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New microsatellite markers allow high-resolution taxon delimitation in critically endangered Asian box turtles, genus *Cuora*

Ralph Tiedemann[1], Anja R. R. Schneider[1], Katja Havenstein[1], Torsten Blanck[2], Elmar Meier[3], Martina Raffel[4], Henk Zwartepoorte[5] & Martin Plath[6]

[1)] University of Potsdam, Institute for Biology and Biochemistry, Unit of Evolutionary Biology/Systematic Zoology, Karl-Liebknecht-Str. 24–25, Haus 26, 14476 Potsdam, Germany

[2)] Cuora Conservation Center, 8530 Deutschlandsberg, Austria

[3)] International Centre for the Conservation of Turtles (IZS), c/o Allwetterzoo Münster, Sentruper Str. 315, 48161 Münster, Germany

[4)] Allwetterzoo Münster, Sentruper Str. 315, 48161 Münster, Germany

[5)] Rotterdam Zoo, Blijdorplaan 8, 3041 JE Rotterdam, The Netherlands

[6)] Goethe University Frankfurt, Institute for Ecology, Evolution & Diversity, Unit of Evolutionary Ecology, Max-von-Laue-Str. 13, 60438 Frankfurt, Germany

Corresponding author: Ralph Tiedemann, e-mail: tiedeman@uni-potsdam.de

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Abstract. We isolated and characterized 16 new di- and tetranucleotide microsatellite markers for the critically endangered Asian box turtle genus *Cuora*, focusing on the "*Cuora trifasciata*" species complex. The new markers were then used to analyse genetic variability and divergence amongst five described species within this complex, namely *C. aurocapitata* (n = 18), *C. cyclornata* (n = 31), *C. pani* (n = 6), *C. trifasciata* (n = 58), and *C. zhoui* (n = 7). Our results support the view that all five species represent valid taxa. Within two species (*C. trifasciata* and *C. cyclornata*), two distinct morphotypes were corroborated by microsatellite divergence. For three individuals, morphologically identified as being of hybrid origin, the hybrid status was confirmed by our genetic analysis. Our results confirm the controversial species (*Cuora aurocapitata*, *C. cyclornata*) and subspecies/morphotypes (*C. cyclornata meieri*, *C. trifasciata* cf. *trifasciata*) to be genetically distinct, which has critical implications for conservation strategies.

Key words. Testudines, *Cuora*, box turtles, captive breeding, conservation units, microsatellites.

Introduction

Asian box turtles of the genus *Cuora* are small to medium-sized species (11–35 cm straight carapace length) that inhabit rainforests in South and East Asia (Fig. 1 shows the morphology and distribution of the species investigated in this paper). Habitat destruction and collection for consumption or as pets are the primary threats to these semi-aquatic freshwater turtles (Turtle Conservation Coalition 2011) and all but one species (*C. amboinensis*) are now listed as 'critically endangered' in the IUCN Red List of Threatened Species (IUCN 2012). Two species, *C. mccordi* and *C. zhoui*, might already have become extirpated in the wild (Turtle Conservation Coalition 2011).

Up to 13 species have been described, but taxonomic uncertainty persists, hampering conservation efforts. Studies on mitochondrial DNA (mtDNA) provided a first taxonomic framework (e.g., Stuart & Parham 2004, Spinks et al. 2004, Parham et al. 2004), but later work using a few nuclear loci suggested that some putative species could be hybrids (Stuart & Parham 2007). High-resolution nuclear markers (like microsatellites) were not previously available for the genus, however, even though these are essential tools for species delimitation and the identification of hybrids, both of which are paramount for conservation projects and legislation. It is also important to develop analytical tools by which confiscated turtles or animals in captive breeding programs can be traced back to their population of origin.

Taxonomic uncertainty has plagued conservation efforts, especially in the *C. trifasciata* species complex. Morphological and mtDNA sequence divergence suggest the existence of two species within what was once considered *Cuora trifasciata* (Blanck et al. 2006): *C. trifasciata* sensu stricto ('clade A') and *C. cyclornata* ('clade C'), but the validity of *C. cyclornata* has been challenged based on

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mtDNA and nuclear single copy loci sequences (Spinks & Shaffer 2007, Spinks et al. 2009, Spinks et al. 2012). Within *C. cyclornata*, Blanck et al. (2006) detected two divergent lineages, which they described as the subspecies *C. c. cyclornata* and *C. c. meieri*. Furthermore, several specimens of unknown origin formed yet another genetically divergent *C. trifasciata* clade ('clade B'), but clades A and B were indistinguishable based on the low-resolution nuclear markers used thus far (Spinks & Shaffer 2007). Even the most recent study (Spinks et al. 2012), which included sequences of 15 nuclear single-copy loci, could not resolve these issues.

Yet another level of taxonomic uncertainty concerns *C. aurocapitata* and *C. pani*, which – although distinguishable by morphology and some nuclear loci (Spinks et al. 2012) – show extensive mt-haplotype sharing (Parham et al. 2004, Spinks et al. 2004). This suggests that they interbreed in secondary contact zones, e.g., in western Anhui, southern Henan or eastern Hubei Province, from where a phenotypically intermediate variety is known (Blanck & Tang 2005, T. Blanck pers. obs.).

Microsatellite analysis is an elegant tool to assess the degree of reproductive isolation, delimit taxonomic units, and detect potential hybridisation between taxa. The aims of this study were (1) to develop a new informative set of highly polymorphic, biparentally inherited molecular markers (i.e., microsatellites) for *Cuora* spp., and (2) to evaluate the degree of reproductive isolation among the distinguishable morphotypes of the *C. trifasciata* species complex. Here, we perform a genetic evaluation of the prediction of the Biological Species Concept (BSC) among the a priori defined morphotypes, i.e., should diagnosable morphotypes occur in sym- or parapatry, then significant genetic divergence among these morphotypes indicates reproductive isolation. We are aware of the fact that genetic distinctiveness between allopatric populations does not necessarily preclude that these populations could in principle interbreed (Milinkovitch et al. 2001).

Material and methods

We obtained blood and tissue samples from 120 *Cuora* specimens, morphologically assigned to 5 focal species, i.e., *C. aurocapitata* (n = 18), *C. cyclornata* (n = 31), *C. pani* (n = 6), *C. trifasciata* (n = 58), and *C. zhoui* (n = 7; Fig. 1). Our samples included the holotypes of *C. trifasciata* and *C. cyclornata* (see Supplementary material).

Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen). Four microsatellite-enriched genomic DNA libraries were constructed from a total DNA extract of a *C. trifasciata* sample following Paulus & Tiedemann (2003): genomic DNA was simultaneously restricted with NheI, HaeII, RsaI, and HaeIII. After treatment with mung bean exonuclease and calf intestinal phosphatase, genomic DNA fragments were blunt-end legated to SNX-linkers and PCR-amplified with linker primers. Fragments were hybridised with 5'-biotin-labelled microsatellite probes ($[GA]_{15}$, $[GT]_{15}$, $[ACTG]_6$, $[ACAT]_6$), conjugated with streptavidin-coated magnetic beads (Dynal), and extracted with a magnetic device. PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). PCR products of recombinants were blotted onto a nylon membrane and again hybridised with the microsatellite probes. Positive

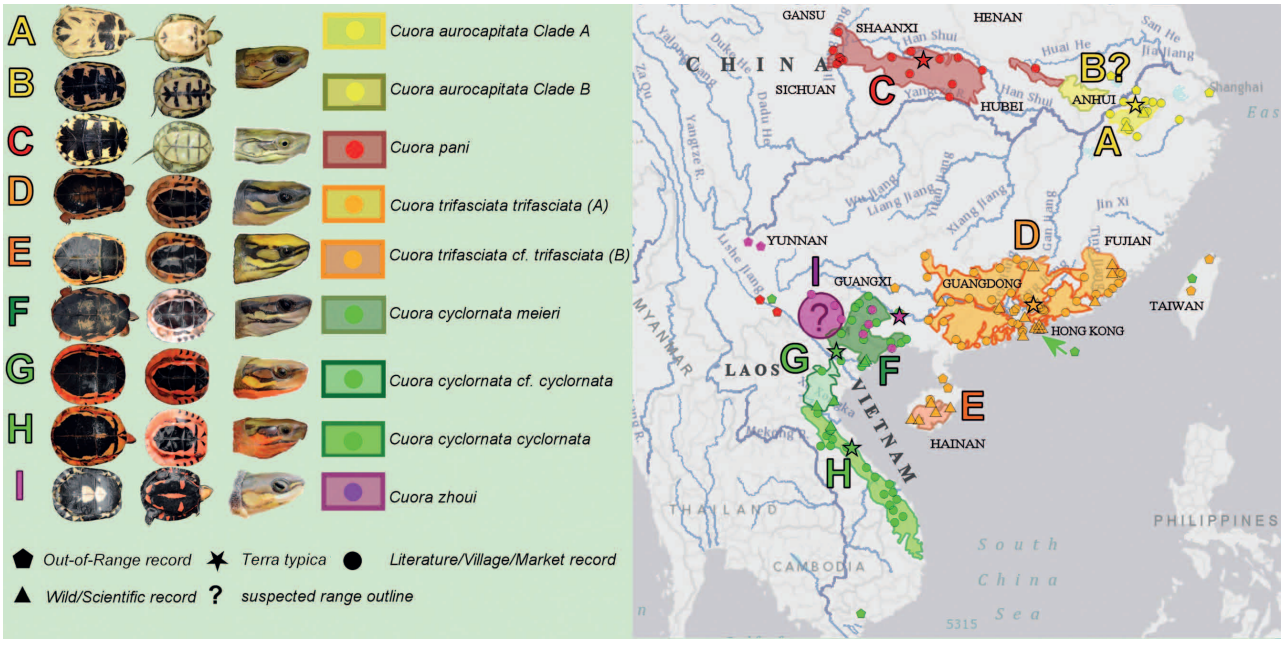

Figure 1. Colouration (left to right: carapace dorsal, carapace ventral, head lateral), distribution, and sample origins of the analysed *Cuora* taxa.

clones were detected using the Phototope-Star chemoluminescent detection kit (New England BioLabs), purified with Exonuclease I and Antarctic Phosphatase (New England BioLabs), sequenced with the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems), and analysed on an ABI Prism 3130 multicapillary automatic sequencer (Applied Biosystems). Primers were designed in microsatellite flanking regions, and 16 primer pairs showed reliable amplifications in all 5 focal species.

The variability of microsatellite loci was analysed by PCR using 5'-fluorescence-labelled forward primers. 25 µl-reaction volumes (buffer solution: 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X100, 0.2 mg/ml BSA) were prepared as follows: 2.5 µl dNTP mixture (2.0 mM each), 0.5 µl of each primer (10 µM), 1 µl DNA template (~20 ng), and 0.1 µl Taq polymerase (5 U/µl, MP Biomedicals). Cycling parameters included initial denaturation at 94°C for 5 min, 5 cycles at 94°C for 30 s, an elevated locus-specific annealing temperature T_a+3°C for 1 min, 72°C for 45 s; 35 cycles at 94°C for 30 s, T_a (Tab. 1) for 1 min, 72°C for 45 s, and a final extension at 72°C for 10 min. Fragment sizes were determined on an ABI Prism 3130 multicapillary automatic sequencer, using the GeneMapper v 4.0 software and an internal size standard (LIZ 500, Applied Biosystems, 250 bp peak omitted).

We conducted an Analysis of Molecular Variance (AMOVA) to test for molecular divergence among *Cuora* species and calculated pairwise F_{ST} values using ARLEQUIN (Excoffier & Lischer 2010); significance was evaluated based on 1,000 permutations. Locus-wise F_{ST} values were assessed with GENALEX 6 (Peakall & Smouse 2006). ARLEQUIN was also used to calculate observed (H_O) and expected (H_E) heterozygosity and to test for deviation from the Hardy-Weinberg equilibrium (HWE) for each locus in each population using Fisher's exact test and the Markov Chain method (1,000 demorisation steps, 100 batches, with 10,000 iterations per batch set). Alpha-levels of HWE and linkage disequilibrium tests were Bonferroni-corrected for multiple comparisons.

