheral bones occur in the posterior shell margin. All other softshell turtles do not possess such ossicles, and have a flexible wide rubbery posterior shell margin. While it was long debated whether the posterior peripheral ossicles of *Lissemys* are homologous to the peripheralia of other chelonians, there is now growing evidence for their homology (DELFINO *et al*., 2010), suggesting that the solid shells of *Lissemys* and the other cyclanorbines represent an ancestral character state.

Even though there was much confusion about the correct genus and species group names of *Lissemys*, there has been for about a century consensus among most authors that the genus embraces three distinct taxa that are currently named *Lissemys punctata punctata*[1], *Lissemys punctata andersoni*, and *Lissemys punctata scutata* or *Lissemys scutata* (BOULENGER, 1889; SIEBENROCK, 1909; SMITH, 1931; DERANIYAGALA, 1939; MERTENS & WERMUTH, 1955; WERMUTH & MERTENS, 1961, 1977; WEBB, 1980, 1982; ERNST & BARBOUR, 1989; ERNST *et al*., 2000; FRITZ & HAVAŠ, 2007; RHODIN *et al*., 2010; but see ANNANDALE, 1912 who recognized five 'races' and DERANIYAGALA, 1953 who treated Sri Lankan flapshell turtles as a distinct

[1] Prior to WEBB's (1980) reappraisal of the nomenclatural history of *Testudo punctata* LACEPÈDE, 1788, this name was generally identified with spotted flapshell turtles from the northern part of the distribution range of *Lissemys*. However, WEBB (1980) concluded that this name was based on unspotted flapshells from the southern part of the range. Although we are convinced that WEBB (1980) erred, we do not want to contribute to further nomenclatural confusion and accept his type locality restriction to Pondicherry, Tamil Nadu, India. WEBB's (1980) arguments were the itinerary of the collector of the type specimen and that the holotype of *Testudo punctata* LACEPÈDE, 1788 is unspotted. However, it is well-known that the provenance of historical museum specimens has to be treated with great caution. Moreover, preserved softshell turtles fade with increasing age, and we have studied bleached old museum specimens of *L. p. andersoni* from the collections of the Natural History Museum, London, and the Senckenberg Museum, Frankfurt, completely void of any spotted pattern.

subspecies). Whilst there is morphological evidence for intergradation between *L. p. punctata* and *L. p. andersoni* (Fig. 1), and thus for their subspecies status (Webb, 1982), the classification of *L. scutata* is still debated. Since Annandale (1912), most authors treated the latter taxon as 'race' or subspecies of what is now named *L. punctata*. Then, Webb (1982) separated *L. scutata* as full species from *L. punctata* based on their allopatric distribution, differences in the configuration of the peripherals, and the earlier development of plastral callosities in *L. scutata*. However, Meylan (1987), Ernst & Barbour (1989), Ernst *et al*. (2000) and Das (2001) disputed the species status of *L. scutata* and continued to treat this taxon as a third subspecies of *L. punctata*. Here we follow provisionally Webb (1982) and the recent turtle checklists by Fritz & Havaš (2007) and Rhodin *et al*. (2010) and regard *L. scutata* as a distinct species.

Lissemys punctata punctata occurs in peninsular India and Sri Lanka, while the Indus, Ganges and Brahmaputra drainages (Pakistan, India, Sikkim, Nepal, Bangladesh) and western Myanmar (Rakhine State) are inhabited by *L. p. andersoni*. *Lissemys scutata* is known from the Ayeyarwady (Irrawady), Sittaung, and Thanlwin (Salween) River systems of Myanmar and perhaps also from westernmost Thailand and Yunnan, China (Ernst & Barbour, 1989; Ernst *et al*., 2000; Fritz & Havaš, 2007; Rhodin *et al*., 2010). Besides the above mentioned osteological characters, the three *Lissemys* taxa differ in colouration and pattern of the head, neck and shell. *Lissemys p. punctata* and *L. scutata* have either uniformly coloured heads and necks or an indistinct striped pattern, whereas *L. p. andersoni* has an intensely yellow spotted head. The carapace of *L. scutata* and, ironically, of *L. p. punctata* is more or less uniformly olive brown to brown coloured, whereas *L. p. andersoni* has a conspicuous pattern of dark-bordered, bright yellow spots (Smith, 1931; Webb, 1980, 1982; Ernst & Barbour, 1989; Ernst *et al*., 2000).

The last investigation addressing the taxonomy of *Lissemys*, based entirely on external morphology (colouration and pattern) and osteological characters, was published by Webb (1982). Although several authors (Meylan, 1987; Ernst & Barbour, 1989; Ernst *et al*., 2000; Das, 2001) voiced later doubts about Webb's conclusion to treat *L. scutata* as distinct species, no additional evidence was obtained to re-examine relationships among *Lissemys* and related taxa.

Based on a nearly range-wide sampling of *Lissemys* and representatives of the two African cyclanorbine genera *Cyclanorbis* and *Cycloderma*, here we use 2286 bp of mitochondrial DNA to elucidate phylogeny and taxonomy of *Lissemys*. In addition, we apply a fossil-calibrated molecular clock to assess the split ages of the three cyclanorbine genera and clades within *Lissemys* for gaining additional insights in their biogeography.

