Is *Chinemys megalocephala* FANG, 1934 a valid species? New insights based on mitochondrial DNA sequence data

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Zusammenfassung

Ist Chinemys megalocephala *FANG, 1934 eine valide Art? Neue Erkenntnisse anhand mitochondrialer DNA-Sequenzen.*

Anhand eines 871 bp langen Fragments des mitochondrialen Cytochrom *b*-Gens wurde die Phylogenie der Schildkröten-Gattung *Chinemys* rekonstruiert. *C. nigricans* ist von den beiden anderen *Chinemys*-Arten klar differenziert und durch eine Sequenzdivergenz von 4,3 bis 5,7 % getrennt. Zwischen zwei *C. megalocephala* und zwei *C. reevesii* wurden keine Unterschiede gefunden. Allerdings zeigte sich bei *C. reevesii* eine beträchtliche Sequenzdivergenz im Bereich von 1,6 bis 1,7 %, da sich eine dritte Sequenz aus der GenBank signifikant von unseren eigenen Daten unterscheidet. Es könnte sein, dass dies auf Sequenzierfehler bei den GenBank-Daten zurückzuführen ist. Zur Erklärung der identischen Sequenzdaten von *C. megalocephala* und *C. reevesii* werden drei Hypothesen formuliert:

(a) Beide nominellen Arten sind identisch, und *C. megalocephala* ist nur eine an besondere Nahrungsverhältnisse adaptierte Form von *C. reevesii*, wie es anhand der Morphologie bereits früher vermutet wurde.

(b) Die übereinstimmende mtDNA von *C. megalocephala* und *C. reevesii* ist das Ergebnis einer introgressiven Hybridisierung zweier nahe verwandter Spezies.

(c) *C. megalocephala* entstand durch die Hybridisierung von nur weitläufig verwandten Arten, wie es für mehrere Schildkrötentaxa bekannt ist.

Der mütterliche Elternteil beziehungsweise Vorfahre bei (b) und (c) wäre dann jeweils *C. reevesii*, da mitochondriale DNA nur in mütterlicher Linie vererbt wird.

Des weiteren werden unsere Ergebnisse im Licht angeblicher Unterschiede im Karyotyp von *C. megalocephala* und *C. reevesii* diskutiert. Die meisten dieser Unterschiede dürften auf subjektiven Chromosomen-Klassifizierungen beruhen. Größenunterschiede der homologen Makrochromosomen von *C. megalocephala* könnten jedoch tatsächlich für einen hybriden Ursprung dieses Taxons sprechen.

Schlagwörter: Testudines: Geoemydidae: *Chinemys megalocephala*, *Chinemys nigricans*, *Chinemys reevesii*; molekulare Systematik; Sequenzdaten; Cytochrom *b*; mitochondriale DNA.

Abstract

An 871 bp long fragment of the mitochondrial cytochrome *b* gene was used for reconstructing the phylogeny of turtles of the genus *Chinemys*. *C. nigricans* is clearly differentiated from the other two species of that genus, separated by a sequence divergence of approximately 4.3 to 5.7 %. No differences were detected between two *C. megalocephala* and two *C. reevesii*. However, considerable sequence divergence ranging 1.6 to 1.7 % was found among *C. reevesii* as an additional sequence obtained from GenBank differed significantly from our own data. It might be that this finding is due to sequencing errors of the GenBank sequence. Three hypotheses are formulated to explain the identical sequence data of *C. megalocephala* and *C. reevesii*:

(a) Both nominal species are identical and *C. megalocephala* is just a "diet variant" of *C. reevesii* as believed earlier based on morphology.

(b) The identity of mtDNA sequence data in *C. megalocephala* and *C. reevesii* is the result of introgressive hybridization of two closely related species.

(c) *C. megalocephala* originated from hybridization of only distantly related species, as known for several chelonians.

In (b) and (c), the maternal parent or ancestor would be *C. reevesii* as mitochondrial DNA is inherited exclusively in a maternal line.

