

# Phylogeography of the endangered black-breasted leaf turtle (*Geoemyda spengleri*) and conservation implications for other chelonians

Shiping Gong<sup>1</sup>, Haitao Shi<sup>2</sup>, Yunming Mo<sup>3</sup>, Markus Auer<sup>4</sup>, Mario Vargas-Ramírez<sup>4</sup>,  
Anna K. Hundsdörfer<sup>4</sup>, Uwe Fritz<sup>4,\*</sup>

**Abstract.** Known-locality samples of *Geoemyda spengleri* from three Chinese provinces (Guangdong, Guangxi, Hainan) and northern Vietnam provide clear evidence for phylogeographic structure in a southern Chinese chelonian species. Within southern China two clades of mitochondrial haplotypes from east and west of the Xi Jiang together form the sister group to haplotypes from northern Vietnam. Turtles from Hainan Island harbour haplotypes of the same clade as turtles from Guangxi, 400 km distant. These findings suggest that phylogeographic differentiation may have once existed in other co-distributed, highly endangered chelonian species. The possibility of phylogeographic structure should be considered in any in-situ and ex-situ conservation program for Chinese chelonians.

**Keywords:** China, conservation, *Geoemyda spengleri*, Geoemydidae, phylogeography, Testudines, Vietnam.

## Introduction

The diverse Chinese chelonian fauna is critically endangered by overexploitation and large-scale habitat alteration (e.g. van Dijk et al., 2000; Gong et al., 2005, 2006, 2007). These factors, along with increasing farm-breeding to cover public demand (Shi et al., 2007, 2008) and translocation by man, contribute to obscured natural distribution patterns. While comprehensive phylogeographic data were found most useful for the conservation of European and North American chelonians (e.g. Lenk et al., 1999;

Fritz et al., 2004; Rosenbaum et al., 2007; Amato et al., 2008), such data have not yet been published for any of the Chinese species due to their rareness in the wild. Recent attempts to produce a genetic assessment of geographic variation for *Mauremys mutica*, *Cuora trfasciata* and allied taxa were hampered by the lack of known-locality samples (Fong et al., 2007; Spinks and Shaffer, 2007). We suspect that natural distribution patterns are already irrecoverably lost for most species. An exception could be the small black-breasted leaf turtle, *Geoemyda spengleri* (Gmelin, 1789). Due to its secretive terrestrial mode of life and small size (carapace length usually below 13 cm; Ernst et al., 2000), *G. spengleri* is still present in many regions where other chelonian species have become extinct or extremely rare.

Using samples from three Chinese provinces and North Vietnam, we examine in this paper whether a phylogeographic structure exists within *G. spengleri*. If this should be the case, it is likely that phylogeographic differentiation once existed also in other co-distributed chelonian species.

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1 - Guangdong Provincial Public Laboratory for Wild Animal Conservation and Management, South China Institute of Endangered Animals, Guangzhou 510260, P.R. China

2 - Department of Biology, Hainan Normal University, Longkunnan Road 99, Haikou 571158, P.R. China

3 - Biology Department, Natural History Museum of Guangxi Zhuang Autonomous Region, No.1-1 East Renmin Road, Nanning 530012, Guangxi, P.R. China

4 - Museum of Zoology (Museum für Tierkunde), Natural History State Collections Dresden, A. B. Meyer Building, D-01109 Dresden, Germany

\*Corresponding author; e-mail:  
uwe.fritz@snsd.smwk.sachsen.de

**Table 1.** Studied known-locality samples of *Geoemyda spengleri*. Sample numbers refer to DNA samples in the collection of the Museum of Zoology Dresden. For voucher numbers of trade specimens without locality data, see fig. 1; locality numbers refer to fig. 2.