Materials and Methods

Sampling and DNA extraction

Saliva, blood or tissue samples of 45 *Lissemys punctata* and four *L. scutata* were obtained, representing all three currently recognized *Lissemys* taxa and covering most of the distribution range of the genus. As representatives of the African cyclanorbine genera served samples of *Cyclanorbis senegalensis* and *Cycloderma aubryi*. Total genomic DNA was extracted using the DTAB method (Gustincich *et al*., 1991), the innuPREP DNA Mini Kit or the innuPREP Blood DNA Mini Kit (both Analytik Jena AG, Jena, Germany). Ethanol-preserved samples and remaining DNA are stored at -80°C in the tissue sample collection of the Museum of Zoology, Senckenberg Dresden (Table 1).

Chosen mitochondrial markers, PCR, and sequencing

Three mitochondrial DNA fragments (in total 2286 bp) that had been shown to reveal differences and phylogenetic relationships among chelonian terminal taxa (e.g., Engstrom *et al*., 2002, 2004; Spinks *et al*., 2004; Stuart & Parham, 2004; Praschag *et al*., 2007; Vargas-Ramírez *et al*., 2008, 2010; Fritz *et al*., 2010, 2011) were chosen. Fragment 1 corresponded to 372 bp of the 12S rRNA gene. Fragment 2 contained 599 bp coding for the NADH dehydrogenase subunit 4 (ND4) and 182 bp of adjacent tRNAs (tRNA-His, 70 bp; tRNA-Ser, 61 bp; tRNA-Leu, 51 bp). The 1133-bp-long fragment 3 represented the nearly complete cyt *b* gene. The targeted DNA fragments were amplified with the primers given in Table 2. PCR was performed in a final volume of 25 µl containing 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany) with the buffer recommended by the supplier and a final concentration of 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.4 µM of the respective primer pair and 10-40 ng of total DNA. PCR cycling was slightly modified for each mtDNA fragment; 35 cycles were performed for the 12S rRNA and cyt *b* fragments, and 40 cycles for the fragment containing the partial ND4 gene and the DNA coding for the tRNAs. After

3 min initial denaturation at 94°C, denaturation times varied, with 30 s for the 12S rRNA fragment and 45 s for the two other mtDNA fragments. Annealing took place at 50°C for 45 s for the 12S rRNA fragment and at 56°C for 30 s for the two other fragments. Extension time at 72°C was 30 s for the 12S rRNA fragment and 60 s for the other two fragments, but 10 min in each final cycle. PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Staufen, Germany; 1:20 dilution; modified protocol: 30 min at 37°C, 15 min at 80°C). PCR fragments were sequenced using the primers given in Table 2 and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyser (Applied Biosystems).

Alignment and phylogenetic analyses

Sequences were aligned in BIOEDIT 7.0.5.2 (HALL, 1999); alignments were further inspected in MEGA 4.0.2 (TAMURA *et al.*, 2007). Previously generated homologous sequences of *Pelodiscus maackii* (Trionychidae: Trionychinae) were downloaded from GenBank (Table 1; FRITZ *et al.*, 2010), included in the alignments and used for tree rooting. For phylogenetic analyses, the three mtDNA fragments were concatenated, resulting in a total length of 2286 bp. Position 1-599 of the alignment corresponded to the second half of the ND4 gene; position 600-669 to the DNA coding for the tRNA-His; position 670-730, to the tRNA-Ser; position 730-781, to the tRNA-Leu; position 782-1153, to the 12S rRNA; and position 1154-2286, to the cyt *b* gene. The best evolutionary model for each of these partitions was determined in MrMODELTEST 2.3 (NYLANDER, 2004) using the Akaike information criterion (AIC; Table 3).

Bayesian analyses using this partition scheme were performed in MrBAYES 3.1.2 (RONQUIST & HUELSENBECK, 2003) with two parallel runs, each with four chains. The chains ran for 10 million generations with every 100th generation sampled. 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 is a measure of clade frequency and credibility, corresponding to the percentage of all trees containing that clade.

Maximum Likelihood (ML) analyses using the same partitioning scheme were conducted with RAxML 7.0.3 (STAMATAKIS, 2006). Five independent ML searches were performed with the fast bootstrap algorithm to explore the robustness of the phylogenies by comparing the best-scored trees. Subsequently, 1000 thorough bootstrap replicates were calculated and plotted on the ML tree with the best likelihood value.

Molecular Clock

Based on the concatenated data set of the three mitochondrial DNA fragments, the split ages of the *Lissemys* clades were estimated by a relaxed molecular clock as implemented in BEAST 1.5.4 (DRUMMOND & RAMBAUT, 2007). There is a considerable number of fossil African cyclanorbines known, mainly representing the two extant genera *Cyclanorbis* and *Cycloderma* and allied forms (reviewed in DE LAPPARENT DE BROIN, 2000). The oldest remains are attributed to *Cycloderma* and originate from the Early Miocene of Kenya and the Sultanate of Oman (18 myr); slightly younger *Cycloderma* fossils (16 myr) were excavated in Saudi Arabia. Compared to African cyclanorbines, the fossil record of *Lissemys* is quite incomplete. Besides a few Plio-Pleistocene findings (LYDEKKER, 1886, 1889; DERANIYAGALA, 1939, 1953; TRIPATHI, 1964; PRASAD, 1974; CORVINUS & SCHLEICH, 1994), the oldest records date back to the Middle Miocene (13-11 myr; GAUR & CHOPRA, 1983).