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Further, our findings are discussed in the light of supposed karyotypic differences between *C. megalocephala* and *C. reevesii*. Most karyotypic differences seem to be based on subjective chromosome classifications. However, differences in the sizes of homologous macrochromosomes of *C. megalocephala* could argue indeed for a hybrid origin of that taxon.

Key words: Testudines: Geoemydidae: *Chinemys megalocephala*, *Chinemys nigricans*, *Chinemys reevesii*; molecular systematics; sequence data; cytochrome *b*; mitochondrial DNA.

1 Introduction

One of the most enigmatic Chinese turtles is *Chinemys megalocephala* FANG, 1934. After FANG's description, virtually nothing of that taxon became known for years (ERNST & BARBOUR 1989). FANG (1934) based his diagnosis of *C. megalocephala* mainly on the distinctly larger and more massive head compared with the otherwise very similar *C. reevesii* (GRAY, 1831) (Figs. 1-2). In the western hemisphere, no specimens of *C. megalocephala* were present in zoological collections until recently. In the 1980s, *C. megalocephala* started to appear regularly in the international pet trade, stimulating a morphological comparison with slender-headed *C. reevesii* specimens (IVERSON et al. 1989). These authors concluded that *C. megalocephala* is "a local variant of *C. reevesii*, adapted or acclimated to a diet of mollusks" and synonymized both.

Later, GUO et al. (1997) reported chromosomal differences between *C. megalocephala* and *C. reevesii*. In the following, *C. megalocephala* was accepted again as a valid species by most authors (e. g., ZHAO 1998, VAN DIJK 2000, LAU & SHI 2000), whereas ERNST et al. (2000) supposed that *megalocephala* may represent a valid subspecies of *C. reevesii*.

Amazingly, all so called *C. megalocephala* males which reached Europe via the pet trade are virtually indistinguishable from *C. reevesii*. In contrast, *C. megalocephala* females match the original description of that taxon well (Fig. 1; U. FRITZ unpubl.: voucher specimens in the collection of the Museum für Tierkunde Dresden; E. MEIER, M. REIMANN pers. comm.). However, FANG (1934) mentioned in the original description of *C. megalocephala* also large-headed males so that the question arose whether *C. reevesii* males are sold under a wrong name to earn more money.

Turtles from Southeast and East Asia are facing extinction by overexploitation for food (see reviews in VAN DIJK et al. 2000). At the moment, several captive breeding programs are established, and it is necessary to single out high-priority species. To clarify the taxonomic state of the *C. megalocephala* males and females, we decided to analyze mitochondrial cytochrome *b* sequences in a specimen of each sex. Here we report our findings for *C. megalocephala* and compare it with the other two *Chinemys* species, i. e., *C. nigricans* (GRAY, 1834) and *C. reevesii*. Finally, we discuss our results in the light of the earlier reported karyotypic differences between *C. megalocephala* and *C. reevesii*.

2 Materials and Methods

2.1 Sampling and DNA Extraction

Tissue samples (thigh muscle) of two *Chinemys megalocephala* (male: MTD 41904, female: MTD 41809), one *C. nigricans* (juv.: MTD 42864), one *C. reevesii* (female: MTD 41905) and as outgroups of one *Malayemys subtrijuga* (SCHLEGEL & MÜLLER, 1844) (MTD 43718) and one *Sacalia bealei* (GRAY, 1831) (MTD 41583) were obtained by dissecting fresh dead turtles. Complete alcohol-preserved specimens are deposited in the herpetological collection of the Museum für Tierkunde Dresden (MTD) under

the catalogue numbers given above. From an additional live *C. reevesii* blood was acquired by coccygeal vein puncture as described in HASKELL & POKRAS (1994). Blood and tissue samples were stored at –70 °C in EDTA buffer (10 % EDTA, 0.5 % sodiumfluoride, 0.5 % thymol, 1 % tris, pH 7.0; ARCTANDER 1988). Total genomic DNA was extracted following the protocol of GUSTINCICH et al. (1991).