Sample	mtDNA clade	Locality
3700	A	1 – China: Guangxi: N Paise (Bose)
3707	A	2 – China: Guangxi: Shiwan Dashan, vicinity of Fulong
4175	A	3 – China: Hainan: vicinity of Dali
5239	A	3 – China: Hainan: vicinity of Dali
5240	A	3 – China: Hainan: vicinity of Dali
5077	B	4 – China: Guangdong: Nanling Nature Reserve: Chadong village
5078	B	4 – China: Guangdong: Nanling Nature Reserve: Chadong village
5079	B	4 – China: Guangdong: Nanling Nature Reserve: Chadong village
5072	B	5 – China: Guangdong: Nanling Forest Park (N Ruyuan)
5073	B	5 – China: Guangdong: Nanling Forest Park (N Ruyuan)
5074	B	6 – China: Guangdong: Nanling Nature Reserve: Tianjingshan village
4153	B	7 – China: Guangdong: NankunShan Nature Reserve
980	C	8 – Vietnam: Tinh Vinh Phuc
4327	C	8 – Vietnam: Tinh Vinh Phuc
4328	C	8 – Vietnam: Tinh Vinh Phuc
4329	C	8 – Vietnam: Tinh Vinh Phuc
4330	C	8 – Vietnam: Tinh Vinh Phuc
4331	C	8 – Vietnam: Tinh Vinh Phuc

## Materials and methods

### Sampling, DNA extraction, PCR and sequencing

Saliva samples (oral swabs) of *Geoemyda spengleri* were collected during fieldwork and stored at  $-80^{\circ}\text{C}$  in pure ethanol until processing. Additional blood and tissue samples of Vietnamese and pet-trade turtles from the collection of the Museum of Zoology Dresden served for comparison (table 1). Total genomic DNA from blood or salivary samples was extracted by overnight incubation at  $55^{\circ}\text{C}$  in lysis buffer (6% DTAB, 1.125 M NaCl, 75 mM Tris-HCl, 37.5 mM EDTA, pH 8.0) including 0.5 mg of proteinase K (Merck, Whitehouse Station, NJ) and subsequent purification following the DTAB method (Gustincich et al., 1991). DNA was precipitated from the supernatant with 0.2 volumes of 4 M LiCl and 0.8 volumes of isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Total genomic DNA from tissue samples was isolated with the peqGOLD Tissue DNA mini-kit (Peqlab, Erlangen, Germany).

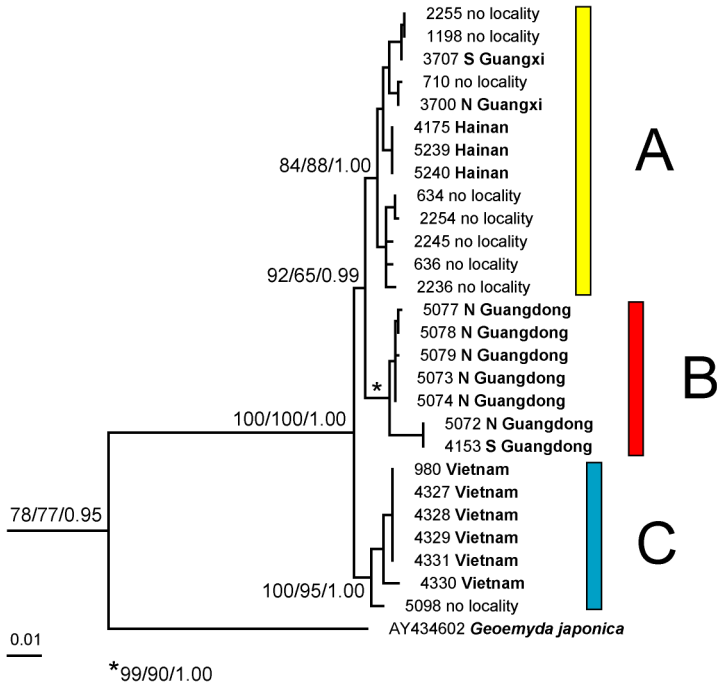
Polymerase chain reaction (PCR) was used to amplify an mtDNA fragment corresponding with the *cyt b* gene. Amplification, PCR product purification and sequencing followed Praschag et al. (2007); for PCR and sequencing the primers *CytbG* (Spinks et al., 2004), *mt-c-For2*, *mt-f-na3*, and *mt-E-Rev2* (Praschag et al., 2007) were used. Sequencing was performed on an ABI 3130 (Applied Biosystems, Foster City, CA). None of the sequences contained internal stop codons and nucleotide frequencies corresponded to those of coding mtDNA; therefore we conclude we amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes. Accession numbers of our new sequences are: FM203341-FM203367.