A Bayesian clustering was performed using the software STRUCTURE v 2.3.2 (Pritchard et al. 2000). Genetic subdivision was evaluated by estimating the likelihood and sample composition of independent runs of subgroups (k = 2–9), assuming an admixture model, with 100,000 iterations discarded as burn-in, and a collection period of 900,000 iterations. To check for convergence of the Markov Chain parameters, 15 replicate runs for each value of k were performed. To estimate relationships among individuals of different geographic origin, we constructed a Neighbour Joining (NJ) tree based on individual Nei's minimum genetic distances and 100 bootstraps over loci (D_m; Nei 1987) using POPULATIONS v 1.2.28 (Langella 2001).

Results and discussion

Our 16 new microsatellites represent the first loci specifically developed for *Cuora* spp. All loci amplified successfully throughout morphologically distinguishable taxa. Locus-wise allele numbers ranged from one to 22 alleles per taxon (Tab. 1); no locus was monomorphic in all species though, and no locus was consistently heterozygote-deficient across species. Genetic variability was high in *C. trifasciata* (mean H_E = 0.735), *C. cyclornata* (0.698) and *C. aurocapitata* (0.621), but low in *C. zhoui* (0.399) and *C. pani* (0.410), alluding to differences in the remaining effective population size (estimates range from 'possibly extinct in the wild' for *C. zhoui*, fewer than 100 specimens for *C. aurocapitata*, to fewer than 1,000 specimens for *C. cyclornata*, *C. trifasciata* and *C. pani*; Turtle Conservation Coalition 2011). The low genetic variability in *C. pani* can be explained by the majority of samples of all other species stemming from wild specimens, while *C. pani* samples mostly came from captive-bred animals where there was a possibility that some individuals could be related. Note that estimates of genetic diversity in *C. zhoui* and *C. pani* are associated with a higher statistical error due to the small sample size. In the case of C. *trifasciata*, consistent heterozygote deficiency (at 14 of 18 loci) hints at the presence of a hidden genetic substructure, leading to a Wahlund effect (see below).

The AMOVA indicated substantial genetic differentiation across taxa, as 27.8% of the variance was apportioned to between-species differences (i.e., global F_{ST} = 0.278). This divergence was also reflected by locus-wise F_{ST} values between 0.323 and 0.732 – remarkably high values for microsatellites. Pairwise F_{ST} values showed highly statistically significant differences amongst all species, ranging from 0.112 (*C. trifasciata* vs. *C. cyclornata*) to 0.628 (*C. pani* vs. *C. zhoui*; Tab. 2). STRUCTURE retrieved k = 5 and k = 7 to have the highest likelihoods; these partitions are in good agreement with the morphological assignment (Fig. 2). For k = 5, five morphospecies (*C. aurocapitata*, *C. cyclornata*, *C. pani*, *C. trifasciata*, and *C. zhoui*) formed well-supported clades to which most specimens were assignable with high likelihoods of 80 to 100%, including the holotypes of *C. trifasciata* and *C. cyclornata* (Fig. 2).

While our findings are generally in agreement with the results of previous studies of the genus *Cuora* (Spinks & Shaffer 2007, Spinks et al. 2009, 2012), they provide additional resolution for the morphospecies *C. cyclornata* (green in Fig. 2) and *C. trifasciata* (orange), which were clearly supported as distinct units by our microsatellite analysis, while previous studies concluded them to be genetically indistinguishable. This is perfectly congruent with their geographic isolation (Fig. 1) and validates the morphological species assignment by Blanck et al. (2006). Only a few samples represent putative hybrids; all of them were of captive origin (Fig. 2).

If we evaluate the results of the STRUCTURE analysis for k = 7 (Fig. 2), further genetic structure within the two major clades becomes apparent: within *C. cyclornata*, two of the three morphologically identified subclusters match the subspecies *C. c. meieri* and *C. c. cyclornata* described by Blanck et al. (2006) and include the respective holotypes, although there is a mismatch for a single individual between morphological (*C. c. cyclornata*) and genetic (*C. c.*

Table 1. Characteristics of 16 microsatellites in the five *Cuora* species investigated herein.

Locus	Forward primer 5'–3' Reverse primer 5'–3'	Primer label	Repeat sequence	T_a	Characteristics in *Cuora* spp.							
					Species	n	Allele No.	Allele size	H_O	H_E	P	F_{ST}
CuofasGAI_D7 GenBank Acc. No. KJ191267	GTCAGCAAAAATCTTCAGAGGG GCTCCCACTAAAGTAAATGCC	FAM	$(GA)_{13}$	56.5	*C. aurocapitata*	12	13	104 –170	0.583	0.913	0.003	0.096
					C. cyclornata	27	16	110–166	0.556	0.834	< 0.001	
					C. pani	6	4	110–130	0.667	0.636	0.211	
					C. trifasciata	54	19	110–160	0.704	0.859	< 0.001	
					C. zhoui	6	5	108–132	0.500	0.742	0.009	
CuofasGAI_C4 GenBank Acc. No. KJ191265	GAATTTGCCTATTTCGTAGGGG GTGTTTGATTTGCTCTTTTGCC	PET	$(GA)_{14}$	56.5	*C. aurocapitata*	16	8	200–226	0.625	0.696	0.140	0.130
					C. cyclornata	29	18	198–290	0.793	0.933	< 0.001	
					C. pani	6	3	200–214	0.333	0.318	1.000	
					C. trifasciata	58	15	198–226	0.672	0.890	< 0.001	
					C. zhoui	6	2	204–206	0.167	0.167	1.000	
CuofasGTI_D7 GenBank Acc. No. KJ191274	GCCTATAAACTACAGCCTGGG GAAAGGCCTGATCTTACTTCCA	VIC	$(GA)_{19}$	56.5	*C. aurocapitata*	18	2	328–336	0.444	0.489	1.000	0.224
					C. cyclornata	28	8	326–352	0.571	0.804	0.006	
					C. pani	6	2	336–340	0.667	0.545	1.000	
					C. trifasciata	55	11	326–354	0.636	0.820	0.028	
					C. zhoui	7	1	328	–	–	–	
CuofasGTI_F5 GenBank Acc. No. KJ191276	GTTAAAAAGGCCCAAATCAAAT GTCATTTCTCCAGCAACTGTCC	FAM	$(GT)_{11}$	56.5	*C. aurocapitata*	16	4	448–460	0.188	0.573	< 0.001	0.236
					C. cyclornata	31	6	448–476	0.452	0.600	0.027	
					C. pani	5	4	448–454	0.400	0.644	0.238	
					C. trifasciata	57	6	446–474	0.368	0.585	0.001	
					C. zhoui	7	2	448–470	0.143	0.143	1.000	
CuofasGTI_F8 GenBank Acc. No. KJ191277	GAGGGAGGCTAGGGTATGCTAT GAATCCTGTTAATTTTTGCTGA	FAM	$(GT)_{10}$	55	*C. aurocapitata*	17	1	120	–	–	–	0.396
					C. cyclornata	28	11	124–182	0.643	0.791	0.013	
					C. pani	6	1	120	–	–	–	
					C. trifasciata	57	8	120–150	0.526	0.745	< 0.001	
					C. zhoui	6	1	128	–	–	–	
CuofasGAI_F6 GenBank Acc. No. KJ191268	GTGAAAATAAACTGCCCTGGA GTAGCAGTTCAGTGTAGCGGGG	PET	$(GA)_{19}$	55	*C. aurocapitata*	16	8	165–211	0.750	0.728	0.223	0.144
					C. cyclornata	29	16	189–237	0.724	0.892	< 0.001	
					C. pani	6	3	167–191	0.500	0.621	0.395	
					C. trifasciata	55	14	171–221	0.418	0.808	< 0.001	
					C. zhoui	6	3	173–203	0.833	0.682	0.656	
CuofasGTI_B10 GenBank Acc. No. KJ191273	GTTCCCTTTTTTGAAAATTATT GCAAACAAACAAAAAGAAAAAA	FAM	$(GT)_{11}$	50	*C. aurocapitata*	18	9	119–151	0.667	0.830	0.117	0.170
					C. cyclornata	30	10	125–171	0.767	0.805	0.228	
					C. pani	6	2	131–133	0.167	0.167	1.000	
					C. trifasciata	57	20	125–169	0.860	0.904	0.135	
					C. zhoui	7	2	123–127	0.571	0.527	1.000	
CuofasGAII_A5 GenBank Acc. No. KJ191262	GGGAGAGCCCTTAACCAATAT GTCTGTGTCAACTTGCCTTTCC	VIC	$(GA)_{14}$	56.5	*C. aurocapitata*	17	3	154–158	0.529	0.629	0.593	0.273
					C. cyclornata	29	7	156–168	0.655	0.783	0.025	
					C. pani	6	2	154–156	0.167	0.167	1.000	
					C. trifasciata	56	9	160–176	0.304	0.681	< 0.001	
					C. zhoui	7	4	152–196	0.429	0.714	0.122	

