Asian Herpetological Research 2011, 2(1): 21-29
DOI: 10.3724/SP.J.1245.2011.00021

Validity of *Pelodiscus parviformis* (Testudines: Trionychidae) Inferred from Molecular and Morphological Analyses

Ping YANG, Yezhong TANG, Li DING, Xianguang GUO and Yuezhao WANG*

Chengdu Institute of Biology, *Chinese Academy of Sciences*, *Chengdu* 610041, *Sichuan*, *China*

Abstract The validity of *Pelodiscus parviformis* within the genus is still not very clear. In this study, molecular and morphological data were combined to evaluate the validity of *P. parviformis*. The phylogeny of some species in *Pelodiscus* was reconstructed by maximum parsimony, maximum likelihood and Bayesian inference analyses based on five mitochondria DNA fragments (5308 bp of 12S rRNA, 16S rRNA, ND4, CO1 and Cyt *b*). The results of ML, MP and Bayesian analyses suggest that *P. parviformis* might be paraphyletic to *P. sinensis*, whereas the partitioned Bayesian analyses support the reciprocal monophyly of *P. parviformis* and *P. sinensis*. Considering the advantages of heterogenous characteristics of sequence evolution, we choose the result of partitioned Bayesian analyses. Furthermore, the morphological data lend support the distinct species status of *P. parviformis* and *P. sinensis*, such as tubercles on carapace skin, color of plastron skin, dark spots on plastron, basisphenoid characteristics (ratio of the smallest width to the largest width; the smallest width of basisphenoid is restrained by two holes on each side) and the shape of entoplastron. Combining the molecular and morphological data, we inferred that *P. parviformis* is a valid species. In addition, the results of this study suggest a new record of *P. axenaria* in Guangxi, China.

Keywords mtDNA, morphology, *Pelodiscus parviformis*, phylogeny, taxonomy

1.Introduction

Pelodiscus parviformis Tang, 1997 was described and identified as a new species with the following characters: the length of carapace 100–120 mm, smaller than that of *P. sinensis* Wiegmann, 1835; prominent tubercles on the carapace arrayed like the shape of wing; the ratio of the smallest width to the largest width in basisphenoid approximate or more than 1/2; ventral body usually turning red when it is captured about one minute later (Tang, 1997).

Zhao (2000) and Zhou (2006) recognized four distinct species within the genus *Pelodiscus* in China, including *P. sinensis* Wiegmann, 1834, *P. mackii* Brandt, 1857, *P. axenaria* Zhou, 1991, and *P. parviformis* Tang, 1997. Based on the morphological characters and mitochondrial DNA analysis, some argued whether *P. axenaria* and *P. mackii* were valid species (Chen *et al*., 2005, 2006; Chkhikvadze, 1987; Fritz and Hava, 2007; Fritz and Obst, 1999; Zhou *et al*., 1991). But, some authors indicated that only *P. sinensis* was a valid species of *Pelodiscus* (Fritz *et al*., 2010). Furthermore, Zhou and Li (2007) pointed out that the validity of *P. parviformis* was questioned, and no molecular evidence would indicate that *P. parviformis* could be a valid species in this genus. Moreover, *P. sinensis* is widely distributed in large areas of China, but *P. parviformis* is found in part of the distribution areas of *P. sinensis* (Tang, 1997; Zhao, 1997). For these reasons, detailed comparison of those questioned species, analyses of their morphological characters, and study on their molecular data should be done to corroborate whether *P. parviformis* is a valid species.

To evaluate the validity of *P. parviformis*, we reconstructed the phylogenetic relationships of some species in the genus *Pelodiscus* and reevaluated the phylogenetic position of *P. parviformis*. In this study, 12S rRNA, 16S rRNA, NADH dehydrogenase subunit 4 (ND4), cytochrome coxidase subunit 1 (CO1) and cytochrome *b* (cyt *b*) were partially sequenced from 8 samples of soft-shelled turtles collected from the same locality in Quanzhou County, Guangxi, China, and then were analyzed together with the sequences downloaded from GenBank. CO1, ND4 and cyt *b* genes are thought

* Corresponding author: Prof. Yuezhao WANG, from Chengdu Institute of Biology, Chinese Academy of Sciences, with his research focusing on taxonomy, phylogeny and biogeography of reptiles.
E-mail: arcib@cib.ac.cn
Received: 17 November 2010 Accepted: 15 February 2011

to provide a rich source of phylogenetic information for vertebrates (Esposti *et al*., 1993; Zardoya and Meyer, 1996). These genes are believed to evolve quickly and are useful markers for analyzing rapid intraspecific and interspecific changes (Fritz *et al*., 2010; Zardoya and Meyer, 1996). In addition, 12S rRNA and 16S rRNA were chosen to investigate the phylogenetic relationship among *P. sinensis*, *P. axenaria* and *Palea steindachneri*, and this result was consistent with prior studies (Chen *et al*., 2005). CO1 barcodes distinguish more than 95% of species (Ward *et al*., 2005; Hajibabaei *et al*., 2006), so we performed the Neighbour-joining (NJ) analysis of CO1 gene as in this study.

