2004 *Asiatic Herpetological Research* Vol. 10, pp. 120-125

An *Ocadia sinensis* x *Cyclemys shanensis* hybrid (Testudines: Geoemydidae)

Maik Schilde[1], Dana Barth[2] and Uwe Fritz[3]

[1]*Opalstr. 31, D-04319 Leipzig, Germany; E-mail: maik.schilde@ufz.de*

[2]*University of Leipzig, Institute of Zoology, Molecular Evolution & Animal Systematics, Talstr. 33, D-04103 Leipzig, Germany; E-mail: dbarth@rz.uni-leipzig.de*

[3]*Zoological Museum (Museum für Tierkunde), Natural History State Collections Dresden, A. B. Meyer Building, Königsbrücker Landstr. 159, D-01109 Dresden, Germany; E-mail: uwe.fritz@snsd.smwk.sachsen.de*

Abstract. - A captive bred *Ocadia sinensis* x *Cyclemys shanensis* hybrid is described. Its hybrid status was confirmed by a comparison of a 1036 bp fragment of the mitochondrial cytochrome b gene with the putative mother (*C. shanensis*) and genomic ISSR fingerprinting. This is the first report of an intergeneric hybrid between very distantly related geoemydid turtles. All previous geoemydid intergeneric hybrids have been crossings within or between two sister clades containing the currently accepted genera (*Chinemys*, *Mauremys*, *Ocadia*) and (*Cuora*, *Pyxidea*).

Key words. - *Cyclemys*, *Ocadia*, testudines, intergeneric hybrid.

Introduction

Recently several new cases of intergeneric chelonian hybrids became known to science (reviewed in Galgon and Fritz, 2002). Most of them belong to the Southeast Asian family Geoemydidae, long known under its junior synonym Bataguridae. However, current research on the molecular phylogeny of geoemydids has shown that some species traditionally attributed to different genera are more closely related than previously thought (Wu et al., 1999; McCord et al., 2000; Honda et al., 2002a, b; Barth et al., in press; Stuart and Parham, in press), suggesting that they should be better lumped in the same genus. Thus, some of the hybrids may be in fact not intergeneric. In this paper we report a captive bred hybrid between two distantly related Southeast Asian geoemydids, representing an undoubtedly intergeneric cross.

According to the cited studies, there are several major clades among geoemydids. One clade contains the currently recognized genera *Chinemys*, *Mauremys*, *Ocadia*, *Cuora*, and *Pyxidea* (McCord et al., 2000; Honda et al., 2002a, b; Barth et al., in press), and another one, being the sister clade, *Cyclemys*, *Sacalia*, *Heosemys*, *Hieremys*, *Notochelys*, and *Leucocephalon* (McCord et al., 2000; Honda et al., 2002b).

The turtle described herein is the result of a hybridization of an *Ocadia sinensis* male and a *Cyclemys shanensis* female, representatives of two of the major clades of the Geoemydidae. This hybrid demonstrates that very distantly related geoemydids are capable of hybridizing successfully. It underlines the possibility that some recently described Southeast Asian chelonians (*Ocadia glyphistoma*, *O. philippeni*), which are only known from few pet trade specimens, might also be hybrids.

The specimen. - The turtle described below hatched in the live collection of M. Schilde from an egg of a *Cyclemys shanensis*, laid August 13, 2002. The second egg of the same clutch did not develop. The mother was a long term captive, and kept with a *Cyclemys shanensis* male and two *Ocadia sinensis* males. The elongated eggs measured 56.5 x 20.0 mm. One quickly developed a white band as typical for fertile eggs. It was incubated constantly at 28°C on Vermiculite. On October 26, 2002 a healthy turtle with a straight line shell length of 33 mm hatched (Figs. 1-4). Its color pattern resembled *Ocadia sinensis* but the general form was more similar to *Cyclemys* (roofed, distinctly tricarinate shell, serrated posterior marginal scutes), suggesting that it might be a hybrid. We decided to use two molecular methods to test this hypothesis.