Locus	Forward primer 5'–3' Reverse primer 5'–3'	Primer label	Repeat sequence	T_a	Species	n	Allele No.	Allele size	H_O	H_E	P	F_{ST}
					Characteristics in *Cuora* spp.							
CuofasGAI_G10 GenBank Acc. No. KJ191269	GAAGCAACGTTTTTCTCACCAC GCTTCTGAAAGTGCGGGAGAG	PET	$(GA)_{12}$	56.5	*C. aurocapitata*	17	8	210–252	0.765	0.861	0.438	0.246
					C. cyclornata	27	6	198–222	0.481	0.653	< 0.001	
					C. pani	6	3	204–212	0.833	0.682	0.655	
					C. trifasciata	53	5	196–218	0.566	0.651	0.179	
					C. zhoui	7	3	204–218	0.571	0.582	1.000	
CuofasGTII_E6 GenBank Acc. No. KJ191270	GCCTTGGCTTTTGCATTTCTTT GTTTCACCAAAATATGATAAAG	NED	$(GT)_{14}$	50	*C. aurocapitata*	17	4	106–116	0.647	0.651	0.971	0.202
					C. cyclornata	27	15	110–148	0.741	0.926	0.006	
					C. pani	6	1	104	–	–	–	
					C. trifasciata	54	9	104–126	0.500	0.742	< 0.001	
					C. zhoui	6	2	112–114	0.667	0.545	1.000	
CuofasGAI_D4 GenBank Acc. No. KJ191266	GAGGAGCCAAGAGGGGAGACAT GCCCTTCTGGCCTTGGAACCTA	VIC	$(GA)_{13}$	58	*C. aurocapitata*	18	5	148–160	0.556	0.727	0.145	0.202
					C. cyclornata	31	9	136–170	0.677	0.689	0.489	
					C. pani	6	2	148–150	0.500	0.530	1.000	
					C. trifasciata	56	8	142–166	0.911	0.818	0.482	
					C. zhoui	7	3	136–150	0.143	0.275	0.078	
CuofasGTI_F11 GenBank Acc. No. KJ191275	GTTTCTCTCTTTTCAACTCAGG GAAATGCAGACATTAGAAATCC	PET	$(GT)_{14}$	50	*C. aurocapitata*	17	4	224–266	0.706	0.576	0.598	0.185
					C. cyclornata	30	11	214–290	0.600	0.795	0.003	
					C. pani	6	1	222	–	–	–	
					C. trifasciata	54	14	214–298	0.611	0.762	0.034	
					C. zhoui	7	3	212–218	0.714	0.648	0.031	
CuofasGAII_H8 GenBank Acc. No. KJ191264	GTATCACACGGGAACCTGGAGC GCTCCAGAGAAATGTGCCACAG	FAM	$(GA)_{15}$	58	*C. aurocapitata*	17	3	254–264	0.412	0.494	0.231	0.399
					C. cyclornata	26	8	244–266	0.385	0.735	< 0.001	
					C. pani	6	1	252	–	–	–	
					C. trifasciata	53	8	242–262	0.566	0.557	0.635	
					C. zhoui	6	2	254–266	0.500	0.409	1.000	
CuofasGAII_D10 GenBank Acc. No. KJ191263	GAATATCCTCCTTCAGCACGCC GTTTCCACGCATCTTCTTTCTC	FAM	$(GA)_{12}$	56.5	*C. aurocapitata*	18	3	159–165	0.389	0.652	0.025	0.176
					C. cyclornata	31	6	141–167	0.484	0.593	0.136	
					C. pani	6	4	157–169	1	0.712	0.479	
					C. trifasciata	57	8	135–165	0.632	0.738	0.463	
					C. zhoui	7	2	159–161	0.143	0.143	1.000	
CuofasGTII_G10 GenBank Acc. No. KJ191271	GTGGAGGATGGTGATTTCA GAATTCTGCTCTGATTCTC	NED	$(GT)_{12}$	48	*C. aurocapitata*	18	1	127	–	–	–	0.801
					C. cyclornata	31	3	139–165	0.032	0.064	0.016	
					C. pani	6	4	127–171	0.833	0.697	0.655	
					C. trifasciata	58	3	127 –141	0.086	0.116	0.033	
					C. zhoui	7	2	145–147	0.429	0.363	1.000	
CuofasGTII_G6 GenBank Acc. No. KJ191272	GTTGCAAATGCTCACTCAGAAG GAAAGTTTGGGGTTAGATGGA	VIC	$(GT)_{12}$	56.5	*C. aurocapitata*	18	10	271–507	0.778	0.870	0.213	0.400
					C. cyclornata	31	4	271–493	0.065	0.095	0.051	
					C. pani	6	7	429–493	0.500	0.833	0.026	
					C. trifasciata	56	7	269–489	0.518	0.586	0.103	
					C. zhoui	7	2	275–279	0.000	0.440	0.021	

