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## Recent hybrid origin of three rare Chinese turtles

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### **Abstract**

Three rare geoemydid turtles described from Chinese trade specimens in the early 1990s, *Ocadia glyphistoma*, *O. philippeni*, and *Sacalia pseudocellata*, are suspected to be hybrids because they are known only from their original descriptions and because they have morphologies intermediate between other, better-known species. We cloned the

alleles of a bi-parentally inherited nuclear intron from samples of these three species. The two aligned parental alleles of *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* have 5-11.5 times more heterozygous positions than do 13 other geoemydid species.

Phylogenetic analysis shows that the two alleles from each turtle are strongly paraphyletic, but correctly match sequences of other species that were hypothesized from morphology to be their parental species. We conclude that these rare turtles represent recent hybrids rather than valid species. Specifically, “*O. glyphistoma*” is a hybrid of *Mauremys sinensis* and *M. cf. annamensis*, “*O. philippeni*” is a hybrid of *M. sinensis* and *Cuora trifasciata*, and “*S. pseudocellata*” is a hybrid of *C. trifasciata* and *S. quadriocellata*. Conservation resources are better directed toward finding and protecting populations of other rare Southeast Asian turtles that do represent distinct evolutionary lineages.

## **Introduction**

Turtles are heavily exploited in Southeast Asia for food, traditional medicine, and pets (van Dijk et al. 2000), and now more than half of Asian species are listed as endangered or critically endangered in the wild (Stuart and Thorbjarnarson 2003; IUCN 2004). At the same time, these demands have fueled large-scale turtle farming operations in southern China (Shi and Parham 2001; Shi et al. 2004), a development that has greatly increased the potential for hybridization and genetic admixture of turtles found in trade.

During the last two decades, 14 new species of geoemydid turtles were described from China (Parham et al. 2001; Spinks et al. 2004), and these represent a significant

proportion of China's turtle diversity (Stuart and Thorbjarnarson, 2003). Most of these newly described taxa were based on specimens purchased from a Hong Kong pet dealer named Yuk Wah "Oscar" Shiu (Parham et al. 2001; Dalton 2003). Three of these species, "*Mauremys iversoni*," "*M. pritchardi*," and "*Cuora serrata*," are now known to be hybrids based on morphology, mitochondrial DNA, and nuclear DNA data (Parham et al. 2001; Wink et al. 2001; Stuart and Parham 2004; Spinks et al. 2004). Specimens matching one of these hybrids, "*M. iversoni*," have been found in Chinese turtle farms where the parental species *M. mutica* and *C. trifasciata* are housed together, and where farm operators acknowledge producing and selling the hybrids (Parham and Shi 2001; Parham et al. 2001). However, a specimen matching another of these hybrids, "*C. serrata*," has been found in the wild (Shi et al. 2005), and so multiple explanations may be required to account for the origin of all hybrid specimens (Parham et al. 2001; Shi et al. 2005). Reproductive isolating mechanisms of geoemydid turtles appear to be relatively weak. Nineteen hybridizations between geoemydid species are now documented, including between members of distantly related lineages (reviewed in Buskirk et al. 2005).

Three additional Chinese species are now suspected to be hybrids because they are known only from their original descriptions (based on specimens obtained from O. Shiu) and because they have morphologies intermediate between other well-known species (Parham and Shi 2001; Parham et al. 2001; Shi and Parham 2001; Dalton 2003; Spinks et al. 2004; Buskirk et al. 2005; Figure 1). *Ocadia glyphistoma* McCord and Iverson, 1994 was described from ten specimens (one preserved, nine living in the private collection of W. P. McCord) reported to have been purchased by O. Shiu from local

people in Guangxi Province, China. *Ocadia philippeni* McCord and Iverson, 1992 was described from nine specimens (two preserved, seven living in the private collection of W. P. McCord) reported to have been purchased by O. Shiu from local people in Hainan Province, China. *Sacalia pseudocellata* Iverson and McCord, 1992 was described from three specimens (two preserved, one living in the private collection of W. P. McCord) also reported to have been purchased by O. Shiu from local people in Hainan Province, China.