Materials and Methods

We sequenced a 1036 bp portion of the mitochondrial cytochrome b gene (cyt b) of the captive bred turtle for comparison with the putative mother. Because mitochondrial DNA is inherited in the maternal line, the sequence of the putative hybrid should be identical with the mother (*Cyclemys shanensis*). Species identification of the *Cyclemys* was done by morphological means and comparison with the mitochondrial cyt b sequences of Guicking et al. (2002); the *Ocadia sinensis* males were determined morphologically.

Blood samples were obtained by coccygeal vein puncture. Samples were stored as described in Haskell

© 2004 by *Asiatic Herpetological Research*

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Fig. 1-4. The captive bred *Ocadia sinensis* x *Cyclemys shanensis* hybrid, September 2003 (11 months old). The roofed, distinctly tricarinate shell and the serrated posterior marginals resemble *Cyclemys* spp. The neck and facial stripes as well as the plastral pattern are similar to *O. sinensis*. The plastral pattern was more contrasting as a hatchling and has faded during growth. Photos: F. Höhler.

and Pokras (1994) and Arctander (1988). Additional blood samples and photographs (dorsal and ventral aspects) of the captive bred turtle (MTD T1262), the *Cyclemys shanensis* female (MTD T816), and the two *Ocadia sinensis* males (MTD T817-818) are in the tissue collection of the Zoological Museum Dresden. DNA extraction was carried out using the QIAamp Blood Mini Kit (Qiagen). Primers mt-A (Lenk and Wink, 1997) and H15909 (Lenk et al., 1999) were used to amplify a DNA fragment containing 1036 bp of cyt b. PCR and sequencing conditions were as described in Barth et al. (in press). Sequencing reactions were performed on an ABI 3100 Genetic Analyzer. Alignment was carried out with CLUSTAL X, v. 1.8 (Thompson et al., 1997) with default parameters. To demonstrate the considerable genetic difference between *Cyclemys* and *Ocadia* compared to other hybridizing geoemydids, Mega 2.1 (Kumar et al., 2001) was used for estimation of genetic distances. Cyt b sequence data for calculating genetic distances are from Barth et al. (in press) and Guicking et al. (2002).

To exclude the possibility of a gynogenetic or parthenogenetic origin of the specimen and to identify the putative father, we conducted genomic fingerprinting with Inter Simple Sequence Repeats (ISSR) for the captive bred specimen, the *Cyclemys* female and both *Ocadia* males. ISSR PCR produces species-specific genomic fingerprints (Gupta et al., 1994; Zietkiewics et al., 1994; Wink et al., 1998, 2001; Nagy et al., 2003) useful in detecting turtle hybrids (Wink et al., 2001). Hybrid specimens share about 50% of their ISSR PCR products with the respective paternal and maternal species (Wolfe et al., 1998; Wink et al., 2001; Storch et al., 2001). ISSR PCR is a simple and cheap method, and the results are easily reproducible (Bornet and Branchard, 2001). Gynogenesis or pseudogamy, the development of unfertilized eggs by activation through sperm of another species, as well as parthenogenesis is not known in turtles. However, if the captive bred specimen should be of such origin, the ISSR profiles should be identical with its biological mother.

The primer 5´-GACAGACAGACAGACA-3` was used to generate ISSR fingerprints for the captive bred specimen, the putative mother (*Cyclemys shanensis*), and both *Ocadia sinensis* males. Each reaction mix contained 100 ng of genomic DNA, 20 pmol primer, 1 U

Table 1. ISSR fingerprints of the *Ocadia sinensis* x *Cyclemys shanensis* hybrid and parental species (biological mother and father plus the second *O. sinensis* male kept with the mother). Only polymorphic and diagnostic PCR products shown.
MTD T = Museum für Tierkunde Dresden Tissue Collection; + = PCR product present, - = PCR product lacking.

Fragment Length	*Cyclemys shanensis* MTD T816	Hybrid MTD T1262	*Ocadia sinensis* MTD T817	*Ocadia sinensis* MTD T818
100 bp	+	+	-	-
480 bp	-	+	+	+
590 bp	-	+	+	+
810 bp	+	+	-	-
940 bp	+	+	-	-
1100 bp	-	+	+	-
1450 bp	-	+	+	+
1500 bp	+	+	-	-
1550 bp	-	+	+	-
1700 bp	+	+	-	-
2400 bp	-	+	+	+
2700 bp	-	+	+	+
2900 bp	+	+	-	-

Taq-polymerase (SIGMA), 2.5 µl 10x PCR buffer (SIGMA) and 2.5 µl of 200 µM dNTPs in a total volume of 25 µl. Amplification conditions were 4 min initial denaturation (94°C), followed by 31 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C, final extension of 7 min (72°C). PCR reactions were performed on an Eppendorf thermocycler.