T_a (°C) – PCR annealing temperature; H_E – expected heterozygosity; H_O – observed heterozygosity; P – probability of deviation from Hardy-Weinberg Equilibrium.

Table 2. Genetic divergence among the five *Cuora* species investigated. Above diagonal: F_{ST} values; below diagonal: associated P values.

	C. aurocapitata	*C. pani*	*C. trifasciata*	*C. cyclornata*	*C. zhoui*
C. aurocapitata	–	0.355	0.290	0.357	0.486
C. pani	< 0.001	–	0.356	0.404	0.628
C. trifasciata	< 0.001	< 0.001	–	0.112	0.367
C. cyclornata	< 0.001	< 0.001	< 0.001	–	0.425
C. zhoui	< 0.001	0.002	< 0.001	< 0.001	–

meieri) assignments. An additional morphological group (*C. cyclornata* cf. *cyclornata*) was genetically not supported. Likewise, two subclusters can be seen within *C. trifasciata*, one being composed of Chinese mainland specimens including the holotype, the other of animals from Hainan Island, which can be assigned to *C. trifasciata* mtDNA 'Clade B' (*C. trifasciata* cf. *trifasciata*; Blanck et al. 2006; Fig. 2). This substructure within *C. trifasciata* explains the heterozygote deficiency observed when heterozygosity is estimated species-wide (Wahlund effect). While the subclusters of *C. cyclornata* are easily distinguished based on morphological characters, morphological identification is far less straightforward in the cases of the two *C. trifasciata* subclusters. Further studies are needed to better characterize the potentially new subspecies uncovered in this study. All 7 clusters were confirmed in a Neighbour Joining (NJ)

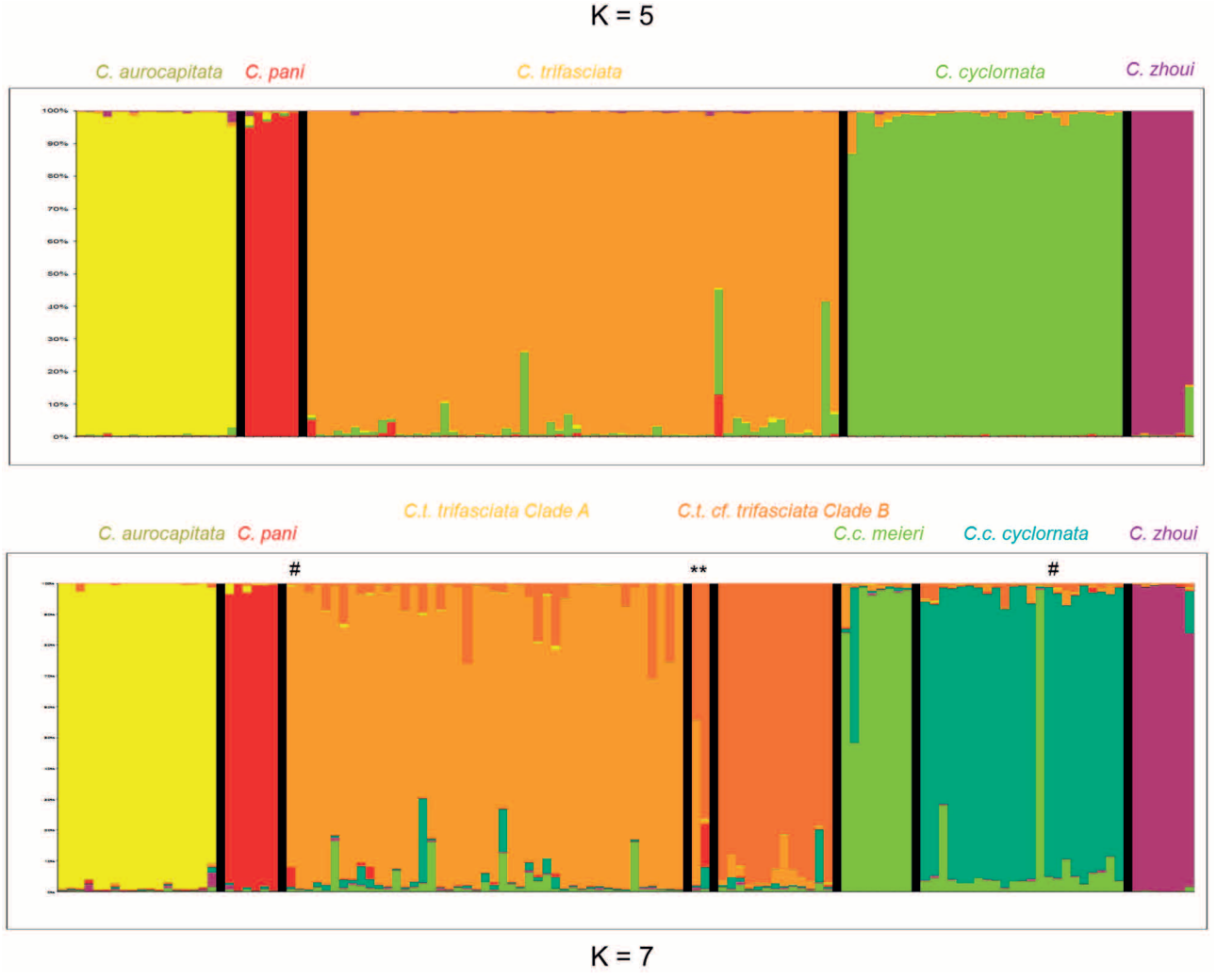

Figure 2. Proportional assignment of *Cuora* specimens to genetically inferred clusters (STRUCTURE results for k = 5 [upper] and k = 7 [lower]). Vertical black bars separate morphologically assigned species/subspecies; * depicts individuals that were morphologically identified as hybrids and # depicts type specimens.

