

## A morphological review of the *Cuora flavomarginata* complex (Testudines: Geoemydidae)

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*Abstract.*—A reevaluation of the morphometric and color pattern differences within the Asiatic box turtle, *Cuora flavomarginata* sensu latu, was conducted in view of determining the taxonomic position of the three currently recognized subspecies: *C. f. flavomarginata* (Taiwan), *C. f. sinensis* (southern mainland China), and *C. f. evelynae* (Ryukyu Islands, Japan). Recent analyses indicate that the allopatric population of *C. f. evelynae* is the most divergent of the three taxa and shares little possibility for gene exchange with the other two populations. In contrast, the populations of *C. f. flavomarginata* and *C. f. sinensis* share many characters. We recommend the recognition of the Ryukyu population as a full species, *C. evelynae*.

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The Asian box turtle, *Cuora flavomarginata*, is currently considered polytypic, consisting of three subspecies: *Cuora flavomarginata flavomarginata* (Gray, 1863) of Taiwan; *C. f. sinensis* (Hsü, 1930) of southern mainland China (Fong et al. 2002); and *C. f. evelynae* Ernst & Lovich, 1990, of the Japanese Ryukyu Islands (Ernst et al. 2000, Hennen 2004, Schilde 2005, Bonin et al. 2006, Fritz & Havaš 2007). The validity of *C. f. sinensis* and the taxonomic rank of *C. f. evelynae*, both of which were described as full species, have been questioned (McCord & Iverson 1991, Zhao & Adler 1993, Fritz & Obst 1999, Yasukawa & Ota 1999, Fritz & Havaš 2007).

The goal of this study is to examine morphometric and color pattern differences between the three populations to reevaluate the taxonomic status of three geographic disjunct populations. We now

possess significantly larger samples of the three separate populations of *C. flavomarginata* sensu latu than in our former analysis (Ernst & Lovich 1990). Our results and those of other studies are presented below.

### Materials and Methods

A total of 125 turtles were examined (Ryukyu Islands, 38; Taiwan, 50; southern mainland China, 37; see Appendix 1). Notes and drawings were made of head, neck, limb, carapacial and plastral patterns of each specimen. Sexes were determined by the characters given by Ernst & Barbour (1989) and Ernst et al. (2000). Straight-line measurements of each specimen were taken with dial calipers accurate to 0.1 mm. Variables included: the greatest carapace length (CL, not midline), carapace width (CW) and depth (CH) at the level of the seam separating vertebral scutes 2 and 3,

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marginal width (MW, the difference between the CW and the width across the carapace taken between the points of juncture of the marginal scutes and pleurals at the level of the seam between vertebrals 2 and 3), greatest plastron length (PL), greatest width and length of both plastral lobes (APW, APL, PPW, PPL), greatest length of the right shell bridge (BL), greatest width and length of the cervical scute (CSW, CSL) and all vertebrals ( $V_1W$ ,  $V_1L$ , etc.), and the medial seam lengths of all plastral scutes (Gul., Hum., Pect., Abd., Fem., An.).

Shell proportions were expressed as ratios of one measurement to another. Several ratios proved useful: width/length of cervical scute, width/length of designated vertebrals, MW/CL, CH/CL, CH/CW, length of light-colored blotch at its medial height on pleural scute 2/total length of pleural scute 2 at the same point (PBL/PSL), length of the light-colored blotch at its medial height on pleural 2/carapace length (PBL/CL), PL/CL, BL/PL, APL/PL, APW/PL, APW/APL, PPL/PL, PPW/PL, and PPW/PPL. The number of rows of large scales at the lateral edge of the antibrachium between the knuckle joint of digit 5 and the first horizontal skin fold proximal to the elbow (FLSR, mistakenly described in Ernst & Lovich 1990 as the scale rows between the claw of digit 5 and the first horizontal skin fold) were counted and recorded.

Only adult turtles (those that displayed secondary sexual characteristics) were used in the analyses. Data of both sexes were combined for further analysis as no characters displayed significant differences between females and males (adjusted for region). Effects due to sexual dimorphism were also minimized since turtles on the Ryukyus and Taiwan appear to have similar levels of size dimorphism (Gibbons & Lovich 1990). Statistical techniques were executed using SAS package 8.2. Levels of significance were set a priori at  $\alpha = 0.05$ .

Continuous variables were checked for normality using q-q plots (Conover 1980). As a result, most were judged to be approximately normal. These variables were then used in a stepwise discriminant function analysis, which identified the variables used in subsequent analyses.

Because FLSR was deemed significantly different in Ernst & Lovich (1990) but not used by McCord & Iverson (1991), this trait was analyzed separately using a Kruskal-Wallis test. Because sample differences were highly significant, multiple comparisons (Conover 1980) were conducted to look for significant differences among all three populations.