Assessing the taxonomic status of *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* is more than academic. These three species are known from only a very small number of specimens collected 15 or more years ago, are reported to be harvested by local people, and are reported to occur in southern China, an area with very high levels of turtle harvest and trade (Lau and Shi 2000). If these three species represent valid taxa, they are likely to be threatened with extinction. However, if these three species represent recent hybrids, then conservation resources should be directed away from them and toward legitimate Southeast Asian turtle taxa that are threatened in the wild (van Dijk et al. 2000; Parham et al. 2001; Stuart and Thorbjarnarson 2003; Parham et al. 2004). Currently, these three species are listed as data deficient in the 2004 IUCN Red List of Threatened Species, meaning “there is inadequate information to make a direct, or indirect, assessment of its risk of extinction” (IUCN 2004).

A recent survey of mitochondrial DNA variation in geoemydid turtles by Spinks et al. (2004) included blood samples from a specimen of *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* living in the private collection of W. P. McCord. Because mitochondrial DNA is maternally inherited, hybridization would be detected by

mitochondrial DNA only if the mother belonged to the “wrong” phylogenetic clade (Spinks et al. 2004). The sample of *O. glyphistoma* was found to have mitochondrial DNA closely related to *M. annamensis*, and the authors concluded it was a hybrid having an *M. annamensis* mother. The father was assumed to be *Mauremys* (formerly *Ocadia*; Feldman and Parham 2004; Spinks et al. 2004) *sinensis* on the basis of the morphological similarity of *O. glyphistoma* with *M. sinensis*. The mitochondrial results from the other two species were ambiguous. The mitochondrial DNA of *O. philippeni* closely matched that of “*M. iversoni*,” a known hybrid of *M. mutica* and *Cuora trifasciata*, within a clade of *Cuora*, but was distinct from another sample of *C. trifasciata*. The mitochondrial DNA of *S. pseudocellata* was closely related to *S. quadriocellata*, a well-known species (Spinks et al. 2004).

We test the recent hybrid origin hypothesis for *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* by cloning a bi-parentally inherited nuclear intron from the same blood samples studied by Spinks et al. (2004). We then reconstruct the phylogenetic position of these parental alleles within relevant geoemydid turtles. A sample of “*Cuora serrata*,” known to be a hybrid between a male *C. mouhotii* and a female *C. bourreti* (Parham et al. 2001; Stuart and Parham 2004), is included as a positive control.

## **Materials and methods**

Total genomic DNA was extracted from tissues (Table 1) using PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems, Inc.). A fragment of nuclear DNA containing an intron and part of flanking exons 1 and 2 of the RNA fingerprint protein 35 (R35)

gene was amplified by EPIC PCR (exon-primed-intron-crossing polymerase chain reaction; 94°C 45s, 54°C 30s, 72°C 1 min) for 35 cycles using the primers R35Ex1 and R35Ex2 (Fujita *et al.* 2004). PCR products of “*C. serrata*,” *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* were cloned using a TOPO TA Cloning kit (Invitrogen) to isolate the two parental alleles. Seven cloned colonies of each turtle were re-amplified. PCR products were electrophoresed in a 1% low melt agarose TALE gel stained with ethidium bromide and visualized under ultraviolet light. The bands containing DNA were excised and agarose was digested from bands using GELase (Epicentre Technologies). PCR products were sequenced in both directions by direct double strand cycle sequencing using Big Dye version 3 chemistry (Perkin Elmer). The amplifying primers and two internal primers, L-R35int (5'-AGCATTACTACATTTTGATGCAATG-3') and H-R35int (5'-CCAGCAAAGGACTCACTTGTA-3'), were used in the sequencing reactions. Cycle sequencing products were precipitated with ethanol, 3 M sodium acetate, and 125 mM EDTA, and sequenced with a 3730 DNA Analyzer (ABI). Sequences were edited and aligned using Sequencher v. 4.1 (Genecodes). The two parental alleles of “*C. serrata*,” *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* were aligned to each other to count heterozygous positions, but were maintained as separate sequences in phylogenetic analyses. The total alignment contained 1156 characters.