In addition, the variation in morphological characters, such as the body size, tubercles on carapace skin, color of plastron skin, dark spots on plastron, basisphenoid characteristics (ratio of the smallest width to the largest width; the smallest width of basisphenoid is restrained by two holes on each side) and the shape of entoplastron unique to *P. sinensis*, *P. parviformis* and *P. axenaria* were assessed according to the characters identified by the authors who first found them (Tang, 1997; Zhou, 1991).

2. Materials and Methods

2.1 Sampling Eight samples of soft-shelled turtles were collected from Quanzhou County in Guangxi, China, the type locality for *P. parviformis*. This location is also intersected by the Xiang River, the type locality for *P. axenaria* (Zhou *et al*., 1991). Among all the samples, three (Nos. CIB95410 – CIB95412; two females and one male) were collected from the Yixiang River in Lishui Village of Wenqiao Town in Quanzhou County, and the others (Nos. CIB95413 – CIB95417; two females and three males) were collected from the Xiang River between Yongsui and Huangshahe villages in Quanzhou County (Figure 1). All specimens were stored at the Chengdu Institute of Biology, Chinese Academy of Sciences (voucher: CIB95410 – CIB95417).

Four samples of *P. sinensis* were used as the references, and three of them (CIB000026, CIB000027, and CIB000028; one female and two males) were collected from Guizhou Province, the other one, which was bought from a local supermarket (Caraford) in Chengdu, was used for preparing a skeletal specimen. Sequences for *P. sinensis* were downloaded from GenBank (Accession numbers: AY962573.1 and NC006132.1; samples were collected from Korea and Anhui, China). Samples of *P. axenaria* were collected from Hunan, China (GenBank accession numbers: AY743421, AY583693 and AY583695).

2.2 Phylogenetic analyses Total genomic DNA was extracted from muscle tissue using a standard sodium dodecyl sufate-proteinase K procedure, as described by Sambrook and Russel (2001). The 12S rRNA gene (876

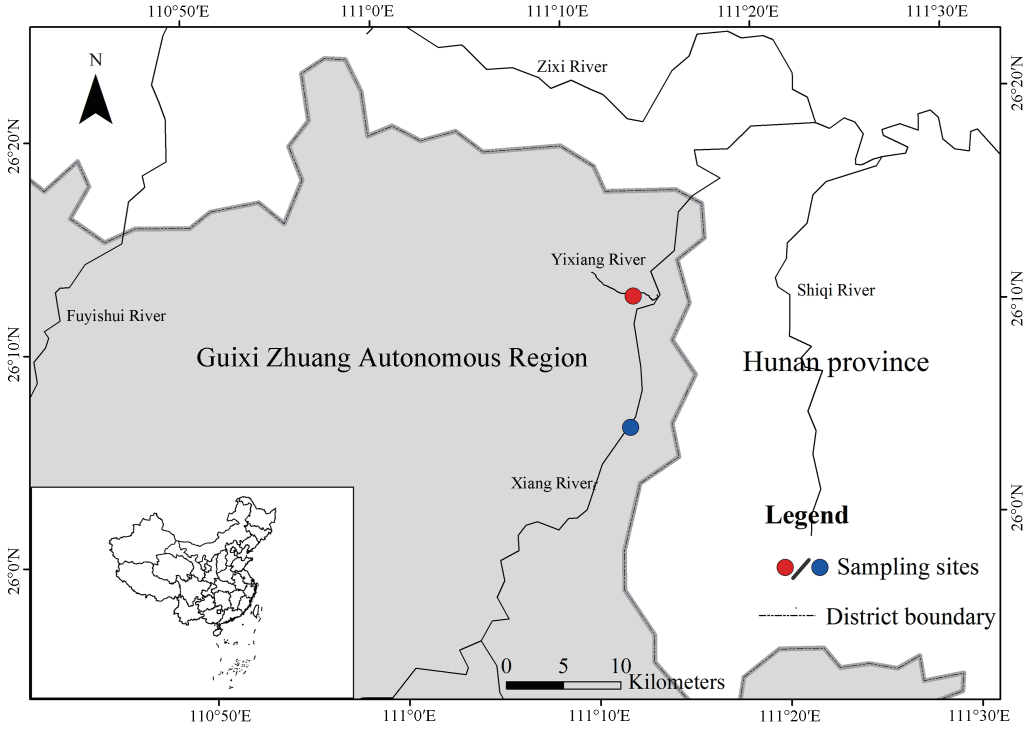

Figure 1 Sampling sites of the eight soft-shelled turtles in Quanzhou County, Guangxi, China

bp) was amplified with the primers 12S_F and 12S_R as described by Chen *et al*. (2005), and 16S_F and 16S_R by Chen (2005) for the 16S rRNA gene (1453 bp). The cytochrome *b* gene (990 bp) was obtained with the primers C-F and C-R as reported by Chen *et al*. (2006). The CO1 gene (1269 bp) was amplified with the primer (CO1-f: TAATCCGAGCAGAACTAAGTCAACC; CO1-r: GTCATTCTACGTTGGTGGTTGT), which was designed based on the conserved regions found in an alignment of complete genome available in GenBank for *P. sinensis*. To sequence the ND4-tRNA^{Leu} gene segment (720 bp), the primers ND4 and Leu used by Arevalo *et al*. (1994) were employed in this study. The primers were synthesized by Sangon Biotech Company in Shanghai. Standard polymerase chain reactions (PCR) were performed with approximately 50 ng genomic DNA and annealed at 50–52°C for different primer sets. Double strand DNA was sequenced by Invitrogen. The DNA sequences of other genera in the family Trionychidae were downloaded from the GenBank. We had only two whole sequences of mitochondria DNA from *P. sinensis,* which were collected from Korea (Jung *et al*., 2006) and Anhui, China (Peng *et al*., 2005), respectively.