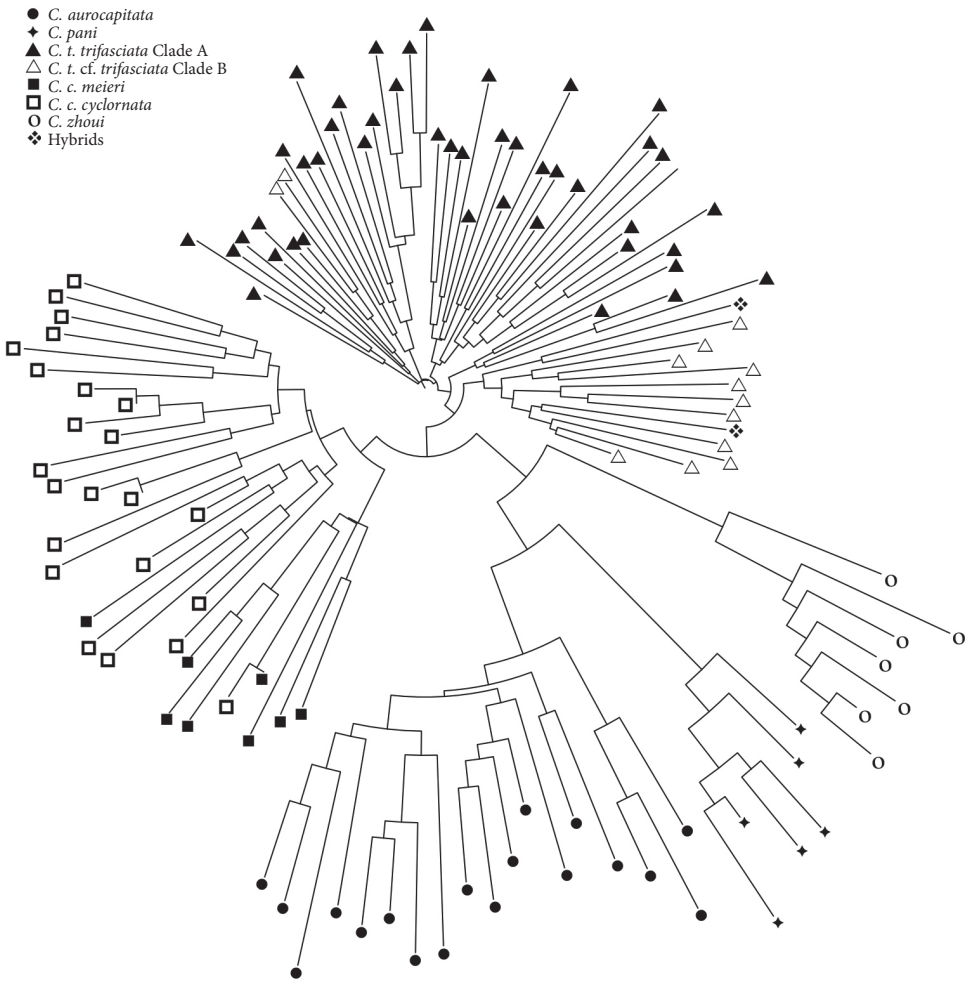

Figure 3. Individually based neighbour-joining tree of Nei's minimum genetic distance for the microsatellite data. Each individual is represented by a symbol corresponding to its morphological species/subspecies assignment as shown in the legend.

tree based on individual Nei's minimum genetic distances (Fig. 3).

We detected only one presumed subspecies hybrid within *C. cyclornata (C. c. cyclornata* × *C. c. meieri),* and two within *C. trifasciata*, equalling only 5% of the 92 specimens of these two morphospecies analysed herein. We are thus inclined to argue that *C. cyclornata* is indeed a valid species with a least two well-distinguishable subspecies and that neither *C. cyclornata* nor *C. aurocapitata* is of hybrid origin as has been hypothesized by Spinks et al. (2012).

In summary, our analysis of 16 new microsatellite loci demonstrated that the previous morphospecies/subspecies assignment in *Cuora* spp. (e.g., Blanck et al. 2006) is fully justified for the taxa examined here. These markers can be used, e.g. to genotype confiscated animals, and should form the backbone of future captive breeding initiatives, particularly for taxa that are morphologically difficult to distinguish. Also, the application of this marker system will help to avoid the accidental inclusion of hybrid specimens in captive breeding programs. We highlight the validity of the species status for *C. cyclornata* as demonstrated in this study and wish to draw attention to the precarious status of the few remaining natural *C. cyclornata* and *C. trifasciata* populations (Blanck et al. 2006, Turtle Conservation Consortium 2011). Our new analytical tools hopefully will prove useful in future reintroduction programs by allowing animals to be genotypified prior to their captive breeding or release.

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Supplementary material

Additional information is available in the online version of this article at http://www.salamandra-journal.com

Supplementary table 1. Information about analysed specimens.

Supplementary table 2. *Cuora* taxa included in study.

Supplementary material

Tiedemann, R., A. R. R. Schneider, K. Havenstein, T. Blanck, E. Meier, M. Raffel, H. Zwartepoorte & M. Plath: New microsatellite markers allow high-resolution taxon delimitation in critically endangered Asian box turtles, genus *Cuora*. – Salamandra, **50**(3): 139–146.

2 Supplementary tables

S1. Information about analysed specimens.