### Genetic distances and phylogenetic analyses

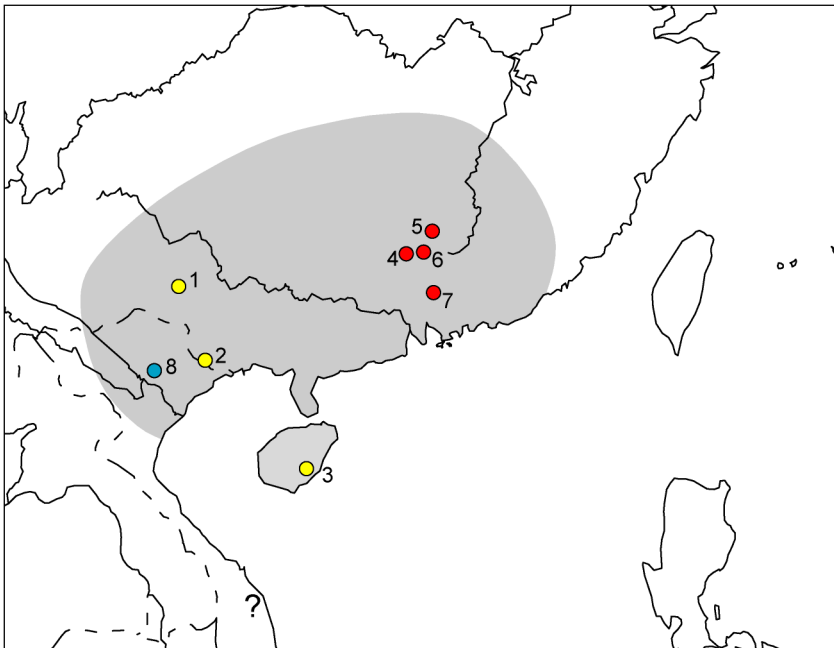
Phylogenetic analyses were run using a 1133-bp-long alignment including all sequences of *Geoemyda spengleri* and a sequence of its sister species *G. japonica* downloaded from GenBank (AY434602). Sequences of the distantly related geoemydid species *Siebenrockiella crassicollis* (AY434571), *S. leytensis* (AY954911) and *Vijayachelys silvatica* (AJ973611) served as outgroups. Data were analysed under the optimality criteria Maximum Parsimony (MP; equal weighting, command: `hs add = cl`) and Maximum Likelihood (ML) using PAUP\*4.0b10 (Swofford, 2002) and under Bayesian inference of phylogeny (BA) using MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003; settings: `ngen = 10000000 nchains = 4 nrun = 1 sample = 500 temp = 0.2 mcmcdiag = yes diagnfreq = 1000 swapfreq = 1 nswaps = 1 printfreq = 500 savebrlens = yes startingtree = random`; the burn-in was set to sample only the plateau of the most likely trees). The best evolutionary model was selected by the Akaike information criterion using MODELTEST 3.06 (best-fit model: GTR+G; Posada and Crandall, 1998). Bootstrap support values were calculated with PAUP\*4.0b10 for MP (`nreps = 1000`) and with GARLI 0.95 for ML (Zwickl, 2006; settings: `bootstrapreps = 100 genthreshfortopoterm = 5000 ratematrix = 6 rate statefrequencies = estimate ratehetmodel = gamma numratecats = 4 invariantsites = none`). Using a 1020-bp-long alignment, uncorrected *p* distances were calculated with MEGA 3.1 (Kumar et al., 2004).

## Results

All phylogenetic analyses resulted in trees with an identical branching pattern for ingroup sequences. Under parsimony, 800 of 1133 char-



**Figure 1.** Maximum likelihood phylogram for *Geoemyda spengleri* and *G. japonica*. Outgroup species (*Siebenrockiella crassicolis*, *S. leytensis*, *Vijayachelys silvatica*) removed for clarity. Numbers along nodes are ML and MP bootstrap values and Bayesian posterior probabilities. Letters and coloured bars on the right denote clades. Code numbers refer to DNA samples in the Museum of Zoology Dresden.