Bioedit and ClustalX v1.83 were used to edit sequence data. The compatibility of the 5 gene fragments included in this study were examined by using the partition homogeneity test (Farris *et al*., 1995), which was executed in PAUP* 4b10 (Swofford, 2003). The sequences of different fragments were analyzed in order to determine if saturation existed in substitution using DAMBE (Xia and Xie, 2001). MEGA3 (Kumar *et al*., 2004) was used to compute the genetic distance among the species of the genus *Pelodiscus*. We performed NJ analysis of COI gene in MEGA3 (Kumar *et al.*, 2004), using the Kimura-two-parameter (K2P) model, the best metric when distances are low (Nei & Kumar 2000).

We performed both maximum parsimony (MP) and maximum likelihood (ML) analyses using PAUP*; Partitioned and non-partitioned Bayesian analyses were both implemented in MrBayes V3.1 (Huelsenbeck and Ronquist, 2001a; Ronquist and Huelsenbeck, 2003). *Dogania subplana* was chosen as outgroup, because its status in Trionychinae was closest to *Pelodiscus* and *Palea* (Iverson and Sheeley, 2007). We used partition-specific evolutionary models to improve phylogenetic inference by accounting for the heterogenous characteristics of sequence evolution among different data partitions (Brandley *et al*., 2005; Guo and Wang, 2007; Nylander *et al*., 2004). The most appropriate evolutionary model for each partition was selected by the Akaike Information Criterion (AIC; Akaike, 1974) implemented in Modeltest 3.7 (Posada and Crandall, 1998) (Table 1). Model parameters were estimated independently for each data partition using the UNLINK command. Eight separate proceedings that acted as the BI partitions were performed with four Markov Chain Monte Carlo (MCMC) processes starting from a random tree. Four chains, one cold and three heated (using default heating values), were run simultaneously with 20,000,000 generations and sampled every 1000 generations. The first 10,000,000 generations (10,000 trees) were discarded as burn-in, and majority-rule consensus phylograms and posterior probabilities for nodes were assembled from the last 10,000,000 generations (10,000 trees). Except for the different models in each partition, BI conducted the same parameters. Bootstrap values (BP) $\geqslant$70% and posterior probabilities (PP) $\geqslant$0.95 are considered statistically significant clade support (Hillis and Bull, 1993; Hulelsenbeck and Rannala, 2004).

Maximum parsimony and maximum likelihood analyses were implemented in PAUP version 4.0b10 (Sworfford, 2002). In MP analyses, heuristic searches were performed with 100 replicates of random taxon addition, accelerated character transformation (ACCTRAN) optimization, tree bisection-reconnection (TBR) branch swapping, and gaps coded as missing data. The GTR +G model (GTR, general time reversible [Tavaré, 1986]; G, the gamma distribution for rate variation among sites) was selected as the best-fit model for both the data sets by likelihood ratio tests implemented in Modeltest 3.7 (Posada and Crandall, 1998) under the corrected Akaike information criterion

Table 1 Models of different partitions under AIC

Partition	12S rRNA	16S rRNA	CO1_1st_2nd	CO1_3rd	ND4_1st_2nd	ND4_3rd	Cyt *b*_1st_2nd	Cyt *b*_3rd
Mode	GTR+G	GTR+I	GTR+I	GTR+I	GTR+I		GTR+I	
Parameter	nst=6 rates=gamma	nst=6 rates=propinv	nst=6 rates=propinv	nst=6 rates=propinv	nst=6 rates=propinv	nst=2 rates=equal	nst=6 rates=propinv	nst=6 rates=equal
Length	876 bp	1453 bp	846 bp	423 bp	480 bp	240 bp	660 bp	330 bp

(AIC; Akaike, 1974). To assess nodal support in MP and ML trees, we used non-parametric bootstrapping with heuristic searches of 1000 replicates for MP and 100 replicates for ML (Felsenstein, 1985; Felsenstein and Kishino, 1993; Hedges, 1992). Nodes with bootstrap values of 70% or greater were regarded as sufficiently supported (Huelsenbeck and Hillis, 1993), and those with the values between 50% and 70% as weakly supported.

2.3 Morphological characters We measured and recorded the following morphological characters: body size, tubercles on carapace skin, color of plastron skin, dark spots on plastron, basisphenoid characteristics (ratio of the smallest width to the largest width; the smallest width of basisphenoid is restrained by two holes on each side) and the shape of entoplastron. These above characters were selected for this study because the authors used them when they identified the species (Tang, 1997; Zhou *et al*., 1991). Besides, the pattern on neck and bottom of marginal carapace were also recorded.