Therefore, the two African genera were used for calibrating the phylogeny. The split between *Cyclanorbis* and *Cycloderma* was constrained by setting the prior for their most recent common ancestor (tMRCA) to a hard upper bound of 18.0 myr and a lower soft value of 23.0 myr (beginning of the Early Miocene; WALKER & GEISSMAN, 2009), with a lognormal distribution that matches the stratigraphic scale (mean = 0, standard deviation = 1.0).

The clock calculation was based on a reduced data set that included only two representatives of each *Lissemys* clade and one representative each of *Cyclanorbis* and *Cycloderma* (Table 1). For the runs with BEAST, the length of the MCMC chain was set to 30 million generations and log parameters were sampled every 1000th generation. A lognormal relaxed clock model (DRUMMOND *et al.*, 2006) was chosen, with the tree prior set to speciation (yule process) and the "auto optimize" option activated to adjust automatically the tuning parameters. Input sequence data were manually partitioned in the XML file generated with BEAUTi according to the estimates with MrMODELTEST (Table 3). Linearized consensus trees including posterior probabilities were obtained using TREEANNOTATOR 1.4.8 (as implemented in the BEAST package) with the burn-in parameter set to 9000. Ninety-five percent confidence intervals for time estimates of lineage splits (highest posterior density intervals) were inferred from the log output files using the TRACER software (RAMBAUT & DRUMMOND, 2007).

Table 1. Samples and sequences used in the present study. MTD = Museum of Zoology, Senckenberg Dresden; samples with three- or four-digit numbers are salivary, blood or tissue samples in the Tissue Collection, the sample with the five-digit number (MTD 42892) is a complete voucher specimen in the Herpetological Collection. The column 'Clade' indicates the clade of the respective *Lissemys* sample.

			GenBank accession numbers			
MTD	**Taxon**	**Locality**	**12S**	**ND4 + tRNAs**	**cyt *b***	**Clade**
929	*Lissemys punctata andersoni*	—	FR850504	FR850556	FR850607	A
42892	*Lissemys punctata andersoni*	—	FR850505	—	FR850608	A
4072	*Lissemys punctata andersoni*	Bangladesh: Dhaka	FR850506	FR850557	FR850609	A
3423	*Lissemys punctata andersoni*	Bangladesh: Khulna	FR850507	FR850558	FR850610	A
3424§	*Lissemys punctata andersoni*	Bangladesh: Khulna	FR850508	FR850559	FR850611	A
4070	*Lissemys punctata andersoni*	Bangladesh: Khulna	FR850509	FR850560	FR850612	A
4071	*Lissemys punctata andersoni*	Bangladesh: Khulna	FR850510	FR850561	FR850613	A
5059	*Lissemys punctata andersoni*	India: Assam: Mangaldai village (northern bank of Brahmaputra)	FR850511	FR850562	FR850614	A
5141	*Lissemys punctata andersoni*	India: Haryana: Yamuna River near Delhi	FR850512	FR850563	FR850615	A
5247	*Lissemys punctata andersoni*	India: Odisha (Orissa): Ghugrai, Subarnarekha River	FR850513	FR850564	FR850616	B*
4039§	*Lissemys punctata andersoni*	India: Uttar Pradesh: between Lucknow and Nepalese border	FR850514	FR850565	FR850617	A
4040	*Lissemys punctata andersoni*	India: Uttar Pradesh: between Lucknow and Nepalese border	FR850515	FR850566	FR850618	A
4069	*Lissemys punctata andersoni*	Myanmar: Rakhine State (Arakan)	FR850516	FR850567	FR850619	A
6069	*Lissemys punctata andersoni*	Myanmar: Rakhine State (Arakan): Sittwe	FR850517	FR850568	FR850620	A
5251	*Lissemys punctata punctata*	India: Andhra Pradesh: Godavari River (10 km inland from river mouth)	FR850518	FR850569	FR850621	B
4032§	*Lissemys punctata punctata*	India: Andhra Pradesh: Godavari River (20 km inland from river mouth)	FR850519	FR850570	FR850622	B
4033	*Lissemys punctata punctata*	India: Andhra Pradesh: Godavari River (20 km inland from river mouth)	FR850520	FR850571	FR850623	B
4034	*Lissemys punctata punctata*	India: Andhra Pradesh: Godavari River (20 km inland from river mouth)	FR850521	FR850572	FR850624	B
5369	*Lissemys punctata punctata*	India: Goa	FR850522	FR850573	FR850625	B
4044	*Lissemys punctata punctata*	India: Gujarat: Rajkot	FR850523	FR850574	FR850626	B
4045	*Lissemys punctata punctata*	India: Gujarat: Rajkot	FR850524	FR850575	FR850627	B
4046	*Lissemys punctata punctata*	India: Gujarat: Rajkot	FR850525	FR850576	FR850628	B
4047	*Lissemys punctata punctata*	India: Gujarat: Rajkot	FR850526	FR850577	FR850629	B
4048	*Lissemys punctata punctata*	India: Gujarat: Rajkot	FR850527	FR850578	FR850630	B
6048§	*Lissemys punctata punctata*	India: Karnataka: Mangalore	FR850528	FR850579	FR850631	B
6045	*Lissemys punctata punctata*	India: Kerala: Kotamangalam, Moyyar River	FR850529	FR850580	FR850632	C
6046§	*Lissemys punctata punctata*	India: Kerala: Kotamangalam, Moyyar River	FR850530	FR850581	FR850633	C
6047	*Lissemys punctata punctata*	India: Kerala: Kotamangalam, Moyyar River	FR850531	FR850582	FR850634	C
4042	*Lissemys punctata punctata*	India: Maharashtra: Pune (Poona)	FR850532	FR850583	FR850635	B
4043	*Lissemys punctata punctata*	India: Maharashtra: Pune (Poona)	FR850533	FR850584	FR850636	B
5259	*Lissemys punctata punctata*	India: Odisha (Orissa): Chilka lake district SW Puri	FR850534	FR850585	FR850637	A*
5260	*Lissemys punctata punctata*	India: Odisha (Orissa): Chilka lake district SW Puri	FR850535	FR850586	FR850638	A*
5261	*Lissemys punctata punctata*	India: Odisha (Orissa): Chilka lake district SW Puri	FR850536	FR850587	FR850639	A*
5262	*Lissemys punctata punctata*	India: Odisha (Orissa): Chilka lake district SW Puri	FR850537	FR850588	FR850640	A*
5258	*Lissemys punctata punctata*	India: Odisha (Orissa): Devi River (20 km inland from river mouth)	FR850538	FR850589	FR850641	A*
6043	*Lissemys punctata punctata*	India: Tamil Nadu: Coimbatore	FR850539	FR850590	—	C
6044	*Lissemys punctata punctata*	India: Tamil Nadu: Coimbatore	FR850540	FR850591	FR850642	C
4041§	*Lissemys punctata punctata*	India: Tamil Nadu: Mahabalipuram	FR850541	FR850592	FR850643	C
6058	*Lissemys punctata punctata*	India: Tamil Nadu: Mahabalipuram	FR850542	FR850593	FR850644	C
6059	*Lissemys punctata punctata*	India: Tamil Nadu: Mahabalipuram	FR850543	FR850594	—	C
6050	*Lissemys punctata punctata*	Sri Lanka: 50 km S Colombo	FR850544	FR850595	FR850645	D
6051	*Lissemys punctata punctata*	Sri Lanka: 50 km S Colombo	FR850545	FR850596	FR850646	D
6052§	*Lissemys punctata punctata*	Sri Lanka: 50 km S Colombo	FR850546	FR850597	FR850647	D
6053	*Lissemys punctata punctata*	Sri Lanka: 50 km S Colombo	FR850547	FR850598	FR850648	D
6056§	*Lissemys punctata punctata*	Sri Lanka: 50 km S Colombo	FR850548	FR850599	FR850649	D
4063§	*Lissemys scutata*	Myanmar	FR850549	FR850600	FR850650	E
4064	*Lissemys scutata*	Myanmar	FR850550	FR850601	FR850651	E
4065§	*Lissemys scutata*	Myanmar	FR850551	FR850602	FR850652	E
4066	*Lissemys scutata*	Myanmar	FR850552	FR850603	FR850653	E
997§	*Cyclanorbis senegalensis*	Benin	FR850553	FR850604	FR850654	—
6313§	*Cycloderma aubryi*	Congo-Brazzaville: Pointe Noire	FR850554	FR850605	FR850655	—
6316	*Cycloderma aubryi*	Congo-Brazzaville: Tchingoli	FR850555	FR850606	FR850656	—
4236§	*Pelodiscus maackii*	Russia: Ussuri Region: Przewalski Peninsula: Lake Khanka	FM999003	FM999019	FM999011	—