15 µl of each PCR reaction was separated on 2% agarose gels (25 cm), stained in ethidium bromide solution (0.5 µg/ml) and visualized under UV light. The 100 bp DNA ladder Plus (MBI Fermentas) was used as a size standard. PCR was repeated under identical conditions to test reproducibility of results. DNA fragments were scored manually. Band sharing coefficients were calculated according to Storch et al. (2001).

Results and Discussion

As expected, the cyt b sequence of the captive bred turtle and the putative mother (*Cyclemys shanensis*; EMBL acc. no. AJ604513) proved to be identical. ISSR fingerprinting produced highly variable profiles which were species-specific and permitted individual identification of both *Ocadia sinensis* males (Table 1). The captive bred turtle shared 6 of its 13 bands with the mother (band sharing coefficient 0.5) and 7 bands with one of the *O. sinensis* males (band sharing coefficient 0.52). Because the captive bred turtle and this *O. sinensis* male exclusively share some fragments, we identified this turtle as the biological father. Thus, both methods confirmed the hybrid origin of the captive bred turtle.

Except for an unconfirmed, anecdotal newspaper record of natural hybrids between *Cuora flavomarginata* and *Geoemyda japonica* in Japan (Anonymous, 1995), all previous geoemydid intergeneric hybrids have been crossings within or between two sister clades containing the currently accepted genera (*Chinemys*, *Mauremys*, *Ocadia*) and (*Cuora*, *Pyxidea*): *Chinemys reevesii* x *Cuora amboinensis kamaroma* (Galgon and Fritz, 2002), *Chinemys reevesii* x *Mauremys japonica* (Yasukawa et al., 1992), *Chinemys reevesii* x *Mauremys mutica* (= "*Mauremys pritchardi*", Wink et al., 2001), *Cuora amboinensis kamaroma* x *Mauremys annamensis* (Fritz and Mendau, 2002), *Cuora bourreti* x *Pyxidea mouhotii* (= "*Cuora serrata*", Parham et al., 2001; Stuart and Parham, in press), *Cuora galbinifrons* x *Pyxidea mouhotii* (= "*Cuora serrata*", Parham et al., 2001; Stuart and Parham, in press), and *Cuora trifasciata* x *Mauremys mutica* (= "*Mauremys iversoni*", Parham et al., 2001; Wink et al., 2001). *Cyclemys* belongs to another major clade, comprising the genera *Cyclemys*, *Sacalia*, *Heosemys*, *Hieremys*, *Notochelys*, and *Leucocephalon* (McCord et al., 2000). *Cyclemys* is separated by a considerable genetic distance from *Ocadia* (Table 2), surpassing the genetic distances of the other hybridizing geoemydids.

Superficially our hybrid *Ocadia sinensis* x *Cyclemys shanensis* resembles *O. sinensis* due to its striped head and neck and the spotted plastral pattern. This leads to the speculation that the morphologically similar *Ocadia philippeni* McCord and Iverson, 1992 and *O. glyphistoma* McCord and Iverson, 1994 might be also intergeneric hybrids, as earlier suggested by van Dijk (2000), Lau and Shi (2000), Parham and Shi (2001), and Galgon and Fritz (2002). Both species were described on the basis of only a few pet trade turtles (McCord and Iverson, 1992, 1994), and until now no

Table 2. Pairwise genetic distances (cyt b) between hybridizing geoemydid species.