## Results

The results of the discriminant function analysis (resubstitution) clearly showed that the three populations were morphologically distinct, and a follow up cross-validation analysis confirmed this. The first two canonical variables (from the resubstitution results) are plotted in Fig. 1 and show the three populations to be basically separated, with very minor overlap between the Ryukyu and China populations. Only one outlier causes the major polygon overlap between the China and Ryukyu Island populations. This specimen has not been identified but may have erroneous locality data associated with it; even with this specimen included, the overlap is no more than expected in such a genetically related complex. Some overlap, possibly due to more recent gene flow, occurs between Taiwan and China. This result is also supported by a MANOVA performed as part of the discriminant function analysis.

Univariate results generally confirm the results of the discriminant analysis, although APW and CH showed no significance. This result is not unexpected as multivariate statistics often show significant differences when univariate statistics on the same data do not (Johnson &

Results using stepwise selected variables, both sexes

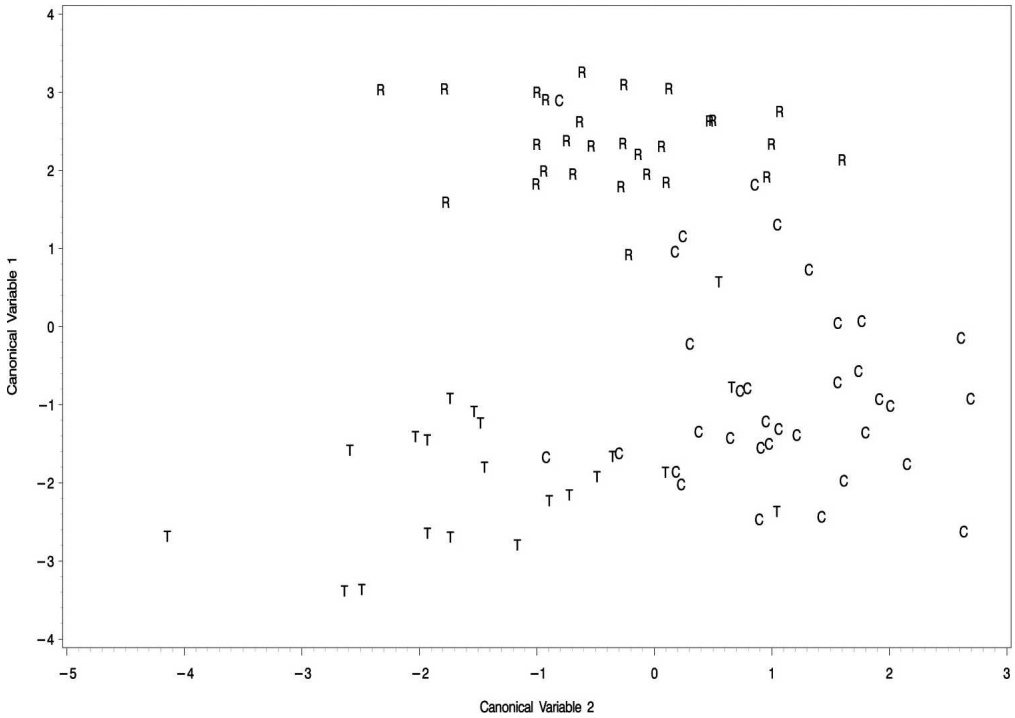


Fig. 1. Discriminant function analysis (resubstitution) using the first two canonical variables of individuals (sexes combined) of *Cuora* populations from southern mainland China (C), the Ryukyu Islands (R), and Taiwan (T).

Wichern 1998). The two most important univariate characters were PBL/PSL (Ryukyu Islands:  $\bar{X} = 64.9$  [58.0–73.7] %; Taiwan:  $\bar{X} = 43.0$  [24.0–69.6] %; China:  $\bar{X} = 38.7$  [31.9–51.1] %) and FLSR (Taiwan:  $\bar{X} = 9.7$  [6–13]; Ryukyu Islands:  $\bar{X} = 8.8$  [6.5–11]; China:  $\bar{X} = 8.3$  [6–10]).

Morphological and pattern data were compared by locality (mainland China, Taiwan, and the Ryukyu Islands) to determine if geographic variation occurs, and to what extent. Univariate test results indicate that PBL/PSL and FLSR were significantly different for the combined and each individual sex samples ( $p = 0.001$ ); in addition, Abd ( $p = 0.01$ ) and PPL ( $p = 0.004$ ) were also significantly different in females, but not for males or for combined sex data. A NPAR1WAY procedure (Table 1) yielded Wilcoxon

scores (rank sums) for FLSR (Kruskal-Wallis Test chi-square 18.07,  $p = 0.0001$ ) that differed significantly. Results of the discriminant analysis are shown in Fig. 1.

