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Molecular evidence for hybrid origin of *Mauremys iversoni* PRITCHARD et MCCORD, 1991, and *Mauremys pritchardi* MCCORD, 1997 (Reptilia: Testudines: Bataguridae)

With 2 figures and 3 tables

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Abstract. Nucleotide sequences of the mitochondrial cytochrome *b* gene demonstrate that *Cuora trifasciata* and *Mauremys iversoni*, or *Mauremys mutica* and *Mauremys pritchardi*, respectively, form closely related species pairs. Furthermore, all four taxa together with *Chinemys reevesii* form a monophyletic group. Since mitochondrial genes are inherited maternally, it is not possible to gain unequivocal evidence for a putative hybrid status for *M. iversoni* or *M. pritchardi* initially suspected because of morphological similarities. Genomic fingerprinting with Inter-Simple-Sequence-Repeat profiles provides good evidence that *M. iversoni* is a hybrid between *Cuora trifasciata* and *M. mutica* and *M. pritchardi* between *M. mutica* and *Chinemys reevesii*.

Kurzfassung. **Molekularer Nachweis für die Entstehung von *Mauremys iversoni* PRITCHARD et MCCORD, 1991 und *Mauremys pritchardi* MCCORD, 1997 durch Hybridisierung (Reptilia: Testudines: Bataguridae).** – Nukleotidsequenzen des mitochondrialen Cytochrom *b* Gens zeigen, daß *Cuora trifasciata* und *Mauremys iversoni* bzw. *Mauremys mutica* und *Mauremys pritchardi* nahe verwandte Artenpaare sind. Alle vier Taxa bilden zusammen mit *Chinemys reevesii* eine monophyletische Gruppe. Da mitochondriale Gene nur in mütterlicher Linie vererbt werden, lassen diese Daten keine klaren Aussagen über einen möglichen Hybridstatus von *M. iversoni* und *M. pritchardi* zu, der aufgrund morphologischer Ähnlichkeiten vermutet wird. Fingerprint-Analysen des Genoms mit der Inter-Simple-Sequence-Repeat-PCR-Methode belegen die Entstehung von *M. iversoni* und *M. pritchardi* durch Hybridisierung von *Cuora trifasciata* und *M. mutica* bzw. *M. mutica* und *Chinemys reevesii* eindeutig.

Key words. Testudines, Bataguridae, *Mauremys*, genomic fingerprinting, ISSR-PCR, cytochrome *b* sequences, hybrids.

1. Introduction

Recently two new turtle species (Testudines: Bataguridae), *Mauremys iversoni* and *Mauremys pritchardi*, have been described (PRITCHARD & MCCORD 1991, MCCORD 1997). However, some researchers have questioned these species assignments because of morphological affinities and the possibility that *M. pritchardi* is a hybrid between *Chinemys reevesii* and *Mauremys mutica*, and *M. iversoni* is a hybrid between *Cuora trifasciata* and *Mauremys mutica* (FRITZ & OBST 1999; E. MEIER, pers. comm.). As both species are consid-

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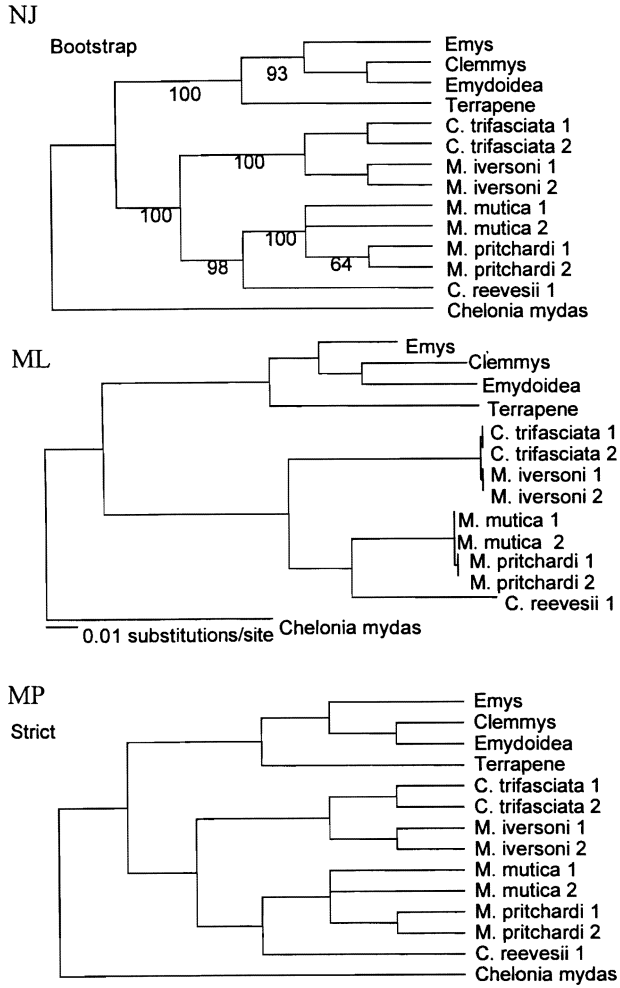