A digital caliper was used to measure the length of the animals. The abbreviations used in this study include HL: head length (from snout tip to posterior margin of parietal), HW: head width (the widest part of temporal region), HH: head height (the topmost part of temporal region), SL: snout length, SW: snout width, EL: eye length (distance between anterior and posterior corners of eyelid), DEL: distance between two eyes (minimum distance between two eyes), BH: body height, CL: carapace length, CW: carapace width, TL: tail length, and BL: body length (from snout tip to tail end).

Skeletal samples were obtained by alkaline degreasing, in which 10% H_2O_2 solution in 0.5% potassium hydroxide (KOH) solution was used to erode muscle tissue at room temperature. The skulls were photographed; including those of *P. parviformis*, *P. sinensis* and *P. axenaria*, and basisphenoid was stood out to emphasize the ratio of the smallest width to the largest width.

3. Results

3.1 Phylogenetic results The final matrix consisted of 5308 bp length DNA sequences: 12S (876 bp), 16S (1453 bp), Co1 (1269 bp), ND4 (720 bp), and Cyt *b* (990 bp). GenBank accession numbers are HQ116584 –HQ116623. The partition-homogeneity test (p=0.5) showed no significant differences among the five genes, and then the sequences were combined and the resulting sequences analyzed as a single matrix. The K2P genetic distance among the voucher specimens of Nos. CIB95410 – CIB95412 is 0.001, 0.024 between two specimens of *P. sinensis*is, and 0.022 between the voucher specimens of Nos. CIB95410 – CIB95412 and *P. sinensis*.

In MP analyses, 3318 equally most parsimonious trees were obtained [tree length = 1376; consistency index (CI) =0.88; retention index (RI) = 0.88]. The topology found under ML analysis was identical to that found in MP analysis (Figure 2A), which shows the voucher specimens of Nos. CIB95410 – CIB95412 and *P. sinensis* form one clade (ML/MP tree, bootstrapping proportion = 65/89); the voucher specimens of Nos. CIB95413, CIB95414 and CIB95417 are clustered with *P. axenaria*, as the sister group of the voucher specimens of Nos. CIB95415 and CIB95416 (ML/MP tree, bootstrapping proportion = 97/100, partitioned-BI tree, posterior possibilities = 1.0). But this topology differed from the Bayesian tree, for the voucher specimens of Nos. CIB95410 – CIB95412 formed one monophyletic group, which was sister to the group of *P. sinensis* (ML/MP tree, bootstrapping proportion = 100/100, partitioned-BI tree, posterior possibilities = 1.0) (Figure 2B). The result of COI under NJ analysis (not shown) showed the same topology as MP and ML.

3.2 Morphological results From the morphological data, the mean length of the body sizes of voucher specimens (Nos. CIB95410 – CIB95412) is 409.3±164.7 mm. The mean length of *P. pelodiscus* is 517.1±195.3 mm (CIB00026, CIB00027, and CIB00028), and that of Nos. CIB95413 – CIB95417 is 408.6±156.2 mm. For the further measurements see Table 2. As for the voucher specimens of Nos.CIB95410 – CIB95412, prominent tubercles arrayed like the shape of wing. However, those tubercles were randomly arranged on *P. sinensis* and the voucher specimens of Nos. CIB95413, CIB95414 and CIB95417, but they were not found on the voucher specimens of Nos. CIB95415 and CIB95416. Color of the plastron skin turned red when captured after one minute, which was found in voucher specimens of Nos. CIB95410 – CIB95412. The voucher specimens of Nos. CIB95410 – CIB95412 and *P. sinensis* had dapples spots on the bottom of marginal carapace and neck, but no on voucher specimens of Nos. CIB95413 – CIB95417. All the samples under comparison were found with no dark spot on plastron, except the voucher specimens of Nos. CIB95413, CIB95414 and CIB95417. The shape of entoplastron in all the species varied from one another, of which the angle grew gradually from 90° to 135° even straight like "⌒" on *P. sinensis*, voucher specimens of Nos. CIB95410 – CIB95412 and voucher specimens of Nos. CIB95413 – CIB95417, respectively (Table 3).