Geomydid species	Cyt b pairwise distances
Chinemys reevesii - Cuora amboinensis	0.104
Chinemys reevesii - Mauremys japonica	0.050
Chinemys reevesii - Mauremys mutica	0.070
Cuora amboinensis - Mauremys annamensis	0.098
Cuora galbinifrons - Pyxidea mouhotii	0.059
Cuora trifasciata - Mauremys mutica	0.104
Ocadia sinensis - Cyclemys shanensis	0.118

additional specimens became known to science. For some individuals of three other pet trade taxa a hybrid status has been unambiguously demonstrated: Two turtles identified as *Mauremys pritchardi* McCord, 1997 proved to be hybrids of *Chinemys reevesii* and *Mauremys mutica* (Wink et al., 2001). Three *Mauremys iversoni* Pritchard and McCord, 1991 originated from crossing *Cuora trifasciata* and *Mauremys mutica* (Parham et al., 2001; Wink et al., 2001), and several *Cuora serrata* Iverson and McCord, 1992, have been demonstrated to be hybrids between *Cuora galbinifrons* and *Pyxidea mouhotii* and of *Cuora bourreti* and *Pyxidea mouhotii* (Parham et al., 2001; Stuart and Parham, in press).

Until now it is unknown whether all specimens of these taxa are of hybrid origin, and if so, whether the crosses occurred in the wild, in captivity, or whether one or the other form might represent a natural, stable hybrid taxon (Parham et al., 2001; Wink et al., 2001). Many Southeast Asian chelonians are facing extinction due to overexploitation (van Dijk et al., 2000). Therefore, many conservation efforts are established around the globe, including CITES listing and captive breeding programs for several species. A correct taxonomy is the prerequisite for any conservation measure. Hence, it is crucial to determine whether the mentioned taxa represent real evolutionary entities and deserve high priority in conservation, this includes also natural hybrid taxa (Allendorf et al., 2001), or whether they are only incidentally occurring hybrids, without any conservation relevance.

Acknowledgments

Special thanks go to Michael Wink and Daniela Guicking, Heidelberg, for sharing unpublished geoemydid sequences with us and to Thomas U. Berendonk, Leipzig, for technical advice. James R. Buskirk, Oakland, provided the newspaper report on *Cuora flavomarginata* x *Geoemyda japonica* hybrids.

Literature Cited

Allendorf, F. W., R. F. Leary, P. Spruell and J. K. Wenburg. 2001. The problems with hybrids: setting conservation guidelines. TREE 16:613-622.

Anonymous. 1995. Hybrids between two protected turtles increase. Okinawa Times 15 August 1995.

Arctander, P. 1988. Comparative studies of Avian DNA by restriction fragment polymorphism analysis. Journal of Ornithology 129:205-216.

Barth, D., D. Bernhard, G. Fritzsch and U. Fritz. in press. The freshwater turtle genus *Mauremys* - a textbook example of an east-west disjunction or a taxonomic misconcept? Zoologica Scripta.

Bornet, B. and M. Branchard. 2001. Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Molecular Biology Reporter 19:209-215.

Fritz, U. and D. Mendau. 2002. Ein Gattungsbastard zweier südostasiatischer Schildkröten: *Cuora amboinensis kamaroma* Rummler and Fritz, 1991 x *Mauremys annamensis* (Siebenrock, 1903). Salamandra 38:129-134.

Galgon, F. and U. Fritz. 2002. Captive bred hybrids between *Chinemys reevesii* (Gray, 1831) and *Cuora amboinensis kamaroma* Rummler and Fritz, 1991. Herpetozoa 15:137-148.

Guicking, D., U. Fritz, M. Wink and E. Lehr. 2002. New data on the diversity of the Southeast Asian leaf turtle genus *Cyclemys* Bell, 1834. Molecular results. Faunistische Abhandlungen, Museum für Tierkunde Dresden 23:75-86.

Gupta, M., Y.-S. Chyi, J. Romero-Severson and J. L. Owen. 1994. Amplification of DNA markers from evolutionary diverse genomes using single primers of simple-sequence repeats. Theoretical Applied Genetics 89:998-1006.

Haskell, A. and M. A. Pokras. 1994. Nonlethal blood and muscle tissue collection from redbelly turtles for genetic studies. Herpetological Review 25:11-12.

Honda, M., Y. Yasukawa and H. Ota. 2002a. Phylogeny of the Eurasian freshwater turtles of the genus *Mauremys* Gray, 1869 (Testudines), with special

reference to a close affinity of *Mauremys japonica* with *Chinemys reevesii*. Journal of Zoological Systematics and Evolutionary Research 40:195-200.