2.2 DNA Amplification and Sequencing

All tissue samples were analyzed by the team members in Leipzig. The blood sample of *Chinemys reevesii* was sequenced by D. GUICKING in Heidelberg. This sample is called "*C. reevesii* HD" in the following. We amplified a fragment of 1083 bp containing 1037 bp of the mitochondrial cytochrome *b* gene and 46 bp of the tRNA threonine gene. The PCR primers were mt-A (LENK & WINK 1997) and H15909 (LENK et al. 1999) or modified versions of these (Tab. 1). PCR conditions were as follows: 5 min at 95 °C, then 40 cycles of 1 min at 95 °C, 1 min at 50 °C, 2 min at 72 °C, and a single extension step of 10 min at 72 °C.

Primer	Direction	Sequence
PCR Primers		
mt-A	forward	5'-CAACATCTCAGCATGATGAAACTTCG-3'
H15909	reverse	5'-CAGTTTTTGGTTTACAAGACCAATG-3'
Sequencing Primers		
mt-A	forward	5'-CAACATCTCAGCATGATGAAACTTCG-3'
mt-c2	forward	5'-TGAGGACAAATATCATTCTGAGG-3'
mt-e	forward	5'-AAACCAGAATGATACTTCCTATTTGC-3'
H15909	reverse	5'-CAGTTTTTGGTTTACAAGACCAATG-3'
mt-E	reverse	5'-GCAAATAGGAAGTATCATTCTGG-3'
mt-B	reverse	5'-ACCTCAAAAGGATATTTGTCCTCA-3'
TestudRi3	reverse	5'-AGTAGGTTGGTGATGACAGTGGC-3'

Tab. 1. Primers used for PCR and sequencing.

PCR- und Sequenzier-Primer.

Sequencing reactions were performed with the 7-deaza-dGTP sequencing kit (Amersham Pharmacia) and separated on an automated LI-COR DNA sequencer. For sequencing the *C. reevesii* HD sample, the Cycle Sequencing Kit (Amersham Pharmacia Biotech, RPN 2438/RPN 2538) was used in combination with fluorescently labeled primers, and fragments were separated on an ALFExpressII automated sequencer (Amersham Pharmacia). The sequencing primers are listed in Table 1. They are modified versions of the primers used by WINK (1995) and LENK et al. (1999), with the exception of the newly designed TestudRi3, which gave better results than the primer mt-B.

2.3 Phylogenetic Analyses

One additional sequence of *Chinemys reevesii* was retrieved from GenBank (Acc. No. U81358). Because this *C. reevesii* sequence is shorter than our sequences, only an 871 bp long alignment of the cytochrome *b* gene could be used for all phylogenetic analyses.

Fig. 1. *Chinemys megalocephala* FANG, 1934. From top to bottom: dorsal, ventral, and lateral aspect (MTD 36692, adult female, straight line carapace length 224.2 mm).

Von oben nach unten: Dorsal-, Ventral- und Lateralansicht von *Chinemys megalocephala* FANG, 1934 (MTD 36692, adultes Weibchen, Stockmaß-Carapaxlänge 224,2 mm).

The program package MEGA (KUMAR et al. 1993) was used to estimate genetic distances and to calculate sequence statistics. Alignment was carried out with CLUSTAL X, v. 1.8 (THOMPSON et al. 1997) with default parameters. Neighbor-joining trees (SAITOU & NEI 1987) were constructed with the program TREECON, v. 1.3b (VAN DE PEER & DE WACHTER 1994) with the KIMURA (1980) two-parameter model and estimated transition/transversion ratio. Maximum-parsimony analyses were carried out with PAUP* 4.0 (SWOFFORD 1998) with the heuristic search method with 10 random stepwise additions of the sequences and the TBR branch swapping option. To test the robustness of the resulting trees, bootstrap analyses with 1000 replicates were performed in both methods. Maximum-likelihood trees were calculated with TREE-PUZZLE, v. 5.0 (STRIMMER & VON HAESELER 1996). The model of TAMURA & NEI (1993)

Fig. 2. *Chinemys reevesii* (GRAY, 1831). From top to bottom: dorsal, ventral, and lateral aspect (MTD 41905, adult female, straight line carapace length 198.5 mm).