Classification by cross validation of combined data from both sexes for the three geographic areas is presented in Table 2. For the Ryukyu sample, 100% of males and 77.8% of females were classified correctly (females, 22.2% as mainland China). Data from mainland China yielded a correct female identification of 57.1% (42.9% as Taiwan), and 85.2% of Taiwanese females were identified correctly (11.1% as mainland China, 3.7% as Ryukyu Islands). Male data from both mainland China and Taiwan yielded 71.4% correct identifications and 28.6% as from the Ryukyu Islands.

Table 1.—Wilcoxon scores (rank sums) for the character FLSR classified by population (Ryukyu Islands, southern mainland China, and Taiwan).

Population ( <i>n</i> )	Sum of scores	Expected under HO	Standard deviation under HO	Mean score
Ryukyu (28)	1114.0	1190.0	102.4	39.8
China (21)	561.5	892.5	94.1	26.7
Taiwan (35)	1894.5	1487.5	107.1	54.1

### Discussion

Discriminant function analysis with cross validation of combined sex data is presented in Table 2. The same test of the same data set but testing each sex separately, properly classified 100% of males and 77.8% of females from the Ryukyu Islands (presently, *Cuora f. evelynae*); 71.4% of males, and 57.1% of females from southern mainland China (*C. f. sinensis*); and 71.4% of males and 85% of females from Taiwan (*C. f. flavomarginata*). Males are less variable than females.

The populations of *C. flavomarginata* from mainland China and Taiwan share enough similarities among the characters to have both males and females incorrectly assigned to the other population or the Ryukyus (however, only 3.7% of Taiwanese females and no females from southern mainland China were identified as from the Ryukyu population). The morphological overlap in the Chinese and Taiwanese populations might result from proximity and relatively recent gene exchange, and strengthens the interpretation that the two populations represent a single subspecies, *C. f. flavomarginata* (Zhao & Adler 1993, Fritz & Obst 1999, Yasukawa & Ota 1999, Fritz & Havaš

2007). In contrast, the more distant Ryukyu population appears less variable, and it is widely separated morphologically from the other two populations.

A plot of the discriminant analysis (resubstitution) using the first two canonical variables of the total adult sample from the three populations (Fig. 1) indicates a sharing of characters between mainland China and Taiwan turtles, but essentially none, except one distant Chinese outlier (see above), between the Ryukyu population and mainland China, and no character overlap between turtles from the Ryukyus and Taiwan.

These results support the concept that the populations from mainland China and Taiwan represent one taxon, and that *C. f. sinensis* (Hsü, 1930) is a junior synonym of *C. f. flavomarginata* (Gray, 1863), as recognized by McCord & Iverson (1991), Zhao & Adler (1993), Fritz & Obst (1999), Yasukawa & Ota (1999), and Fritz & Havaš (2007).

The taxonomic status of the Ryukyu Islands population, *C. f. evelynae*, is another matter. McCord & Iverson (1991) thought elevation of the Ryukyu population to full specific status by Ernst & Lovich (1990) was “unwarranted,” and that it “perhaps” represented a subspecies of *C. flavomarginata*. They did not

Table 2.—Classification by cross validation of individual turtles from the Ryukyu Islands, southern mainland China, and Taiwan.

Population ( <i>n</i> )	Taxon frequency		
	Ryukyu	China	Taiwan
Ryukyu (28)	25 (89.3%)	0	3 (10.7%)
China (21)	0	17 (81.0%)	4 (19.1%)
Taiwan (35)	2 (5.9%)	7 (28.9%)	25 (73.5%)

include in their analysis all characters used in the original diagnosis by Ernst & Lovich (1990). They also criticized Ernst & Lovich (1990) for including juveniles in their original analyses; our present analyses use only adult *Cuora*. Subsequent study of morphological variation within *Cuora flavomarginata* by Yasukawa (1998) and Yasukawa & Ota (1999) demonstrate that *C. f. evelynae* is the most divergent of the three described subspecies.