Fig. 1: Molecular phylogeny of Bataguridae turtles based on nucleotide sequences of the cytochrome *b* gene.
A. Bootstrap cladogram of a neighbor joining tree.
Distance algorithm (Kimura 2 parameter); 1000 bootstrap replicates.
B. Maximum likelihood phylogram.
Nucleotide frequencies: A = 0.294, C = 0.317, G = 0.125, T = 0.264; score of best tree 3867.53; branch lengths correspond to genetic distances.
C. Maximum parsimony strict consensus cladogram of 2 most parsimonious trees (unweighted analysis); tree length: 520 steps; CI = 0.769; RI = 0.832; HI = 0.231.

ered to be endangered in the wild and a captive breeding programme is currently established by the Zoological Garden at Münster, we used molecular markers to assess whether these taxa are hybrids.

Sequences of the mitochondrial cytochrome *b* gene were used to produce a molecular phylogeny, similar to our previous study on the *Emys orbicularis* complex (LENK *et al.* 1999). A maternally inherited locus such as mtDNA cytochrome *b* cannot detect hybrids with certainty. Therefore, we analysed the structure of the nuclear genome with Inter-Simple-

Table 1: Documentation of parsimony informative characters in the cytochrome *b* data set. . = nucleotide identical to that in the first line.

```
                    1 111 112 222 222 222 233 333 333 333 344 444 444 444 445 555 555
                  356 792 345 680 001 134 447 812 234 456 899 902 234 557 788 890 034 446
                  640 589 846 801 270 344 693 594 702 843 446 920 385 091 706 954 510 691

C. trifasciata 1  CGC GCC TGG AGC ACT TCC CCT TTC ACT TCA CGT TTA ACT TCC TTC CTT ATT AAG
C. trifasciata 2  ... ... ... ... ... ... ... .?. ... ... ... ... ... ... ... ... ... ...
M. iversoni 1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ... ...
M. iversoni 2     ... ... ... ... ... ... ... ..? ... ... ... ... ... ... ... .C. ... ... ...
M. mutica 1       TAT ATT CAT TAT CTC CTT ATC CCA GTC ?TC TAC CCG GTC CTT CCT TCC GCC TCA
M. mutica 2       TAT ATT CAT TAT CTC CTT A?C CCA GTC CTC TAC CCG GTC CTT CCT TCC GCC TCA
M. pritchardi 1   TAT ATT CAT TAT CTC CTT ATC CCA GTC CTC TAC CCG GTC CTT CCT TCC GCC TCA
M. pritchardi 2   ??? ATT CAT TAT CTC C?? ??C CCA GTC CTC TAC CCG GTC CTT CCT TCC GCC TCA
C. reevesii 1     .AT AT. CAT ... ..C CTT A.C CCA .TC CTC TA. C.G GTC C.T CCT T.. GCC ..A

                  555 566 666 666 666 666 677 777 778 888 888 888 888 999 999 999 99
                  688 911 234 445 667 899 903 446 890 112 234 478 899 011 225 556 67
                  758 456 390 499 672 406 955 475 928 393 841 930 204 423 274 573 95

C. trifasciata 1  TCT TTT TAC CAC TAA TCC CAA TCT GTT CTT TAC CCT ATC TTG ACG GTC TC
C. trifasciata 2  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
M. iversoni 1     ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ..
M. iversoni 2     ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ..
M. mutica 1       CTC CCC CTT TGT CCG C.A TCG CAC ACG TCC CGT ATC GCA CC. GTA ACT CT
M. mutica 2       CTC CCC CTT TGT CCG C.A TCG CAC ACG TCC CGT ATC GCA CC. GTA ACT CT
M. pritchardi 1   CTC CCC CTT TGT CCG C.A TCG CAC ACG TCC CGT ATC GCA CCA GTA ACT CT
M. pritchardi 2   CTC CCC CTT TGT CCG C.A TCG CAC ACG TCC CGT A?? ??? ?CA GTA ACT ?T
C. reevesii 1     C.C CCC ... TGT CC. C.A T.. C.C ACG T.C .GT A.C CCA C.. G.A ACT C?