Von oben nach unten: Dorsal-, Ventral- und Lateralansicht von *Chinemys reevesii* (GRAY, 1831) (MTD 41905, adultes Weibchen, Stockmaß-Carapaxlänge 198,5 mm).

was used with both nucleotide frequencies and the transition/transversion ratio being estimated by the program. Quartet puzzle support values were calculated for each branch, which are comparable to bootstrap values (STRIMMER & VON HAESELER 1996).

3 Results

Complete nucleotide sequence data are deposited under accession numbers AJ519496-AJ519502 in the EMBL database. An 871 bp long region of the cytochrome *b* gene was used to reconstruct the phylogeny within *Chinemys*. Of these nucleotide sites, 203 were variable and 59 parsimony informative. The genetic distances between *C. nigricans* and the two other *Chinemys* species range approximately 4.3 to 5.7 %, clearly arguing for its distinctiveness and validity (Tab. 2). However,

no significant differences were found between our sequences of two *C. reevesii* and two *C. megalocephala*. Between the *C. reevesii* samples we detected a sequence divergence of 0.115 % (1 bp), whereas the sequence data of both *C. megalocephala* were identical with one of the *C. reevesii* samples. In contrast, the sequence data of a third *C. reevesii* (GenBank U81358; SHAFFER et al. 1997) exhibit a considerable divergence ranging 1.6 to 1.7 % (14 substitutions) compared to our two highly congruent samples (Tab. 2).

	1	2	3	4	5	6	7	8
1 *C. megalocephala* 1	–							
2 *C. reevesii*	0.00000	–						
3 *C. megalocephala* 2	0.00000	0.00000	–					
4 *C. reevesii* HD	0.00115	0.00115	0.00115	–				
5 *C. reevesii* GB	0.01628	0.01628	0.01628	0.01747	–			
6 *C. nigricans*	0.04306	0.04306	0.04306	0.04432	0.05685	–		
7 *Sacalia bealei*	0.12658	0.12658	0.12658	0.12813	0.14167	0.12184	–	
8 *Malayemys subtrijuga*	0.19212	0.19212	0.19212	0.19031	0.19290	0.19437	0.20299	–

Tab. 2. Genetic distances (TAMURA & NEI 1993) between the samples studied. Abbreviations: GB: data from GenBank; HD: sample analyzed in Heidelberg.

Genetische Distanzen (TAMURA & NEI 1993) zwischen den untersuchten Proben. Abkürzungen: GB: Daten aus der GenBank; HD: in Heidelberg analysierte Probe.

All phylogenetic analyses yielded the same tree topology (Fig. 3). *Malayemys subtrijuga* and *Sacalia bealei* were used as outgroups to root the tree. They are separated from each other as well as from all *Chinemys* species by large genetic distances. The monophyly of the genus *Chinemys* is supported with bootstrap or puzzle support values of 100 %. The same values are found for the group containing the 5 sequences of *C. reevesii* and *C. megalocephala*. The sequence of *C. reevesii* from GenBank (SHAFFER et al. 1997) is separated from the other *C. reevesii* and *C. megalocephala* sequences with slightly lower values. The branching pattern of this remaining group is not resolved because there are no significant differences between the sequences.

4 Discussion

Research on Southeast and East Asian turtles is severely handicapped by the fact that virtually all specimens originate from the animal trade and lack reliable locality data. Even in cases where locality data were provided by animal dealers, the data were often wrong or even intentionally falsified (FRITZ & OBST 1998, 1999, PARHAM et al. 2001). Unfortunately, also for the *Chinemys* specimens studied we cannot provide an exact geographic provenance. The turtles originated from the pet trade in Hong Kong (China).

The detected identity of the sequences of the *C. megalocephala* male and *C. reevesii* could argue for a wrong species designation, i. e., that the alleged *megalocephala* specimen is indeed a *C. reevesii* and not a *C. megalocephala* (see introduction). However, this interpretation cannot be true for the female, which is morphologically a typical representative of *C. megalocephala*.