Phylogenies were reconstructed using the maximum parsimony and maximum likelihood optimality criteria implemented in PAUP\* 4.0b10 (Swofford 2002). Maximum parsimony analyses were performed with equal weighting of nucleotide substitutions using the branch-and-bound search option. The model of sequence evolution that best described the data was inferred using the Akaike Information Criterion as implemented in

Modeltest 3.7 (Posada and Crandall 1998). The selected model was HKY + I, with base frequencies A = 0.2801, C = 0.1816, G = 0.2054, and T = 0.3328, ti/tv ratio = 2.3601, and proportion of invariable sites = 0.7487. Maximum likelihood analyses were performed under this model using the heuristic search option, stepwise addition with 500 random addition replicates, and TBR branch swapping. Nodal support was evaluated with 1000 nonparametric bootstrapping pseudoreplications (Felsenstein 1985). The parsimony bootstrapping was limited to 5 min of branch swapping per pseudoreplication. Trees were rooted with the testudinid *Testudo hermanni*.

The two parental alleles should be closely related if the samples of “*C. serrata*,” *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* do not have a recent hybrid origin. To test the null hypotheses that these taxa do not have a recent hybrid origin, likelihood trees were reconstructed using the same model of sequence evolution but with the two parental alleles of one taxon constrained to be monophyletic in each tree. The likelihood scores of the four constrained trees were compared against the unconstrained tree using the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) with RELL optimization implemented in PAUP\* 4.0b10. Mann-Whitney U tests were performed using SPSS 13.0.

## **Results**

The two aligned parental alleles of “*C. serrata*,” *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* contained 38, 18, 10, and 23 heterozygous positions (including insertion-deletions), respectively, whereas the 22 sequences of the other 13 geoemydid species



contained 0-2 heterozygous positions. The difference in number of heterozygous positions between these two groups is highly significant ( $p < 0.001$ , Mann-Whitney U test), with or without the inclusion of the known hybrid “*C. serrata*.”

Of the 1156 aligned characters, 85 were variable and 37 were parsimony-informative. Maximum parsimony and maximum likelihood analyses recover the same hypothesis of phylogenetic relationships (Figure 2), except that one of the two equally most parsimonious trees places the clade containing *S. quadriocellata* and *S. pseudocellata* Allele B as sister to the genus *Cuora*. The two parental alleles of “*C. serrata*,” *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* are strongly paraphyletic (Figure 2). “*Cuora serrata*” has alleles closely related to *C. bourreti* and *C. mouhotii*. *Ocadia glyphistoma* has one allele closely related to *M. cf. annamensis* and one allele identical to *M. sinensis*. *Ocadia philippeni* has alleles closely related to *C. trifasciata* and *M. sinensis*. *Sacalia pseudocellata* has alleles closely related to *C. trifasciata* and *S. quadriocellata*. SH tests showed the unconstrained tree ( $-\ln L$  2261.79875; Figure 2) had a significantly better likelihood score than any of the trees constrained to have monophyletic parental alleles of “*C. serrata*” ( $-\ln L$  2293.14984,  $p = 0.005$ ), *O. glyphistoma* ( $-\ln L$  2294.07329,  $p = 0.003$ ), *O. philippeni* ( $-\ln L$  2299.61171,  $p = 0.008$ ), or *S. pseudocellata* ( $-\ln L$  2324.40705,  $p = 0.000$ ).