	Taxa		Taxon according to STRUCTURE	European Studbook Foundation No.	Additional field name/information
1	A and B	1	*Cuora aurocapitata*	ESF-AUR-HH	CA Hersche1/Switzerland; wild-caught
2		2	*Cuora aurocapitata*	ESF-AUR-1	CA MS1; wild-caught
3		3	*Cuora aurocapitata*	ESF-AUR-2	CA MS2; wild-caught
4		4	*Cuora aurocapitata*	ESF-AUR-3	CA MS3; wild-caught
5		5	*Cuora aurocapitata*	ESF-AUR-4	CA MS4; wild-caught
6		6	*Cuora aurocapitata*	ESF-AUR-5	CA MS5; wild-caught
7		7	*Cuora aurocapitata*	ESF-AUR-6	CA MS6; wild-caught
8		8	*Cuora aurocapitata*	ESF-AUR-7	CA MS7; wild-caught
9		9	*Cuora aurocapitata*	ESF-AUR-8	CA MS8; wild-caught
10		10	*Cuora aurocapitata*	ESF-AUR-9	CA MS9; wild-caught
11		11	*Cuora aurocapitata*	ESF-AUR-10	CA MS10; wild-caught
12		12	*Cuora aurocapitata*	ESF-AUR-11	CA MS11; wild-caught
13		13	*Cuora aurocapitata*	ESF-AUR-12	CA MS12; wild-caught
14		14	*Cuora aurocapitata*	ESF-AUR-13	CA MS13; wild-caught
15		15	*Cuora aurocapitata*	ESF-AUR-15	CA MS15; captive-bred
16		16	*Cuora aurocapitata*	ESF-AUR-20	CA MS20; captive-bred
17		17	*Cuora aurocapitata*	ESF-AUR-24	CA MS24; captive-bred
18		18	*Cuora aurocapitata*	ZT-JI1	C.A.1, Jingxian, Anhui; wild-caught
19	C	1	*Cuora pani*	ZT-PA1	PANI; wild-caught
20		2	*Cuora pani*	ESF-PAN-1	Cp MS1; wild-caught
21		3	*Cuora pani*	ESF-PAN-2	Cp MS2; wild-caught
22		4	*Cuora pani*	ESF-PAN-3	Cp MS3; captive-bred
23		5	*Cuora pani*	ESF-PAN-5	Cp MS5; captive-bred
24		6	*Cuora pani*	ESF-PAN-9	Cp MS9; captive-bred
25	D	1	*C. t. trifasciata* Clade A	OUM8557	OUM 8557 *Cuora fasciata* exchno 2009001, Luofoshan, Guangdong, China; wild-caught
26		2	*C. t. trifasciata* Clade A	ESF-TRI-1	Rotterdam 701253
27		3	*C. t. trifasciata* Clade A	ESF-TRI-2	Rotterdam 703160 male; wild-caught
28		4	*C. t. trifasciata* Clade A	ESF-TRI-3	Cuora trifasciata 703875; wild-caught
29		5	*C. t. trifasciata* Clade A	ESF-TRI-7	Cuora trifasciata 704309; wild-caught
30		6	*C. t. trifasciata* Clade A	MNHN9102	MNHN 9102, Macao; wild-caught, obtained on a market
31		7	*C. t. trifasciata* Clade A	2006-16	TB4 Mio / CT4; wild-caught
32		8	*C. t. trifasciata* Clade A	2006-13	TB7 HK2 Hong Kong / CT7; wild-caught
33		9	*C. t. trifasciata* Clade A	2006-19	CT13 (Bill E/ 19); wild-caught
34		10	*C. t. trifasciata* Clade A	2006-20	CT14 (Bill H / 20); wild-caught
35		11	*C. t. trifasciata* Clade A	2006-10	CT17 (Bill L / 17); wild-caught
36		12	*C. t. trifasciata* Clade A	2006-11	CT19 (Bill N / 15); wild-caught
37		13	*C. t. trifasciata* Clade A	2006-12	CT20 (Bill O / 16); wild-caught
38		14	*C. t. trifasciata* Clade A	2006-18	TB15 NMW Canton / CT26, Guangzhou, China; wild-caught, obtained on a market
39		15	*C. t. trifasciata* Clade A	ESF-TRI-12	CT MS8 (=CM8 Poschadel); wild-caught
40		16	*C. t. trifasciata* Clade A	ESF-TRI-13	CT MS13 (=CM13 Poschadel); wild-caught
41		17	*C. t. trifasciata* Clade A	ESF-TRI-42	Pinky; wild-caught
42		18	*C. t. trifasciata* Clade A	ESF-TRI-43	Perky; wild-caught
43		19	*C. t. trifasciata* Clade A	ESF-TRI-61	wild-caught
44		20	*C. t. trifasciata* Clade A	ESF-TRI-62	wild-caught
45		21	*C. t. trifasciata* Clade A	ESF-TRI-63	wild-caught
46		22	*C. t. trifasciata* Clade A	ESF-TRI-64	wild-caught
47		23	*C. t. trifasciata* Clade A	ESF-TRI-65	wild-caught