A study of variations in 882 base positions of mitochondrial DNA (mtDNA) 12S and ribosomal RNA (rRNA) 16S genes of Asian *Cuora* by Honda et al. (2002) also revealed that Ryukyu *C. f. evelynae* are genetically distinct from the Taiwan population of *C. flavomarginata*. Both groups were shown to be monophyletic in three separate cladistic analyses: a neighbor-joining dendrogram derived from the distance matrix from mtDNA 12S and rRNA 16S sequence data, a maximum-likelihood dendrogram, and a maximum parsimony dendrogram using heuristic option. In addition, two additional recent studies also reported genetic differences between the populations from China and Ryukyus. Spinks et al. (2004), use protein coding cytochrome b, mtDNA, 12 s ribosomal RNA, and a ~1 kb intron from the R35 neural transmittter gene. Spinks & Shaffer (2006), although concentrating on other species of *Cuora*, use cytochrome c oxidase subunit I mtDNA, the nicotinamide adenine dinucleotide dehydrogenase subunit 4 gene, plus the flanking histidine and serine tRNAs and part of the leucine tRNA in their sequencing. Unfortunately, the turtle sample sizes in all three of these studies are small, but differences between the populations tested were indicated in every test.

Our data clearly strengthen the observations of Yasukawa (1998), Yasukawa & Ota (1999), Honda et al. (2002), Spinks et al. (2004), and Spinks & Shaffer (2006) and further indicate that the *Cuora* on the Ryukyu Islands should be considered a

separate species. In addition, the Ryukyu turtles are geographically separated (allopatric) and have been at least since the mid-Pleistocene (Inger 1947, Ota 1998, 2003; Takahashi et al. 2003). Currently, natural gene exchange is highly unlikely with either the Taiwan or mainland China populations of *C. flavomarginata*, adding to the argument for specific status.

Discussions about what constitutes a "species" flourished in scientific journals during the last two decades, occasionally mired in both theoretical and operational conceptualizations and criteria (Frost & Hillis 1990). Alternative species explanations have been proposed, including the biological, evolutionary, phylogenetic and unified species concepts, all of which define species as separately evolving metapopulation lineages (De Queiroz 2005, 2007). As reiterated by Berry et al. (2002), the species is the fundamental unit of evolution, forged through separation of ancestral lineages by mechanisms including vicariance, dispersal, ecology, or behavior. We made no attempt in our analysis to recover the phylogenetic history of this complex. However, our morphological analyses once again (Ernst & Lovich 1990) demonstrate the existence of internally cohesive, diagnosable taxonomic units with actual or potential gene flow occurring between some but not other metapopulations: a characteristic of the evolutionary species concept (Berry et al. 2002). The congruence of our findings with those of independent researchers using molecular techniques provides additional strong support for recognition of *C. evelynae* as a separate species. Similar to Frost & Hillis (1990), we do not believe that recognition of subspecies in this complex advances our understanding of variation in the *Cuora flavomarginata* complex. We, therefore, recommend that *Cuora flavomarginata evelynae* Ernst & Lovich, 1990, be reinstated as a full species, *Cuora evelynae* Ernst & Lovich, 1990.

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#### Appendix I

##### Turtles of the *Cuora flavomarginata* Complex Examined

Specimens (125) from the following collections were examined: American Museum of Natural History, New York (AMNH); California Academy of Sciences, San Francisco (CAS); Carl H. Ernst, personal collection (CHE); Field Museum of Natural History, Chicago (FMNH); George Mason

University, Fairfax, Virginia (GMU); Harvard University Museum of Comparative Zoology, Cambridge (MCZ); John F. McBreen, personal collection (JFM); William P. McCord, personal collection (MPM); Peter Miller, personal collection (PM); Museum National d'Histoire Naturelle, Paris (MNHN); Naturhistorisches Museum Wien, Vienna (NHMW); Natur-Museum und Forschungs-Institut Senckenberg, Frankfurt (SMF); Staatliches Museum für Tierkunde, Dresden (MTKD); Jerry J. Stemler, personal collection (JJS); The Natural History Museum, London (BMNH); National Museum of Natural History, Washington, D.C. (USNM); and William H. Randel, personal collection (WHR). The specimens examined are listed below by geographic region. Numbers in parentheses following the collection number indicate multiple specimens catalogued under that number.

Ryukyu Islands, Japan (38): AMNH 50804; CAS 26102–26124, 26026–26029, 26801; GMU 730–731; MCZ 7997, 56064; NMW 29513; USNM 34076–34079.

Southern mainland China (37): AMNH 110181; BMNH 1947-3.4.50, 1983.58; CHE 500; FMNH 216515; JJS 1–4; MTKD 24689–24690, 29011–29012, 30703; NMW 5914, 30700(2); PM 1; SMF 69683, 70506; USNM 32103–32107; WPM 100–104; WHR 1–7.

Taiwan (50): BMNH 1922.6.16.8–1922.6.1.6.11, 1947.3.5.68; CAS 18034–18040; FMNH 121224–121226; JFM 1–2; MNHN 1988–2063; MTKD 31164; NMW 1784, 29218, 29515(3), 29516(2), 29517(4), 29518(3), 29519(2), 29520(4), 29521(5), 29522(3); SMF 34342, 64638–64639; USNM 140825.