§ Sample used for molecular dating; * haplotype conflicting with morphology

Table 2. Primers used for amplification and sequencing. For the fragment containing the partial ND4 gene plus adjacent DNA coding for the tRNAs, the reverse primer H-Leu was combined either with the forward primer L-ND4 or ND4 672.

Fragment	Primer	Sequence (5′-3′)	Reference
12S rRNA	L1091	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	KOCHER *et al.* (1989)
	H1478	TGACTGCAGAGGGTGACGGGCGGTGTGT	KOCHER *et al.* (1989)
ND4 + tRNAs	L-ND4	GTAGAAGCCCCAATCGCAG	STUART & PARHAM (2004)
	ND4 672	TGACTACCAAAAGCTCATGTAGAAGC	ENGSTROM *et al.* (2004)
	H-Leu	ATTACTTTTACTTGGATTTGCACCA	STUART & PARHAM (2004)
cyt *b*	CytbG	AACCATCGTTGTWATCAACTAC	SPINKS *et al.* (2004)
	mt-f-na	AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG	FRITZ *et al.* (2006)

Table 3. Best evolutionary models and their parameters selected by MrMODELTEST 2.3 (AIC).

Partition	Model	Nst	+G	+I	Free parameters
ND4	GTR+G+I	6	yes	yes	8
tRNA-His	HKY+G	2	yes	no	3
tRNA-Ser	HKY+G	2	yes	no	3
tRNA-Leu	SYM	6	no	no	6
12S rRNA	GTR+G	6	yes	no	7
cyt *b*	GTR+I+G	6	yes	yes	8

Nst: number of substitution types, +G: gamma correction, +I: correction for points of invariance