Morphological comparison of skulls of different

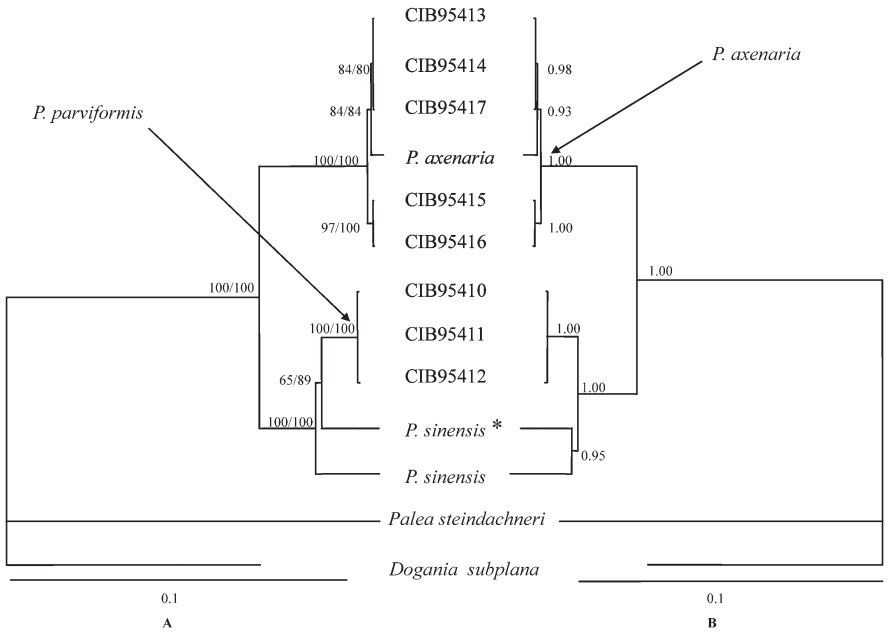

Figure 2 ML and MP tree (A) and Partitioned Bayesian tree (B). Numbers near corresponding branches indicate percentages out of 1,000 bootstrap replicates for ML on the numerators and MP on denominators position in (A), and posterior probabilities for Bayesian analysis in (B). Note: The numbers (CIB95410 – CIB95412; Red font means samples collected from the Yixiang River in Lishui Village of Wenqiao Town in Quanzhou County; Blue font means samples collected from Xiang River between Yongsui and Huangshahe villages in Quanzhou County) are the voucher specimens numbers; *P. sinensis** is collected from Korean (GenBank accession No: AY962573.1), *P. sinensis* from Anhui, China (GenBank accession No: NC006132.1) and *P. axenaria** from Hunan, China (GenBank accession Nos: AY743421.2; AY583695.1; AY583693.1).

species was shown in Figure 3, and the feature we chose for this study was the ratio of the smallest width to the largest width in the basisphenoid, and this feature was compared with that of Tang (1997). Of *P. sinensis*, the ratio was less than 1/2 (Figure 3A), but almost equal to the 1/2 ratio of the voucher specimens of Nos. CIB95410 – CIB95412 or larger than that of Nos. CIB95413 – CIB95417 (Figure 3B, C).

4. Discussion

4.1 Evaluation of phylogenetic analyses methods

Methods based on parsimony do not depend on evolutionary models of nucleotide substitution but rather on attempting to find the minimum number of mutations, thus long-branch attraction will be more probable since sequences which have converged on gaining an increased

Table 2 Measurements of *P. sinensis*, *P. parviformis* and *P. axenaria* (mm)

	P. sinensis				*P. parviformis*				*P. axenaria*			
	CIB 00026	CIB 00027	CIB 00028	Mean±SD n=3	CIB 95410	CIB 95411	CIB 95412	Mean±SD n=3	CIB 95413	CIB 95414	CIB 95415	Mean±SD n=3
Sex	Male	Female	Male		Male	Female	Female		Male	Male	Female	
HL	49.9	40.8	43.1	44.6±3.6	35.2	27.1	32.3	31.5±2.8	33.8	33.5	34.9	34.1±0.7
HW	26.7	22.5	24.3	24.5±1.3	19.4	16.7	17.5	17.9±0.6	14.5	16.1	19.2	16.6±1.7
HH	21.6	16.4	17.7	18.6±2.0	15.2	14.3	15.1	14.9±0.4	14.7	15.3	16.4	15.5±0.6
SL	5.8	5.6	5.3	5.6±0.3	4.0	3.3	3.5	3.6±0.2	4.4	4.1	4.3	4.3±0.1
SW	5.4	4.0	4.1	4.5±0.7	4.2	3.2	3.6	3.7±0.3	3.6	3.9	4.2	3.9±0.2
EL	6.7	5.6	5.8	6.0±0.5	7.8	6.0	5.9	6.6±0.4	5.8	5.0	5.0	5.3±0.2
DEL	4.0	3.2	3.2	3.5±0.4	3.1	2.9	3.2	3.1±0.2	3.0	2.8	3.0	2.9±0.1
BH	39.7	32.6	34.0	35.4±3.0	29.2	23.4	27.6	26.7±2.2	26.8	26.2	28.1	27.0±1.0
CL	122.2	101.4	97.8	107.1±12.3	98.2	71.8	81.1	83.7±6.3	79.4	79.1	86.7	81.7±3.9
CW	101.9	92.0	86.3	93.4±7.8	80.4	60.2	68.3	69.6±5.1	67.8	67.2	75.3	70.1±4.1
TL	26.9	6.6	11.7	15.1±8.0	23.3	15.4	14.1	17.6±1.8	14.3	15.2	18.9	16.1±1.9
BL	204.8	154.4	157.9	517.1±195.3	160.7	117.6	131	409.3±164.7	131.9	131.9	144.8	408.6±156.2

For abbreviations, see the Materials and Methods.