The considerable sequence divergence of a *C. reevesii* (GenBank U81358; SHAFFER et al. 1997) compared to our samples of *C. reevesii* and *C. megalocephala* (14

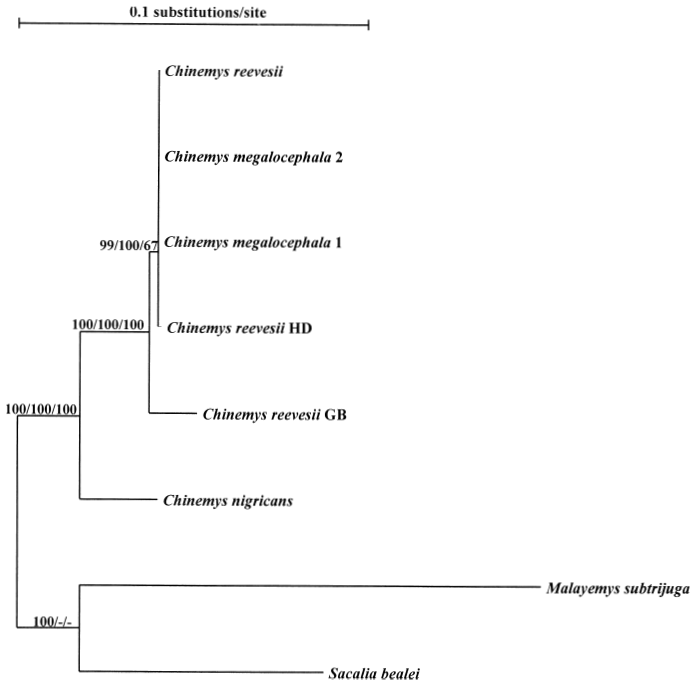

Fig. 3. Distance matrix tree of the genus *Chinemys* inferred from 871 bp of the mitochondrial cytochrome *b* gene. The consensus tree of 1000 bootstrap replicates was constructed by the neighbor-joining method. The first number at a node represents the bootstrap value for the group. The second number gives the quartet puzzle support value from the maximum-likelihood analysis and the third number the bootstrap value out of 1000 trees in maximum-parsimony analysis. The scale bar equals 0.1 substitutions per site. In all methods *Sacalia bealei* and *Malayemys subtrijuga* were used as outgroups. Abbreviations: GB: data from GenBank; HD: sample analyzed in Heidelberg.

Distanz-Matrix-Baum der Gattung *Chinemys*, abgeleitet von 871 bp des mitochondrialen Cytochrom *b*-Gens. Der auf 1000 Bootstrap-Replikaten beruhende Consensus-Baum wurde mit der Neighbor-Joining-Methode konstruiert. An den Knoten gibt die erste Zahl jeweils den Bootstrap-Wert für die Gruppe an, die zweite Zahl den Quartet-Puzzle-Support-Wert der Maximum-Likelihood-Analyse und die dritte Zahl den in der Maximum-Parsimony-Analyse ermittelten Bootstrap-Wert aus 1000 Bäumen. Der Maßstab entspricht 0,1 Substitutionen pro Position. Bei allen Methoden wurden *Sacalia bealei* und *Malayemys subtrijuga* als Außengruppen verwendet. Abkürzungen: GB: Daten aus der GenBank; HD: in Heidelberg untersuchte Probe.

substitutions) seems to highlight at the first glance an extensive intraspecific variation and differentiation within *C. reevesii*. However, we believe most of these differences are due to sequencing errors as nearly all of these alterations occur at the end of the sequence. Furthermore, the translation of the genes into aminoacid sequences show five aminoacid substitutions between the sequence of the *C. reevesii* from GenBank and our sequences of *C. reevesii* and *C. megalocephala.* The nucleotide sequence of *C. nigricans* shows 35 substitutions compared with our sequences of *C. reevesii* and *C. megalocephala*, also leading to five aminoacid substitutions.