**Figure 2.** Approximate range of *Geoemyda spengleri* (shaded) and collection sites of samples. For numbers, see table 1. Colours of collection sites correspond with clades of fig. 1.

acters were constant and 196 were variable and parsimony-informative; 137 variable characters were parsimony-uninformative. For *Geoemyda spengleri* only, 1060 characters were constant and 73 sites were variable of which 59 were parsimony-informative. The MP analysis resulted in four equally parsimonious trees (tree length = 515; CI = 0.7495, RI = 0.7696); the ML analysis in one most likely tree ( $-\ln L = 3776.09$ ). Within *G. spengleri*, three well-supported clades occur that strongly correlate with geography (figs 1, 2). Clade A comprises sequences from Hainan and Guangxi, as well as sequences from trade turtles that cluster in two subclades. One of the subclades, with bootstrap support of 79 and 82 (ML, MP) and a Bayesian posterior probability of 1.00 corresponds with our known-locality samples from Guangxi and Hainan and three pet-trade samples. The other subclade (support values of 95/91/1.00) consists only of sequences from trade specimens. Clade A is suggested as sister group to clade B (sequences from Guangdong) and both constitute together the sister group of clade C (sequences from northern Vietnam and one sequence from a trade turtle). On average, the three clades differ by uncorrected *p* distances of 1.96–2.70%, corresponding with approximately 20% of the distance between *G. spengleri* and *G. japonica* (table 2).

## Discussion

Our results provide evidence for clear phylogeographic structure in *Geoemyda spengleri*, with

two clades (A, B) occurring in southern China, and a third (C) in Vietnam (figs 1, 2). While closely-related Chinese clades A and B are separated by the Xi Jiang, suggesting that this river could act as barrier, any geographical barrier separating Vietnamese clade C from the two Chinese clades is not obvious. This striking situation warrants further research. With respect to the two Chinese clades, clade A was found in Guangxi and, approximately 400 km distant and separated by the Gulf of Tonkin, on the island of Hainan. Considering that Hainan was connected with the mainland during Pleistocene low sea level stands (Voris, 2000), this pattern is not surprising however.

The ranges of several other endangered chelonians resemble the geographical distribution of *G. spengleri*, viz. *Cuora g. galbinifrons*, *C. trifasciata*, *Mauremys mutica*, *M. nigricans*, *M. sinensis*, *Palea steindachneri*, *Sacalia bealei* and *S. quadriocellata* (Iverson, 1992), suggesting that phylogeographic differentiation occurs or once occurred in these taxa as well. Most of these endangered chelonians are now captive-bred in moderate to large numbers and it is expected that this factor contributes to admixture of original genetic signatures (Shi et al., 2007, 2008). Fong et al. (2007) used three known-locality samples of *M. mutica*, as well as 29 specimens without locality data, in their study and identified a clade containing two samples from Hainan that was significantly different from another clade containing the only known-locality sample from eastern China, reminiscent of differentiation we found in *G. spengleri*. Us-

**Table 2.** Average uncorrected *p* distances (percentages) of the three clades of *Geoemyda spengleri* (A, B, C) and the other investigated species. Below the diagonal, between-group distances; on the diagonal in bold, within-group distances.

	A	B	C	<i>Geoemyda japonica</i>	<i>Siebenrockiella crassicollis</i>	<i>Siebenrockiella leytenis</i>	<i>Vijayachelys silvatica</i>
A	<b>0.88</b>						
B	1.96	<b>0.65</b>					
C	2.19	2.70	<b>0.42</b>				
<i>G. japonica</i>	11.06	11.25	11.22	–			
<i>S. crassicollis</i>	16.77	17.05	16.04	16.67	–		
<i>S. leytenis</i>	14.81	14.65	14.54	14.71	14.12	–	
<i>V. silvatica</i>	15.23	15.57	14.83	14.80	18.43	15.88	–

ing 10 field-collected *C. trifasciata* from Hong Kong, and many turtles lacking locality data, Spinks and Shaffer (2007) found a complicated pattern of genetic variation being obscured by mtDNA introgression from related species, and were unable to reach any conclusions regarding geographical variation in *C. trifasciata*.

Our study demonstrates that phylogeographic differentiation needs to be addressed in any conservation project for Chinese chelonians in order to avoid lineage admixture and preserve genetic diversity. Most species co-distributed with *G. spengleri* are aquatic (see Ernst et al., 2000), and distinct phylogeographic patterns are likely. Given that many species are already rare in the wild, reconstructing their phylogeography is impossible using wild-living turtles and further studies should focus on well-documented historical museum specimens and aDNA methods for deciphering former genetic differentiation. This approach has been useful for delineating the historical ranges of two critically-endangered South Asian river terrapins (*Batagur affinis*, *B. baska*; Präsach et al., 2008) and will offer a sound starting point for in-situ and ex-situ conservation projects involving Chinese turtles.

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