Table 3 Comparison of P. parviformis and P. axenaria in morphological characters

	Body size (mm)	Tubercles on carapace skin	Color of plastron skin	Dapples on ventral calipash	Dark spot on plastron	Dapples on neck	Basisphenoid*	Shape of entoplastron
P. sinensis	517.1±195.3	Randomly arranged tubercles	Yellow white & grey white	Yes	No	Yes	Smaller than 1/2	"^", included angle is 90°
Voucher specimens of Nos. CIB95410–CIB95412	409.3±164.7	Prominent tubercles arraying like wing	White & pale yellow; turning red when caught	Yes	No	Yes	Approximate or more than 1/2	"^", included angle is 135°
Voucher specimens of Nos. CIB95413–CIB95417	408.6±156.2	Nos. CIB95413, CIB95414 and CIB95417 have randomly arranged tubercles; Nos. CIB95415 and CIB95416 are smooth	Yellow white	No	Yes, on Nos. CIB95413, CIB95414 and CIB95417; No, on Nos. CIB95415 and CIB95416	No	Approximate or more than 1/2	Straight, present"^"

Basisphenoid* means the ratio of the smallest width to the largest width

numbers of nucleotide subsitutions may be clustered together (Holder and Lewis, 2003; Felsenstein, 2003). So the method based on maximum likelihood is better than parsimony to analyze the topological structure for the phylogenetic relationships, especially when looking at quickly evolving genes (Philippe *et al*., 2005). Bayesian statistics have a strong connection to the maximum likelihood method, even faster than the bootstrapping of likelihood (Zhang and Nei, 1997). Besides, MCMC-based processes can be used to approximate the probability distributions (Holder and Lewis, 2003). It is worth noting that accurate posterior probability estimates could be enhanced when the systematic error has been reduced as well as the partitioned Bayesian analyses been executed to explore the partition-specific evolutionary (Brandley *et al*., 2005; Nylander *et al*., 2004). Consequently we prefer the Bayesian result to others for the above advantages.

4.2 The validity of *P. parviformis*

4.2.1 Molecular data MP, ML, and Bayesian analyses differ on the exact placement of the voucher specimens of Nos. CIB95410 – CIB95412: MP and ML suggest *P. parviformis* might be paraphyletic to *P. sinensis*, whereas Bayesian analysis suggests a sister-group relationship with *P. sinensis*. The relationship of nodal support based on ML analyses is weak (bootstrapping proportion = 65), which is below 70% (Huelsenbeck and Hillis, 1993), but the posterior probabilities for Bayesian analysis are better (posterior probabilities=1.00). According to the advantages of heterogenous characteristics of sequence evolution, we chose the result from our partitioned Bayesian analyses which support *P. parviformis* (voucher specimens of Nos. CIB95410 – CIB95412) as a valid new species. In addition, all the molecular phylogenetic analyses show that the voucher specimens of Nos.

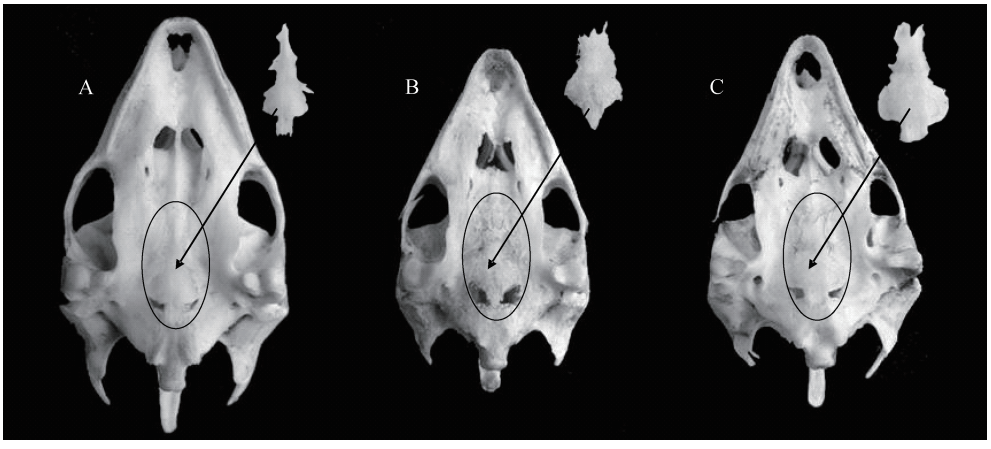

Figure 3 Difference in basisphenoids of skulls. The top right corner shows the different basisphenoids (Proportional scale is 1:2).
Note: A: *P. sinensis* with its ratio of the smallest width to the largest width being smaller than 1/2; B: *P. parviformis*; C: *P. axearia* with their ratio of the smallest width to the largest width almost equal to or larger than 1/2.

CIB95413 – CIB95417 are *P. axenaria*.

In this study, the available molecular data did not resolve the phylogenetic relationship of *Pelodiscus*. By adding more samples of *P. parviformis,* we may be able to resolve this problem. As for barcoding and genetic distance in this study, we could not distinguish the voucher specimens of Nos. CIB95410 – CIB95412 from *P. sinensis*. The topology of NJ tree using CO1 gene did not support that voucher specimens of Nos. CIB95410 – CIB95412 could form a clade either. Meanwhile, the genetic distance between the specimens of *P. sinensis* (0.024) was slightly larger than that among the voucher specimens of Nos. CIB95410 – CIB95412 (0.022). One reasonable explanation for this phenomenon might be due to the geographic distance between the sampling localities of *P. sinensis*, which are in Anhui (China) and Korea, and this distance is larger than that of the voucher specimens of Nos. CIB95410 – CIB95412.