## Discussion

Nuclear DNA sequence data strongly support the hypothesis of a recent hybrid origin of *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata*. First, the sequences of these taxa

have 5-11.5 times more heterozygous positions than do 13 other geoemydid taxa, of which 12 are well-known species. A high number of heterozygous positions are also found in the known hybrid “*C. serrata*.” Second, the two parental alleles of *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* are more closely related (or identical) to sequences of other geoemydid species than to each other. The two parental alleles of *O. glyphistoma*, *O. philippeni*, and *S. pseudocellata* correctly match sequences of species that were hypothesized to be the parental species based on morphology (Parham et al. 2001; Dalton 2003) and, in the case of *O. glyphistoma*, on morphology and mitochondrial DNA (Spinks et al. 2004). Likewise, the two parental alleles of the known hybrid ‘*C. serrata*’ correctly match sequences of its parental species, *C. mouhotii* and *C. bourreti* (Parham et al. 2001; Stuart and Parham 2004). Using published mitochondrial DNA data from the same samples (Spinks et al. 2004) to distinguish the maternal species from the paternal species, we conclude that the sample of “*O. glyphistoma*” is the progeny of a male *M. sinensis* and a female *M. cf. annamensis*, the sample of “*O. philippeni*” is the progeny of a male *M. sinensis* and a female *C. trifasciata*, and the sample of “*S. pseudocellata*” is the progeny of a male *C. trifasciata* and a female *S. quadriocellata*. Ironically, the maternal lineage of “*O. glyphistoma*,” referred to here as *M. cf. annamensis*, is itself a Southeast Asian geoemydid of uncertain taxonomic status in need of study. These turtles are closely related to *M. annamensis* in mitochondrial DNA, but are readily distinguished from *M. annamensis* in morphology (J. J. Fong, J. F. Parham and B. L. Stuart, unpublished data). Our findings support the observation that most geoemydid hybridizations involve at least one member of the genera *Mauremys* and *Cuora*, and that hybridization can occur between these genera and the distantly related

genus *Sacalia* (Buskirk et al. 2005). The method proved simple and effective for identifying hybridization, and demonstrates a new utility for the R35 intron in turtles.

The parental species of “*O. glyphistoma*,” “*O. philippeni*,” and “*S. pseudocellata*” have overlapping geographic ranges (Iverson 1992) and are raised in Chinese turtle farms (Shi and Parham 2001; Shi et al. 2004), and consequently these hybrids may have originated in the wild or in captivity. Some authors have openly questioned the veracity of locality information provided by O. Shiu (Parham and Li 1999; de Bruin and Artner 1999; Dalton 2003; Shi et al. 2005). We treat “*O. glyphistoma*,” “*O. philippeni*,” and “*S. pseudocellata*” as invalid species, and unless they are discovered to be members of natural hybrid zones, we suggest that their conservation value is limited to serving as substitutes for wild-caught individuals of legitimate taxa in the food, traditional medicine, and pet trade (Parham and Shi 2001; Spinks et al. 2004). Conservation resources are better directed toward finding and protecting populations of other rare Southeast Asian turtle taxa that do represent distinct evolutionary lineages. For example, the box turtles *C. mccordi*, *C. picturata*, *C. yunnanensis*, and *C. zhoui* represent distinct evolutionary lineages described from pet trade and market specimens, but remain unknown in the wild (Parham et al. 2004; Stuart and Parham 2004).

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Figure 1. Images of living *Ocadia glyphistoma*, *O. philippeni*, and *Sacalia pseudocellata* modified from their original descriptions with permission of the Proceedings of the Biological Society of Washington. Each of these taxa possesses a combination of characters found in other geoemydid species. A) *O. glyphistoma* has the numerous small neck stripes (right arrow) of *Mauremys* (formerly *Ocadia*) *sinensis*, but also has the wide temporal stripes (left arrow) of the *M. annamensis*-*M. mutica* species complex. B) *O. philippeni* has the numerous small neck stripes (right arrow) of *M.* (formerly *Ocadia*) *sinensis*, but also has the yellow head, brown postorbital blotch (left arrow), and red plastron of *Cuora trifasciata*. C) *S. pseudocellata* has the diagnostic dorsal “eye spots” on its head (center arrow) and few, thick neck stripes (right arrow) of *S. quadriocellata*, but also has the yellow head, brown postorbital blotch (left arrow), and red plastron of *C. trifasciata*.