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Sequence-Repeat profiles. ISSR-PCR usually provides species-specific genomic fingerprints (GUPTA *et al.* 1994, ZIETKIEWICS *et al.* 1994, WINK *et al.* 1998). ISSR-PCR of nuclear DNA is a PCR method to map the nuclear genome and to discover rearrangements. ISSR employs a single PCR primer only. These PCR primers bind directly to microsatellites, such as $(CA)_n$, which are abundant in eukaryotic genomes. Since sequences of microsatellites are conserved over wide ranges of organisms, ISSR-PCR can use universal primers which do not need to be adapted to individual species as in microsatellite PCR. In ISSR-PCR stretches of DNA between adjacent microsatellite elements are amplified. Since we use a single PCR primer only, a necessary prerequisite is the inversion of a microsatellite motive in the neighbourhood (up to 2000 bp distance) of an existing microsatellite element. Because hybridization often includes major genomic changes, ISSR-PCR should provide a critical test of hybrid origin for *M. iversoni* and *M. pritchardi*.

2. Material and Methods

PCR and sequencing

Polymerase chain reaction (PCR) was used to amplify a fragment containing the target sequence (1036 nt of the cytochrome *b* gene and 38 nt of the $tRNA_{THR}$) using the primers L-14846 in combination with H-15909 (LENK *et al.* 1999). PCR was performed in 50 µl volume containing 0.75 units of Amersham Pharmacia Biotech *Taq* DNA Polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9), and 0.01 % BSA. After an initial denaturing step for 4 min at 94 °C, 31 cycles were performed with annealing 50 sec at 47 °C, primer extension 120 sec at 72 °C, and denaturing 45 sec at 94 °C.

Table 2: Pairwise genetic distances (uncorrected "p" distance).
1.0000 = 100 % nucleotide substitutions.

		1	2	3	4	5	6	7	8	9
1	*Chelonia mydas*	-								
2	*C. trifasciata* 1	0.16109	-							
3	*C. trifasciata* 2	0.15971	0.00000	-						
4	*M. iversoni* 1	0.16180	0.00210	0.00209	-					
5	*M. iversoni* 2	0.16163	0.00209	0.00209	0.00000	-				
6	*M. mutica* 1	0.15795	0.10609	0.10482	0.10565	0.10471	-			
7	*M. mutica* 2	0.15966	0.10654	0.10526	0.10609	0.10515	0.00000	-		
8	*M. pritchardi* 1	0.15954	0.10785	0.10658	0.10752	0.10647	0.00105	0.00105	-	
9	*M. pritchardi* 2	0.16121	0.10788	0.10680	0.10788	0.10680	0.00122	0.00121	0.00000	-
10	*C. reevesii* 1	0.15950	0.11907	0.11882	0.11975	0.11870	0.07579	0.07505	0.07660	0.07805

Table 3: ISSR-Fingerprint profiles of hybrids and their corresponding parent species, only polymorphic and diagnostic PCR products are tabulated.
+ = PCR product present, - = PCR product absent.

PCR Product	*C. trifasciata*	*M. iversoni*	*M. mutica*
$(GATA)_4$			
7	+	+	-
8	+	-	-
9	-	-	+
12	-	+	+
13	-	+	+
14	+	-	-
15	-	-	+
17	+	+	-
18	+	+	+
21	-	+	+
22	+	+	-
23	-	+	+
24	+	-	+
$(CA)_{10}$			
a	-	+	+
b	+	+	-
c	+	+	-
d	-	+	+
e	-	-	+
f	+	+	-
g	+	+	-
h	+	+	-
i	-	+	+
j	-	+	+
$(GACA)_4$			
A	+	+	-
B	-	+	+
C	+	+	-
D	+	+	-
E	-	+	+
F	+	-	-
G	-	+	+
H	+	+	-
I	-	+	+
J	+	+	-
K	-	+	+
L	+	+	-
M	-	+	+
N	-	+	+

PCR Product	*M. mutica*	*M. pritchardi*	*C. reevesii*
$(GATA)_4$			
2	-	+	+
6	+	+	-
8	+	+	+
9	-	+	+
11	+	+	-
13	+	+	-
14	+	+	-
18	-	+	+
19	-	+	+
21	+	+	-
22	-	+	+
23	-	+	+
24	+	+	-
$(CA)_{10}$			
b	-	+	+
c	-	+	+
d	-	+	+
e	-	+	+
h	+	+	-
j	-	+	+
$(GACA)_4$			
A	-	+	+
B	+	+	+
E	-	-	+
F	-	+	+
F	+	+	-
I	+	+	-
L	-	+	+
M	+	+	-

PCR products were sequenced directly using the dideoxy chain termination method (SANGER *et al.* 1977) using the Cycle Sequencing Kit (Amersham Pharmacia Biotech, RPN 2438/RPN 2538) in combination with fluorescently labelled primers. For cycle sequencing an initial denaturing step at 94 °C for 4 min was followed by 26 cycles at 55 °C (60 sec) and 94 °C (45 sec). The primers employed are those listed in LENK *et al.* (1999). Fluorescent labelled fragments were analysed on an automated Sequencer (Amersham Pharmacia Biotech, ALF-Express II). Deletions, insertions or inversions were not encountered. New nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Database. Sequences from *Terrapene*, *Emydoidea*, *Clemmys*, *Emys*, and *Chelonia* were either published before (LENK *et al.* 1999) or taken from GenBank.