Additionally, we have aligned the aminoacid sequences of 25 species belonging to the families Geoemydidae (formerly known under the younger synonym Bataguridae, BOUR & DUBOIS 1986) and Emydidae (unpubl. data). At least three aminoacid substitutions in the sequence of the *C. reevesii* from GenBank seem very unlikely as they occur in highly conservative positions where in none of the other species any substitution was found. Therefore, such a high degree of variation within one species or closely related taxa seems unlikely.

Our findings regarding sequence variation in the mitochondrial cytochrome *b* gene may be summarized as follows:

(1) *Chinemys nigricans* is a well differentiated species, separated by a sequence divergence of approximately 4.3-5.7 %. If a molecular clock of 0.4 % sequence divergence per 1 million years is applied, as accepted for the cytochrome *b* gene of many chelonians (AVISE et al. 1992, BOWEN et al. 1993, LAMB & LYDEARD 1994, WALKER & AVISE 1998, LENK et al. 1999), the separation of *C. nigricans* from other lineages dates back to the Middle or Upper Miocene (11-14 million years ago).

(2) *Chinemys megalocephala* and *C. reevesii* seem to be not differentiated.

Regarding the second result, three hypotheses have to be discussed:

(a) Both nominal species are identical and *C. megalocephala* is just a "diet variant" of *C. reevesii* (see introduction).

(b) The identity of mtDNA sequence data in *C. megalocephala* and *C. reevesii* is the result of introgressive hybridization of two closely related species. If so, natural hybridization would have caused introgression of *reevesii* mtDNA into the gene pool of *C. megalocephala* (or, less likely, of *megalocephala* mtDNA into the gene pool of *C. reevesii*).

(c) *C. megalocephala* originated from hybridization of only distantly related taxa, as known for several chelonians (reviews in FRITZ 1995, FRITZ & BAUR 1995, PARHAM et al. 2001, GALGON & FRITZ in press). *C. reevesii* is known to hybridize successfully with *Cuora amboinensis* (GALGON & FRITZ in press), *Mauremys japonica* (YASUKAWA et al. 1992), and *Mauremys mutica* (WINK et al. 2001). Hence, as mtDNA is inherited exclusively in a maternal line, *Chinemys megalocephala* could be the result of crossing female *C. reevesii* with an unknown paternal species. The rareness of *C. megalocephala* specimens and the enigmatic gap between its description by FANG (1934) and the next records (ZONG & MA 1985, IVERSON et al. 1989) could perhaps argue for such a hybrid origin, as hybrids may occur only from time to time.

We wish to point out that size differences in the homologous larger chromosomes of *C. megalocephala* as shown in GUO et al. (1997: Fig. 1) could support indeed a hybrid origin of that taxon. It might be that the two different size classes in the homologous chromosomes originate from different parental species.

In this context, the earlier reported karyotypic differences between *Chinemys megalocephala* and *C. reevesii* have to be considered. Like many representatives of the Geoemydidae, *C. megalocephala* (GUO et al. 1997) and *C. reevesii* (SASAKI & ITOH 1967, KILLEBREW 1977, CARR & BICKHAM 1986, GAO et al. 1986, GUO et al. 1997) possess a karyotype of 2n = 52 chromosomes, thought to be the primitive character state in this family (CARR & BICKHAM 1986).

For *C. reevesii* a wide range of different subjective classifications for chromosomes has been published (Tab. 3) which have to be discussed here. The chromosomal arrangements by SASAKI & ITOH (1967) and KILLEBREW (1977) are not based on detailed chromosome measurements. These authors divided the karyotype in different numbers of larger and smaller chromosomes (macro- + microchromosomes: 17 + 9, SASAKI & ITOH