Results

Phylogeny

Both tree-building methods yielded the same branching pattern with similar support values (Fig. 2). The African cyclanorbines *Cyclanorbis senegalensis* and *Cycloderma aubryi* together constituted the well-supported sister group to the Asian *Lissemys*. Contrary to expectation, the *Lissemys* sequences clustered not in three clades corresponding to the currently recognized taxa, but in five well-supported clades (A, B, C, D, E). One maximally supported, more inclusive clade contained the three well-supported terminal clades A, B, and C, whose exact sister group relations were weakly resolved. One of these clades, clade A, contained nearly all flapshell turtles morphologically identified as the spotted subspecies *Lissemys punctata andersoni*. However, also five unspotted individuals from the Indian state of Odisha (formerly Orissa, samples MTD 5258–5262), morphologically identified as the unspotted subspecies *L. p. punctata*, occurred in this clade (Figs 2–3). Sequences of the latter subspecies were scattered over three distinct clades. Clade B, from the northern part of the distribution range of *L. p. punctata*, was with weak support sister to clade A. In clade B occurred also one spotted flapshell turtle from Odisha (MTD 5247), morphologically identified as *L. p. andersoni*. The successive sister group was clade C, from the southern peninsular part of the distribution range of *L. p. punctata*. It contained sequences of unspotted flapshell turtles from Kerala and Tamil Nadu, India. The sequences of the unspotted *L. p. punctata* from Sri Lanka constituted the deeply divergent clade D being sister to (A + B) + C, and the sequences of *L. scutata* clustered in the most basal clade E. All nodes of the more inclusive clades received high support, except for the sister group relation of clades A + B. Clade B contained maximally supported subclades, one comprising two sequences from the southernmost localities of clade B (Goa and Karnataka, India); the other subclade embraced sequences from the more northern localities in the Indian states of Andhra Pradesh, Gujarat, Maharashtra, and Odisha

Uncorrected *p* distances (cyt *b*) among clades A, B and C range from 3.9% to 4.6%. In contrast, the values between clades D and E (10.3%), and when clades D and E are compared with the three other clades, are about twice of that (8.6% to 10.2%; Table 4).

Molecular Clock

The molecular clock calculation suggested that the split between African and Asian cyclanorbines occurred between the Early Oligocene and the Early Miocene, with a mean split age of about 22 myr ago (Fig. 4). The split between the two African genera *Cyclanorbis* and *Cycloderma* was estimated to have

Table 4. Average uncorrected *p* distances (percentages) and their standard errors for mitochondrial clades of *Lissemys*, *Cyclanorbis senegalensis* and *Cycloderma aubryi*, based on a 1133-bp-long mtDNA fragment coding for cyt *b* and calculated with MEGA 4.0.2. Below the diagonal, values among clades are given; on the diagonal, values within each clade in bold.

	A	**B**	**C**	**D**	**E**	***Cyclanorbis***	***Cycloderma***
	n = 18	*n* = 14	*n* = 6	*n* = 5	*n* = 4	*n* = 1	*n* = 2
A	**0.1 ± 0**						
B	4.6 ± 0.6	**0.5 ± 0.1**					
C	3.9 ± 0.6	4.3 ± 0.6	**0.3 ± 0.1**				
D	9.0 ± 0.8	8.6 ± 0.8	8.6 ± 0.8	**0.3 ± 0.1**			
E	10.2 ± 0.8	9.9 ± 0.9	9.7 ± 0.9	10.3 ± 0.9	**0.3 ± 0.1**		
Cyclanorbis	16.5 ± 1.0	17.1 ± 1.0	15.6 ± 1.0	17.2 ± 1.1	15.2 ± 1.0	—	
Cycloderma	15.3 ± 1.1	15.5 ± 1.0	15.9 ± 1.1	16.8 ± 1.1	14.9 ± 1.1	15.6 ± 1.0	**0**