Figure 2. The single maximum likelihood tree (-ln L 2261.79875) based on 1156 aligned characters of the nuclear R35 gene using the HKY + I model of sequence evolution, base frequencies A = 0.2801, C = 0.1816, G = 0.2054, and T = 0.3328, ti/tv ratio = 2.3601, and proportion of invariable sites = 0.7487. The same topology was recovered in one of two equally most parsimonious trees (L = 97), but the alternative most parsimonious tree places the clade containing *Sacalia quadriocellata* and *S. pseudocellata* Allele B as the sister clade to the genus *Cuora*. Four hybrid taxa (bold-face) appear twice in the tree because their two parental alleles were isolated by cloning. Numbers above and below nodes are non-parametric likelihood and parsimony bootstrap values >50, respectively. Numbers in parentheses refer to ID number in Table 1.

Table 1. Samples used in this study and the GenBank accession numbers of their R35 sequences. ID refers to the terminal taxon in figure 1. Abbreviations used are FMNH = The Field Museum, Chicago; HBS = tissue collection of H. Bradley Shaffer at University of California, Davis; MTD T = Staatliches Museum für Tierkunde, Dresden; MVZ = Museum of Vertebrate Zoology, Berkeley; ROM = Royal Ontario Museum, Toronto.

ID	Species	Sample	Provenance	GenBank
1	<i>Cuora amboinensis</i>	FMNH 262239	Pet trade	DQ386653
2	<i>Cuora amboinensis</i>	FMNH 255262	Champasak Prov., Laos	DQ386654
3	<i>Cuora bourreti</i>	FMNH 261578	Pet trade	DQ386657
4	<i>Cuora bourreti</i>	FMNH 261579	Pet trade	DQ386658
5	<i>Cuora flavomarginata</i>	MVZ 230464	Zhejiang Prov., China	DQ386659
6	<i>Cuora galbinifrons</i>	FMNH 261580	Pet trade	DQ386660
7	<i>Cuora galbinifrons</i>	FMNH 256544	Khammouan Prov., Laos	DQ386661
8	<i>Cuora mouhotii</i>	MVZ 230482	Hainan Prov., China	DQ386667
9	<i>Cuora picturata</i>	FMNH 261575	Pet trade	DQ386671
10	<i>Cuora picturata</i>	FMNH 261576	Pet trade	DQ386672
11	<i>Cuora trifasciata</i>	MVZ 230636	Pet trade	DQ386679
12	<i>Cuora trifasciata</i>	MVZ 230467	Pet trade	DQ386680
13	<i>Cuora trifasciata</i>	MTD T 1082	Pet trade	DQ386681
14	<i>Mauremys annamensis</i>	MVZ 230462	Market in Hainan Prov., China	DQ386655
15	<i>Mauremys annamensis</i>	FMNH 262238	Pet trade	DQ386656
16	<i>Mauremys cf. annamensis</i>	MVZ 230476	Market in Hainan Prov., China	DQ386664

17	<i>Mauremys cf. annamensis</i>	MVZ 230477	Turtle farm in Hainan Prov., China	DQ386665
18	<i>Mauremys cf. annamensis</i>	ROM 25614	Trader in Yen Bai Prov., Vietnam	DQ386668
19	<i>Mauremys mutica</i>	MVZ 230487	Zhejiang Prov., China	DQ386666
20	<i>Mauremys sinensis</i>	MVZ 230479	Hainan Prov., China	DQ386678
21	<i>Sacalia quadriocellata</i>	FMNH 256542	Khammouan Prov., Laos	DQ386675
22	<i>Siebenrockiella crassicollis</i>	FMNH 259055	Koh Kong Prov., Cambodia	AY954913
23	<i>Testudo hermanni</i>	MVZ 244866	Edirne Prov., Turkey	DQ386652
24	“ <i>Cuora serrata</i> ”	MVZ 230628	Pet trade	DQ386676-77
25	“ <i>Ocadia glyphistoma</i> ”	HBS 38414	Pet dealer in Hong Kong, China	DQ386662-63
26	“ <i>Ocadia philippeni</i> ”	HBS 38400	Pet dealer in Hong Kong, China	DQ386669-70
27	“ <i>Sacalia pseudocellata</i> ”	HBS 38432	Pet dealer in Hong Kong, China	DQ386673-74

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