4.2.2 Morphological data *Pelodiscus parviformis* is most similar to *P. sinensis* in morphology, which is widely distributed in China (Yang, 2010). Of the *P. sinensis* samples collected from Guizhou, China, the carapace length and width are about 107.1±12.3 mm and 93.4±7.8 mm, respectively, which are similar to those described in Fauna Sinica Reptilia (Zhang *et al*., 1998). Hence, the voucher specimens (Nos. CIB95410 – CIB95417) collected from Guangxi, China differ from those collected from Guizhou by smaller carapace length and width. The tubercles are randomly arranged on carapace skin of the samples of *P. sinensis* from Guizhou, same as those of the voucher specimens of Nos. CIB95413, CIB95414 and CIB95417. Whereas, dorsal tubercles on the voucher specimens of Nos. CIB95410 – CIB95412 more or less fused with one another in longitudinal series, consistent with the description of *P. parviformis* (Tang, 1997). The voucher specimens of Nos. CIB95413, CIB95414 and CIB95417 have a significant black dot on the plastron, which is in accordance with the description of *P. axenaria* (Zhou, 1991). Color of plastral skin became light red when captured about one minute later in the voucher specimens of Nos. CIB95410 – CIB95412 and it did not happen in the other samples, which is a diagnostic character for *P. parviformis* (Tang, 1997). We found that all the samples had the spots on the bottom of marginal carapace and neck, except the voucher specimens of Nos. CIB95413 – CIB95417, which had never been studied before. The angle of entoplastron and the smallest width to the largest width in the basisphenoid found in this study are consistent with the description by Tang (1997) and Zhou (1991). Consequently, our results indicated that the voucher specimens of Nos. CIB95410 – CIB95412 were *P. parviformis*, which was consistent with the description by Tang (1997), and the voucher specimens of Nos. CIB95413 – CIB95417 were *P axenaria*.

With respect to ecological conditions, *P. parviformis* requires fine quality of water, which means that river water is clear and river bed is sandy (Tang, 1997). And the populations of *P. parviformis* are known to have declined since the 1960s in Guangxi (Tang, 1997), which might be related to their geographic isolation or possibly to speciation.

4.3 New record of *P. axenaria* As described by Zhou (1991), *P. axenaria* has the feature of smooth carapacial skin and is recognized as a separate species in the Hunan Province (Zhou, 2006). The morphological characters of the voucher specimens of Nos. CIB95413 – CIB95417 differ from those of *P. sinensis* and the voucher specimens of Nos. CIB95410 – CIB95412. However, of the eight samples collected from Guangxi, five (Nos. CIB95413 – CIB95417) of them showed a molecular similarity with *P. axenaria*, but three (Nos. CIB95413, CIB95414 and CIB95417) of them were different on the basis of the existence of a carapacial protuberances. At the same time, in the phylogenetic tree, Nos. CIB95415 and CIB95416 formed a single monophyletic group, as the sister group of Nos. CIB95413, CIB95414, CIB95417 and *P. axenaria*. So the existence of tubercles on carapacial skin may be considered as being plastic and variable across individuals the phylogenetic placement for the samples of Nos. CIB95415 and CIB95416 may be caused by individual variation or the subspecies of *P. axenaria*. These data indicate not only the morphological variation in *P. axenaria*, but also a new record of *P. axenaria* in Guangxi, China. In addition, our data suggest that the smooth carapace, absence of spots on neck, should be considered as an identifying character for *P. axenaria*.

Acknowledgements We are grateful to the following people for discussion and / or comments on early drafts of this article: Steven BRAUTH, Jinzhong FU, Jiatang LI, Yong HUANG, and two anonymous reviewers. This work was supported by the grants from the National Natural Science Foundation of China (31071892) to Yuezhao WANG and the "Hundred Talents Program" of Chinese Academy of Sciences (KSCX2-YW-R-077) to Yezhong TANG.

References

Akaike H. 1974. A new look at the statistical model identification. IEEE Trans Automat Contr, 19: 716–723

Arèvalo E., Davis S. K., Sites Jr. J. 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. Syst Biol, 43: 387–418

Brandley M. C., Schmitz A., Reeder T. 2005. Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. Syst Biol, 54: 373–390

Castoe T. A., Doan T. M., Parkinson C. L. 2004. Data partitions and complex models in Bayesian analysis: the phylogeny of gymnophthalmid lizards. Syst Biol, 53: 448–469

Chen H. G. 2005. Comparative analysis on some genes of mitochondrial DNA and their molecular identification markers in three species of soft-turtles. M. S. Thesis. Hunan Normal University. 77 pp (In Chinese with English abstract)

Chen H. G., Liu W. B., Li J. Z., Zhang X. J. 2006. Comparative analysis of mitochondrial DNA cyt *b* gene and their molecular identification markers in three species of soft-turtles. Acta Hydrobiol Sin, 30: 380–385 (In Chinese with English abstract)

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. J Fish China, 29: 318–322 (In Chinese with English abstract)

Chkhikvadze V. M. 1987. On systematic position of the USSR Far East soft-shelled turtle. Bull Acad Sci of Georgian SSR, 128: 609–611

Esposti M. D., De Vries S., Crimi M., Ghelli A., Patarnello T., Meyer A. 1993. Mitochondrial cytochrome *b*: Evolution and structure of the protein. Biochim Biophys Acta, 1143: 243–271