n = number of sequences

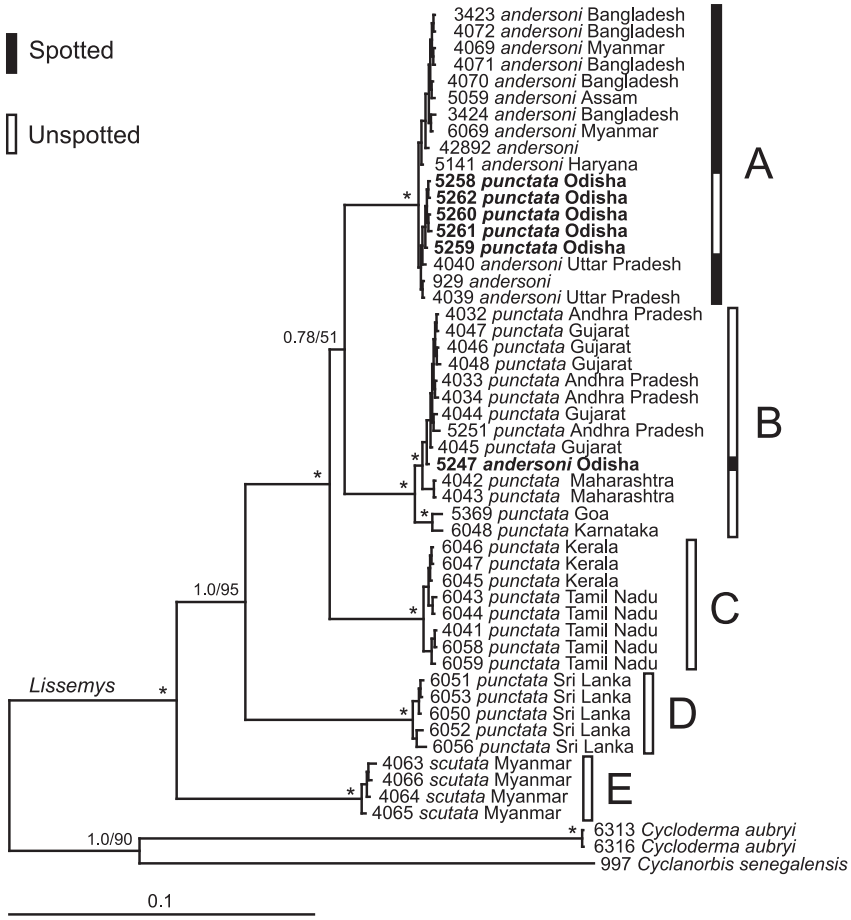

Fig. 2. Bayesian tree based on 2286 bp of mtDNA for *Lissemys* and the two allied African cyclanorbine species *Cyclanorbis senegalensis* and *Cycloderma aubryi*. Outgroup (*Pelodiscus maackii*) removed for clarity. Numbers along branches are posterior probabilities and ML bootstrap values (not presented for most internal clades with short branch lengths). Asterisks indicate maximum support under both methods. Numbers preceding taxon names are MTD numbers (Table 1); samples with haplotypes conflicting with taxonomic allocation in bold. Letters of *Lissemys* clades (A-E) correspond to Table 1 and Figure 3.

occurred in the Early Miocene, with a mean age of about 19 myr. The ages for the two basal splits within *Lissemys* corresponded to 95% confidence intervals embracing a range between the Early Miocene and the Lower Pliocene, with a mean estimate of about 11 myr for the dichotomy between *Lissemys scutata* (clade E) and all the other *Lissemys* clades, and about 8 myr for the Sri Lankan clade D. The weakly resolved branching events among clades A, B and C were calculated to have occurred between the uppermost Miocene and

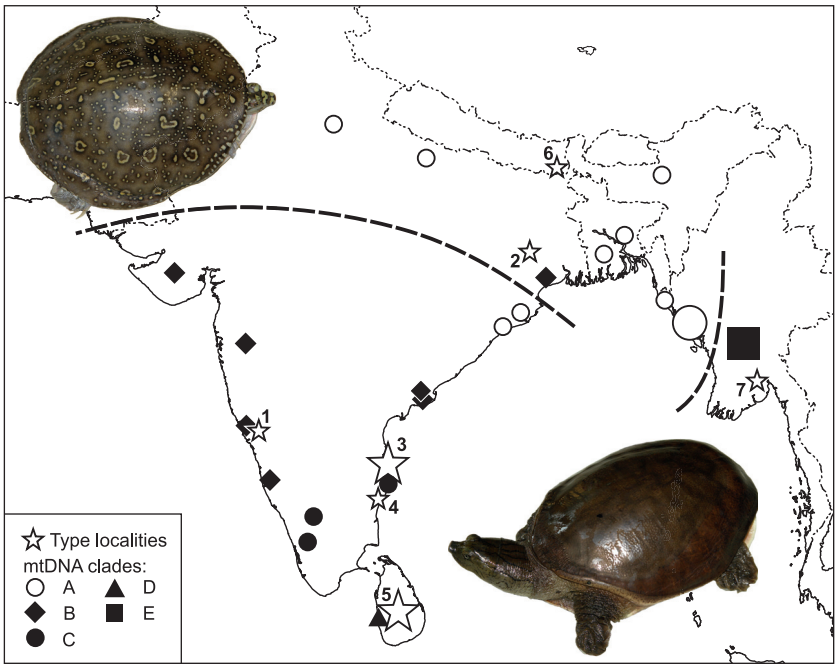

Fig. 3. Distribution of mitochondrial clades of *Lissemys* and type localities. Large symbols indicate imprecise locality data (Table 1). Broken lines denote approximate borders between spotted and unspotted flapshell turtles. Clade A matches more or less the distribution range of *L. punctata andersoni*, clade E corresponds to *L. scutata*, but the range of *L. p. punctata* harbours the three distinct clades B, C, and D (compare Figs 1–2). Type localities: (1) *Emyda vittata* PETERS, 1854 – Goa; (2) *Emyda granosa intermedia* ANNANDALE, 1912 – Purulia, Manbhum District; (3) *Testudo granosa* SCHOEPFF, 1801 – coast of Coromandel; (4) restricted type locality (WEBB, 1980) of *Testudo punctata* LACEPÈDE, 1788 (nomen suppressum; ICZN, 2005: Opinion 2104) = *Testudo punctata* BONNATERRE, 1789 – Pondicherry, South Arcot District, Tamil Nadu, India; (5) *Emyda ceylonensis* GRAY, 1856 – Ceylon; (6) *Lissemys punctata andersoni* WEBB, 1980 – Belbari, Terai, southeastern Nepal; (7) *Emyda scutata* PETERS, 1868 – Pegu. The name *Emyda granosa intermedia* ANNANDALE, 1912 was based on intergrades and cannot be used as the valid name for any of the involved taxa (ICZN, 1999: Article 23.8).

the Early Pleistocene, with mean ages in the Lower Pliocene (4.5 and 4.2 myr).

Discussion

In the present paper we investigated relationships among cyclanorbine flapshell turtles using 2286 bp of mitochondrial DNA. Previous morphological and molecular results (MEYLAN, 1987; ENGSTROM *et al.*, 2004) suggested a sister group relationship of *Lissemys* to an African clade comprising the genera *Cyclanorbis* and *Cycloderma*. However, based on a reanalysis of MEYLAN's (1987) morphological 66-character data set, JOYCE & LYSON (2010) recently proposed that the African taxa are paraphyletic with respect to the Asian *Lissemys*. Our results contradict the latter hypothesis and support the phylogenetic topology as suggested by MEYLAN (1987) and ENGSTROM *et al.* (2004).