	Chinemys reevesii					*Chinemys megalocephala*
Chromosome types	**Sasaki & Itoh (1967)**	**Killebrew (1977)**	**Gao et al. (1986)**	**Carr & Bickham (1986)**	**Guo et al. (1997)**	**Guo et al. (1997)**
Pairs of large(r) chromosomes or "macrochromosomes"	**17**	**13**	**15**	**14**	**14**	**14**
Subdivision of macrochromosomes	none	none	**Group A: 11** (6 metacentric, 1 submetacentric) 2 telomeric, 2 subtelomeric)	**Group A: 9** (metacentric or submetacentric)	**Group A: 9** (8 metacentric, 1 submetacentric)	**Group A: 9** (8 metacentric, 1 submetacentric)
			Group B: 4 (3 metacentric, 1 submetacentric)	**Group B: 5** (telocentric or subtelocentric)	**Group B: 5** (2 subtelocentric, 3 telocentric)	**Group B: 5** (4 subtelocentric, 1 telocentric)
Pairs of smaller chromosomes or "microchromosomes"	**9**	**13**	**Group C: 11**	**Group C: 12**	**Group C: 12**	**Group C: 12**
NOR position	–	–	–	terminal on pair 9 in group A	terminal on pair 7 in group A	terminal on pair 7 in group A

Tab. 3. Review of karyotypic data for *Chinemys megalocephala* and *C. reevesii*. Differences within *C. reevesii* are likely to originate from different resolution and individual karyotype arrangements by authors. Note that the main discrepancy between *C. megalocephala* and *C. reevesii* (Guo et al. 1997) is a different subdivision of the five pairs of group B chromosomes.

Literaturangaben zum Karyotyp von *Chinemys megalocephala* und *C. reevesii* im Überblick. Abweichende Angaben für *C. reevesii* beruhen wahrscheinlich auf einer verschiedenen Darstellungsgenauigkeit und individuell unterschiedlichen Karyotyp-Arrangements der einzelnen Autoren. Man beachte, dass der Hauptunterschied zwischen *C. megalocephala* und *C. reevesii* (Guo et al. 1997) eine andere Feineinteilung der fünf Chromosomenpaare der Gruppe B ist.

1967; 13 + 13, KILLEBREW 1977). But, by chromosome measurements GAO et al. (1986) "preliminarily" divided the *C. reevesii* karyotype into three groups, resulting in a third figure of macro- (A + B = 11 + 4) and microchromosomes (C = 11). The fourth deviating report on the *C. reevesii* karyotype is provided by CARR & BICKHAM (1986: macrochromosome groups A + B = 9 + 5, microchromosome group C = 12).

Like CARR & BICKHAM (1986) for *C. reevesii*, GUO et al. (1997) arranged the chromosomes of this species and *C. megalocephala* into three groups with a karyotypic formula of 9 + 5 + 12. CARR & BICKHAM (1986) found the NOR to be situated terminally on chromosome pair 9, whereas GUO et al. (1997) report it to be on pair 7. Concerning all these differences we wish to quote CARR & BICKHAM (1986): "It seems most likely that the discrepancies result from different levels of resolution achieved by investigators".

According to GUO et al. (1997: Tab. 2, p. 98), *C. megalocephala* has four pairs of subtelocentric chromosomes in group B (called "submetacentric" in their abstract) whereas *C. reevesii* has only two pairs. Merely this arrangement resulted in a difference of the fundamental number (NF, the number of chromosome arms in a karyotype): 78 in *C. megalocephala* but 74 in *C. reevesii*. Last not least, GUO et al. (1997) claimed that the third macrochromosome has a secondary constriction on the short arm in *C. megalocephala*, which is lacking in *C. reevesii*.

Generally, evolution of chromosomes is very slow in turtles (e. g., BICKHAM 1981, MUHLMANN-DIAZ et al. 2001). Conventional staining methods as well as partial G-banding failed to reveal differences even in the karyotypes of distantly related and morphologically well differentiated geoemydid taxa (*Callagur*, *Chinemys*, *Hieremys*, *Malayemys*, *Ocadia*; CARR & BICKHAM 1986). In the light of this evolutionary conservatism and the wide range of different subjective chromosome arrangements in *C. reevesii*, we doubt the supposed chromosomal differences between *C. reevesii* and *C. megalocephala* (Tab. 3).

Our mitochondrial DNA data favor a conspecifity of *C. megalocephala* and *C. reevesii*. However, taking all evidence into account, a hybrid origin of *C. megalocephala* seems possible. Hence, further research, involving nuclear genes, is needed to clarify the taxonomic status of this enigmatic turtle.

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