	Taxa		Taxon according to STRUCTURE	European Studbook Foundation No.	Additional field name/information
48	D	24	*C. t. trifasciata* Clade A	ESF-TRI-300	wild-caught
49		25	*C. t. trifasciata* Clade A	ESF-TRI-57	wild-caught
50		26	*C. t. trifasciata* Clade A	ESF-TRI-56	wild-caught
51		27	*C. t. trifasciata* Clade A	ESF-TRI-59	wild-caught
52		28	*C. t. trifasciata* Clade A	ESF-TRI-58	wild-caught
53		29	*C. t. trifasciata* Clade A	ESF-TRI-60	wild-caught
54		30	*C. t. trifasciata* Clade A	ESF-TRI-49	wild-caught
55		31	*C. t. trifasciata* Clade A	ESF-TRI-344	wild-caught
56		32	*C. t. trifasciata* Clade A	ESF-TRI-100	wild-caught
57		33	*C. t. trifasciata* Clade A	ESF-TRI-112	wild-caught
58		34	*C. t. trifasciata* Clade A	ESF-TRI-123	captive-bred
59		35	*C. t. trifasciata* Clade A	ESF-TRI-124	captive-bred
60		36	*C. t. trifasciata* Clade A	ESF-TRI-113	wild-caught
61		37	*C. t. trifasciata* Clade A	ESF-TRI-114	wild-caught
62		38	*C. t. trifasciata* Clade A	ESF-TRI-115	wild-caught
63		39	*C. t. trifasciata* Clade A	ESF-TRI-116	wild-caught
64		40	*C. t. trifasciata* Clade A	ESF-TRI-117	wild-caught
65		41	*C. t. trifasciata* Clade A	ESF-TRI-129	captive-bred
66		42	*C. t. trifasciata* Clade A	ESF-TRI-134	captive-bred
67		43	*C. t. trifasciata* Clade A	ESF-TRI-81	wild-caught
68		44	*C. t. trifasciata* Clade A	ESF-TRI-47	wild-caught
69		45	*C. t. trifasciata* Clade A	ESF-TRI-219	captive-bred
70			Morph. hybrid	ESF-TRI-220	captive-bred
71			Morph. hybrid	JL1	captive-bred
72	E	1	*C. t.* cf. *trifasciata*	ESF-TRI-4	*Cuora trifasciata* 703564; wild-caught
73		2	*C. t.* cf. *trifasciata*	ESF-TRI-6	*Cuora trifasciata* 704308; wild-caught
74		3	*C. t.* cf. *trifasciata*	MNHN9103	MNHN 9103, Macao; wild-caught, obtained on a market
75		4	*C. t.* cf. *trifasciata*	2006-22	CT15 (Bill J / 22); wild-caught
76		5	*C. t.* cf. *trifasciata*	2006-23	CT16 (Bill K/ 21); wild-caught
77		6	*C. t.* cf. *trifasciata*	2006-21	CT18 (Bill M / 23); wild-caught
78		7	*C. t.* cf. *trifasciata*	ESF-TRI-9	CT MS10 (=CM10 Poschadel); wild-caught
79		8	*C. t.* cf. *trifasciata*	ESF-TRI-10	CT MS11 (=CM11 Poschadel); wild-caught
80		9	*C. t.* cf. *trifasciata*	ESF-TRI-11	CT MS12 (=CM12 Poschadel); wild-caught
81		10	*C. t.* cf. *trifasciata*	ESF-TRI-37	Chavornay; wild-caught
82		11	*C. t.* cf. *trifasciata*	ESF-TRI-48	captive-bred
83		12	*C. t.* cf. *trifasciata*	ESF-TRI-44	wild-caught
84		13	*C. t.* cf. *trifasciata*	ESF-TRI-111	wild-caught
85	F	1	*C. c. meieri*	2006-8	CT23 (Bill S / 8); wild-caught
86		2	*C. c. meieri*	2006-9	CT24 (Bill U / 9); wild-caught
87		3	C. c. meieri	ESF-CYC-20	CT MS4 (=CM4 Poschadel); wild-caught
88		4	*C. c. meieri*	ESF-CYC-21	CT MS5 (=CM5 Poschadel); wild-caught
89		5	*C. c. meieri*	ESF-CYC-22	CT MS6 (=CM6 Poschadel); wild-caught
90		6	*C. c. meieri*	ESF-CYC-24	CT MS29; wild-caught?
91		7	*C. c. meieri*	MHNT T69	Tam Dao, Vietnam; wild-caught
92		8	*C. c. meieri*	ESF-CYC-23	wild-caught
93	G	1	*Cuora cyclornata* cf. *cyclornata*	2006-5	TB12 Ting / CT12; captive-bred
94		2	*Cuora cyclornata* cf. *cyclornata*	2006-6	CT21 (Bill P / 4); wild-caught
95		3	*Cuora cyclornata* cf. *cyclornata*	2006-4	CT22 (Bill R / 5); wild-caught
96		4	*Cuora cyclornata* cf. *cyclornata*	V1	Vu Quang, Vietnam; wild-caught
97		5	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-15	CT MS1 (=CM1 Poschadel); wild-caught
98		6	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-16	CT MS2 (=CM2 Poschadel); wild-caught

	Taxa		Taxon according to STRUCTURE	European Studbook Foundation No.	Additional field name/information
99	G	7	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-17	CT MS3 (=CM3 Poschadel); wild-caught
100		8	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-18	CT MS7 (=CM7 Poschadel); wild-caught
101		9	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-19	CT MS41; wild-caught
102		10	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-55	wild-caught
103		11	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-53	captive-bred
104		12	*Cuora cyclornata* cf. *cyclornata*	ESF-CYC-54	captive-bred
105		13	*Cuora cyclornata* cf. *cyclornata*	ZT-CYC2	TB5 Farm Schuppe / CT5; captive-bred
106	H	1	*C. c. cyclornata*	2006-2	"1", Hue, Vietnam; wild-caught
107		**2**	*C. c. cyclornata*	ESF-CYC-50	"3", Vietnam; wild-caught
108		3	*C. c. cyclornata*	ESF-CYC-52	"5"; wild-caught
109		4	*C. c. cyclornata*	ZFMK 71348	TB2 ZFMK / CT2, Phong Nha Ke Bang, Vietnam; wild-caught
110		5	*C. c. cyclornata*	ZT-CYC1	TB11 Ting / CT11s; wild-caught
111		6	*C. c. cyclornata*	ESF-CYC-14	CT MS9 (=CM9 Poschadel); wild-caught
112		7	*C. c. cyclornata*	ESF-CYC-66	captive-bred
113		8	*C. c. cyclornata*	ESF-CYC-67	captive-bred
114		9	*C. c. cyclornata*	ESF-CYC-68	captive-bred
115		10	*C. c. cyclornata*	ESF-CYC-51	captive-bred
116	I	1	*Cuora zhoui*	ESF-ZHO-1	CZ MS1; wild-caught
117		2	*Cuora zhoui*	ESF-ZHO-2	CZ MS2; wild-caught
118		3	*Cuora zhoui*	ESF-ZHO-3	CZ MS3; wild-caught
119		4	*Cuora zhoui*	ESF-ZHO-4	CZ MS4; wild-caught
120		5	*Cuora zhoui*	ESF-ZHO-5	CZ MS5; wild-caught
121		6	*Cuora zhoui*	ESF-ZHO-6	CZ MS6; wild-caught
122		7	*Cuora zhoui*	NTM-9001	ZHOUI2x, Pingxiang, China; wild-caught

S2. *Cuora* taxa included in study.

	Taxon	Number of specimens
A	*Cuora aurocapitata* Clade A	18
B	*Cuora aurocapitata* Clade B	
C	*Cuora pani*	6
D	*C. t. trifasciata* Clade A	45
	Morph. hybrid	2
E	*C. t.* cf. *trifasciata*	13
F	*Cuora cyclornata meieri*	8
G	*Cuora cyclornata* cf. *cyclornata*	13
H	*Cuora cyclornata cyclornata*	10
I	*Cuora zhoui*	7