Based on a cladistic analysis of Eocene fossils, JOYCE & LYSON (2010) concluded that cyclanorbines originated in North America and that the fossil North American family Plastomenidae represents either an early offshoot of stem-cyclanorbines or a paraphyletic assemblage that gave rise to modern cyclanorbines. According to their considerations, the ancestors of the extant cyclanorbines spread from North America to Asia, and from there in the Miocene to the Indian subcontinent and Africa. This scenario is in rough agreement with our estimate for the split age between African and Asian cyclanorbines (Early Oligocene to Early Miocene, with a mean age of approx. 22 myr).

Currently, three extant taxa of *Lissemys* are recognized (ERNST & BARBOUR, 1989; ERNST *et al.*, 2000; FRITZ & HAVAŠ, 2007; RHODIN *et al.*, 2010). The spotted subspecies *Lissemys punctata andersoni* occurs mainly in the Indus, Ganges and Brahmaputra drainages of the Indian subcontinent and in western Myanmar. The unspotted subspecies *L. p. punctata* is thought to occur in peninsular India and Sri Lanka, and it intergrades with *L. p. andersoni* where their ranges meet (WEBB, 1982). The allopatrically distributed, unspotted *L. scutata* is known from Myanmar and its range may extent into adjacent Thailand and

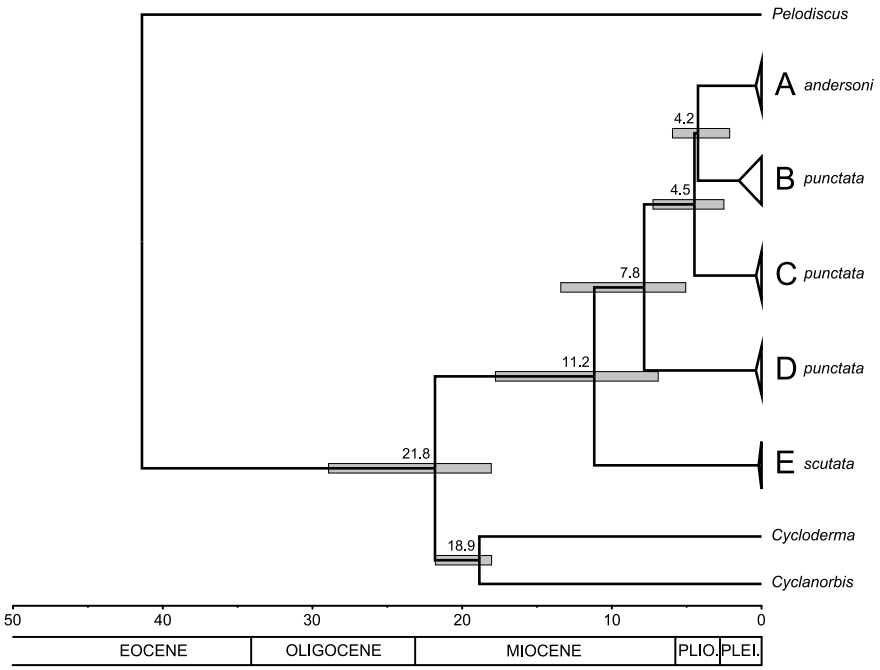

Fig. 4. Estimated split ages of African and Asian cyclanorbines and their 95% confidence intervals (grey bars), obtained with BEAST 1.5.4. Letters of *Lissemys* clades (A–E) correspond to Table 1 and Figures 2–3. Note that the lower boundary of the Pleistocene is according to Walker & Geissman (2009).

Yunnan, China. This classification is mainly based on external morphology (colouration and pattern) and distribution data (Smith, 1931; Webb, 1982; Ernst *et al*., 2000). Osteological characters were only used for the elevation of the allopatric *L. scutata* to species level (Webb, 1982). The latter author suggested that *L. scutata* has a larger number of peripheral bones than the other two taxa, and that the peripheralia are smaller sized in *L. scutata* than in *L. p. punctata* and *L. p. andersoni*. Moreover, according to Webb (1982) the plastral callosities of *L. scutata* develop earlier during ontogeny than in the other two taxa. However, Meylan (1987) pointed out that plastral callosities are highly variable in softshell turtles and doubted the species status of *L. scutata*. In fact, the usage of external morphology for taxonomy and systematics is severely impeded by a high degree of homoplasy and individual variability in softshell turtles. Therefore, recent molecular investigations contributed to a substantial advancement in systematics and taxonomy (Weisrock & Janzen, 2000; Engstrom *et al*., 2002, 2004; Praschag *et al*., 2007; McGaugh *et al*., 2008; Fritz *et al*., 2010).

If the current classification of *Lissemys* is correct, it should be expected that each of the three taxa *L. p. punctata*, *L. p. andersoni* and *L. scutata* represents a distinct genetic lineage, and a more pronounced divergence of *L. scutata* were then supportive of its species status. However, we found five, and not three, well-supported mitochondrial clades. Only one of these clades (clade E), being sister to all other *Lissemys*, corresponds perfectly to one of the currently recognized taxa (*L. scutata*; compare Figs 1–3). Another clade (A) comprises nearly all sequences from spotted flapshell turtles (*L. punctata andersoni*), but also sequences from some unspotted individuals from the Indian state of Odisha. Notably, sequences of flapshell turtles morphologically identified as the unspotted subspecies *L. p. punctata* occur in the three deeply divergent clades B, C, and D; clade B contains also one sequence from a spotted turtle from Odisha, identified as *L. p. andersoni*. Two of the three clades (B and C) with unspotted flapshells are phylogenetically more closely related to the spotted *L. p. andersoni* than to clade D from Sri Lanka.