Honda, M., Y. Yasukawa, R. Hirayama and H. Ota. 2002b. Phylogenetic relationships of the Asian Box turtles of the genus *Cuora* sensu lato (Reptilia: Bataguridae) inferred from mitochondrial DNA sequences. Zoological Science 19:1305-1312.

Kumar, S., K. Tamura, I. B. Jakobsen and M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis Software. Bioinformatics 17:1244-1245.

Lau, M. and H. Shi. 2000. Conservation and trade of terrestrial and freshwater turtles and tortoises in the People's Republic of China. Chelonian Research Monographs 2:30-38.

Lenk, P., U. Fritz, U. Joger and M. Wink. 1999. Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). Molecular Ecology 8:1911-1922.

Lenk, P. and M. Wink. 1997. A RNA/RNA heteroduplex cleavage analysis to detect rare mutations in populations. Molecular Ecology 6:687-690.

McCord, W. P., J. B. Iverson, P. Q. Spinks and H. B. Shaffer. 2000. A new genus of geoemydid turtle from Asia. Hamadryad 25:20-24.

McCord, W. P. and J. B. Iverson. 1992. A new species of *Ocadia* (Testudines: Bataguridae) from Hainan Island, China. Proceedings of the Biological Society of Washington 105:13-18.

McCord, W. P. and J. B. Iverson. 1994. A new species of *Ocadia* (Testudines: Batagurinae) from southwestern China. Proceedings of the Biological Society of Washington 107:52-59.

Nagy, Z. T., U. Joger, D. Guicking and M. Wink. 2003. Phylogeography of the European whip snake *Coluber* (*Hierophis*) *viridiflavus* as inferred from nucleotide sequences of the mitochondrial cytochrome b gene and ISSR genomic fingerprinting. Biota 3:109-118.

Parham, J. F., W. B. Simison, K. H. Kozak, C. R. Feldman and H. Shi. 2001. New Chinese turtles: endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme electrophoresis and known-locality specimens. Animal Conservation 4:357-367.

Parham, J. F. and H. Shi. 2001. The discovery of *Mauremys iversoni*-like turtles at a turtle farm in Hainan province, China: the counterfeit golden coin. Asiatic Herpetological Research 9:71-76.

Storch, V., U. Welsch and M. Wink. 2001. Evolutionsbiologie. Springer Verlag Berlin, Heidelberg, New York, 450 pp.

Stuart, B. L and J. F. Parham. in press. Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). Molecular Phylogenetics and Evolution.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24:4876-4882.

van Dijk, P. P. 2000. The status of turtles in Asia. Chelonian Research Monographs 2:15-23.

van Dijk, P. P., B. L. Stuart and A. G. J. Rhodin (eds.). 2000. Asian Turtle Trade. In Proceedings of a Workshop on Conservation and Trade of Freshwater Turtles and Tortoises in Asia. Chelonian Research Monographs 2:1-164.

Wink, M., H. Sauer-Gürth, F. Martinez, G. Doval, G. Blanco and O. Hatzofe. 1998. Use of GACA-PCR for molecular sexing of Old World vultures (Aves: Accipitridae). Molecular Ecology 7:779-782.

Wink, M., D. Guicking and U. Fritz. 2001. Molecular evidence for hybrid origin of *Mauremys iversoni* Pritchard et McCord, 1991, and *Mauremys pritchardi* McCord, 1997. Zoologische Abhandlungen, Staatliches Museum für Tierkunde Dresden 51:41-49.

Wolfe, A. D., Q.-Y. Xiang and S. R. Kephart. 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Molecular Ecology 7:1107-1125.

Wu, P., K. Zhou and Q. Yang. 1999. Phylogeny of Asian freshwater and terrestrial turtles based on sequence analysis of 12S rRNA gene fragment. Acta Zoologica Sinica 45:260-267.

Yasukawa, Y., N. Kamezaki and N. Ichikawa. 1992. On hybrids between *Mauremys japonica* and *Chinemys reevesii*. Japanese Journal of Herpetology 14:206-207.

Zietkiewicz, E., A. Rafalski and D. Labuda. 1994. Genome fingerprinting by simple sequence repeat (ISSR)-anchored polymerase chain reaction amplification. Genomics 20:176-183.