Faith J. J., Pollock D. D. 2003. Likelihood analysis of asymmetrical mutation bias gradients in vertebrate mitochondrial genomes. Genetics, 165: 735–745

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783–791

Felsenstein J., Kishino H. 1993. Is there something wrong with the bootstrap on phylogeny? A reply to Hillis and Bull. Syst Zool, 42: 193–200

Fritz U., Gong S. P., Auer M., Kuchling G., Schneeweiß N., Hundsdörfer A. K. 2010. The world's economically most important chelonians represent a diverse species complex (Testudines: Trionychidae: *Pelodiscus*). Org Div Evol, 1–16

Fritz U., Havaš P. 2007. Checklist of chelonians of the world. Vert Zool, 57: 149–368

Fritz U., Obst F. J. 1999. Neue Schildkröten aus Südostasien. Teil II. Bataguridae, 11–26

Hajibabaei M., Janzen D. H., Burns J. M., Hallwachs W., Hebert P. D. N. 2006. DNA barcoding distinguishes species of tropical Lepidoptera. Proc Natl Acad Sci USA, 103: 968–971

Hedges S. B. 1992. The number of replications needed for accurate estimation of the bootstrap *P*-value in phylogenetic studies. Mol Biol Evol, 9: 366–369

Hillis D. M, Bull J. B. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol, 42: 182–192

Holder M., Lewis P. 2003. Phylogeny estimation: Traditional and Bayesian approaches. Nat Rev Genet, 4: 275–284

Huelsenbeck J. P., Hillis D. M. 1993. Success of phylogenetic methods inthe four-taxon case. Syst Biol, 42: 247–264

Huelsenbeck J. P., Rannala B. 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Syst Biol, 53: 904–913

Huelsenbeck J. P., Ronquist F. 2001. MrBayes: A program for the Bayesian inference of phylogeny. Bioinformatics, 17: 754–755

Iverson J. B., Brown R. M., Akre T. S., Near T. J., Le M., Thomson R. C., Starkey D. E. 2007. In search of the tree of life for turtles. Chelonian Res Monogr, 4: 85–106

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* (Testudines, Trionychidae). DNA Seq, 17: 471–483

Kumar S., Tamura K., Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform, 5: 150–163

Nei M., Kumar S. 2000. Molecular evolution and phylogenetics. New York: Oxford University Press

Nylander J., Ronquist F., Huelsenbeck J., Nieves-Aldrey J. 2004. Bayesian phylogenetic analysis of combined data. Syst Biol, 53: 47–67

Peng Q. L., Pu Y. G., Wang Z. F., Nie L. W. 2005. Complete mitochondrial DNA sequence of Chinese soft shell turtle (*Pelodiscus sinensis*). Chin J Biochem Mol Biol, 21: 591–596 (In Chinese with English abstract)

Penny D. 1982. Towards a basis for classification: the incompleteness of distance measures, incompatibility analysis and phenetic classification. J theor Biol, 96: 129–142

Philippe H., Zhou Y., Brinkmann H., Rodrigue N., Delsuc F. 2005. Heterotachy and long-branch attraction in phylogenetics. BMC Evol Biol, 5: 50

Posada D., Crandall K. A. 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics, 14: 817–818

Ronquist F., Huelsenbeck J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572–1574

Sambrook J., Russell D. 2001. Molecular cloning: A laboratory manual, 3rd ed. New York: Cold Spring Harbor Laboratory Press

Swofford D. L. 2003. PAUP*. Phylogenetic analysis using parsimony (* and other methods), version 4. Sunderland, MA: Sinauer Associates

Tang Y. Z. 1997. Research on a new species of *Pelodiscus*, Trionychidae in China. Zool Res, 18: 13–18 (In Chinese with English abstract)

Tavaré S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. In Miura R. M. (Ed.), Some mathematical questions in biology–DNA sequence analysis. Providence, RI: Amer Math Soc, 57–86

Xia X., Xie Z. 2001. DAMBE: Data analysis in molecular biology and evolution. J Hered, 92: 371–373

Yang P., Tang Y. Z., Wang Y. Z. 2011. Review of the classification history of Chinese soft shelled turtles (*Trionychidae:Pelodiscus*). Sichuan J Zool, 30(1): 156–159 (In Chinese with English abstract)

Zardoya R., Meyer A. 1996. Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. Mol Biol Evol, 13: 933–942

Zhang J., Nei M. 1997. Accuracies of ancestral amino acid sequences inferred by parsimony, likelihood, and distance

methods. J Mol Evol, 44: 139–146

Zhang M., Zong Y., Ma J. 1998. Fauna Sinica Reptilia Vol. I. Beijing: Science Press (In Chinese)

Zhao E. M. 1997. Studies on taxonomy and distribution of Chinese chelonians. Chin Chelonian Res, 9: 1–26

Zhou G. J., Zhang X. J., Fang Z. G. 1991. Bulletin of a new species trionyx. J Nat Sci Hunan Norm Univ, 14: 379–382 (In Chinese with English abstract)

Zhou T. 2006. Distribution of Chinese chelonians. Sichuan J Zool, 25: 272–276 (In Chinese with English abstract)

Zhou T., LI P. P. 2007. Chelonian species diversity and current status in China. Sichuan J Zool, 26: 463–467 (In Chinese with English abstract)