This situation allows the following conclusions: (1) The morphologically distinctive *L. scutata* is also with respect to mtDNA variation the most divergent taxon; (2) the occurrence of spotted and unspotted individuals in clades A and B provides evidence for gene flow between the spotted subspecies *L. p. andersoni* and adjacent populations with unspotted flapshells, in agreement with morphological data (Webb, 1982); (3) the unspotted subspecies *L. p. punctata* from peninsular India and Sri Lanka represents in reality three deeply divergent cryptic lineages; and (4) the taxonomy of *Lissemys* as it currently stands needs revision.

The sequence divergence of the cyt *b* gene has often been used to assess the species status of chelonians, and the values among all *Lissemys* clades (3.9–10.3%; Table 4) fall into the range as ob-

Fig. 5. Asian flapshell turtles. (a) *Lissemys punctata andersoni* (trade specimen) – photo: Roland Zirbs; (b) spotted intergrade (Subarnarekha River, Odisha, India), bearing mitochondrial haplotype of the unspotted subspecies *L. p. vittata* – photo: Peter Praschag; (c) unspotted intergrade (Chilka lake district, Odisha, India), bearing mitochondrial haplotype of the spotted subspecies *L. p. andersoni* – photo: Peter Praschag; (d) *Lissemys punctata vittata* (Godavari River, Andhra Pradesh, India) – photo: Peter Praschag; (e) *Lissemys punctata punctata* sensu stricto (Moyyar River, Kerala, India) – photo: Peter Praschag; (f) *Lissemys ceylonensis* (50 km S Colombo, Sri Lanka) – photo: Peter Praschag; (g) *Lissemys scutata* (trade specimen) – photo: Roland Zirbs.

Fig. 6. Head and neck pattern in Asian flapshell turtles. (a) *Lissemys punctata andersoni* (trade specimen) – photo: Roland Zirbs; (b) *Lissemys punctata vittata* (Rajkot, Gujarat, India) – photo: Peter Praschag; (c) *Lissemys punctata punctata* sensu stricto (Moyyar River, Kerala, India) – photo: Peter Praschag; (d) *Lissemys ceylonensis* (50 km S Colombo, Sri Lanka) – photo: Peter Praschag; (e) *Lissemys scutata* (trade specimen) – photo: Peter Praschag. In juveniles of *L. ceylonensis* facial stripes may be present, resembling *L. p. punctata*, *L. p. vittata* and *L. scutata*.

served in distinct species of other chelonian genera (2.8–18.3%; see the review in Vargas-Ramírez *et al*., 2010). However, species delineations should not be based on rigid thresholds of sequence divergence alone (see also Vieites *et al*., 2009; Vargas-Ramírez *et al*., 2010). Moreover, the uncorrected *p* distances of the two allopatrically distributed clades E (*L. scutata*, Myanmar) and D (Sri Lanka) to their closest relatives are distinctly higher than the values among clades A, B and C (Table 4). This suggests two levels of differentiation that are also reflected by the different age estimates for these clades (Fig. 4). The clades from Myanmar and Sri Lanka represent old lineages that diverged between the Early Miocene and the Lower Pliocene (mean estimates: approx. 11 and 8 myr), whereas the three other clades A, B and C are roughly half as old (95% confidence interval ages ranging between the uppermost Miocene and the Early Pleistocene, mean ages of 4.5 and 4.2 myr).

In any case, the pronounced distinctness of *L. scutata* (clade E) is in line with Webb's (1982) conclusion that this allopatric taxon should be treated as a distinct species. The similar degree of differentiation

of clade D suggests, however, that Sri Lankan flapshell turtles should be tentatively regarded as another distinct species, for which the name *Lissemys ceylonensis* (GRAY, 1856) is available (Fig. 3). Furthermore, we propose that the remaining two clades B and C, comprising unspotted turtles, could be treated as distinct subspecies along with the spotted subspecies *L. p. andersoni*. Their classification as conspecific evolutionary lineages is suggested by similar genetic divergences, the observation of mismatches between morphology (spotted *vs*. unspotted) and mitochondrial haplotypes in clades A and B, and earlier reported extensive morphological intergradation of spotted and unspotted flapshell turtles (WEBB, 1982). Accordingly, we propose to restrict the name *L. p. punctata* (LACEPÈDE, 1788) = *L. p. punctata* (BONNATERRE, 1789) to clade C. For clade B the name *L. p. vittata* (PETERS, 1854) is available. The well-supported mitochondrial subclades within *L. p. vittata* suggest pronounced geographic variation of this subspecies.

We are aware that further research is needed for corroborating this tentative classification. In particular, population genetic investigations focussing on gene flow among peninsular populations harbouring distinct mitochondrial lineages were crucial, and a re-evaluation of morphological characters, including osteology. Despite having studied the gross morphology of several hundred live and preserved Asian flapshell turtles (collections of the Natural History Museums in London and Vienna, the Senckenberg Museum, Frankfurt, and the Zoological Museum, Dresden), we are unable to correctly identify unspotted flapshells of any of the three peninsular Indian and Sri Lankan lineages based on external morphology alone (compare Figs 5–6). This situation requires further research to reveal whether these taxa are really cryptic or whether a refined morphological assessment will unravel previously overlooked diagnostic characters.

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