Phylogenetic and statistical analysis

Phylogenetic relationships among taxa and haplotypes were analysed by different methods. The program package MEGA (KUMAR *et al.* 1993) was used to estimate genetic distances and to calculate sequence statistics. Maximum parsimony and maximum likelihood searches were conducted with the heuristic search approach of PAUP* 4.0 (SWOFFORD 1998) using the tree-bisection-and-reconnection swapping algorithm. For maximum parsimony the default settings were applied. For maximum likelihood the following parameters were specified: (number of substitution types = 2, estimated transition/transversion ratio via maximum likelihood, estimated base frequencies). Bootstrap analyses (1000 replicates) were performed to examine the robustness of tree furcations with neighbour joining and Kimura-2 parameter as distance algorithm.

ISSR-PCR

Single PCR primer $(GACA)_4$, $(GATA)_4$ or $(CA)_{10}$ were employed for ISSR-PCR. For amplifications 50 ng of total DNA was used as template, plus 6 pmol $(GACA)_4$, 12 pmol $(GATA)_4$ or 20 pmol $(CA)_{10}$ PCR primer, 1.5 mM $MgCl_2$, 0.1 mM of dGTP, dCTP, and dTTP, 0.045

mM dATP, 1 μCi ^{33}P-alpha-dATP, 2.5 μl 10x amplification buffer and 1 unit *Taq* Polymerase (Amersham Pharmacia Biotech; Freiburg) in a total volume of 25 μl. Different PCR programmes were used for each primer. With the $(GACA)_4$ primer the initial denaturation (94 °C, 5 min) was followed by 34 cycles of 60 sec at 94 °C, 120 sec at 55 °C and 120 sec at 72 °C, with the $(GATA)_4$ primer the initial denaturation (94 °C, 2 min) was followed by 34 cycles of 120 sec at 46 °C, 120 sec at 72 °C and 60 sec at 94 °C, and with the $(CA)_{10}$ primer the initial denaturation (94 °C; 4 min) was followed by 31 cycles of 60 sec at 40 °C, 120 sec at 72 °C and 45 sec at 94 °C. After completion of the cycles, the temperature was maintained at 72 °C for 5 min and then lowered to 4 °C for further storage. The reactions were performed on a Biometra thermocycler. PCR products were separated on a denaturing Sequagel matrix at 65 W for 2.5 h (length 40 cm). After drying, the gel was exposed to an X-ray film for one day and developed.

3. Results

Phylogenetic relationships

Using *Chelonia* as outgroup and *Terrapene*, *Emys*, *Clemmys*, and *Emydoidea* as an unrelated sister group a unique phylogenetic tree was obtained from cytochrome *b* sequences independent of the methods used for phylogeny reconstruction (MP, NJ or ML) (Fig. 1). Two individuals were analysed for each taxon (*Chinemys reevesii, Cuora trifasciata, Mauremys iversoni, M. mutica, M. pritchardi*); samples from the same species were identical in all instances (Table 1). *M. iversoni*, the putative hybrid, appears to be closely related to *Cuora trifasciata*; pairwise distances between these taxa d = 0.2 % (Table 2), based on transitions at positions 480 and 690 (Table 1). The other putative hybrid, *M. pritchardi*, is apparently closely related to *M. mutica* (d = 0.1 %) and is distinguished by a single transition at position 913 (Table 1).

M. pritchardi and *M. mutica* share common ancestry with *Chinemys reevesii* (98 % bootstrap support). This monophyletic group clusters as a sister to the *Cuora trifasciata/M. iversoni* pair (d = 10-11 %; Table 1) and is monophyletic relative to outgroups (100 % bootstrap support).

ISSR-PCR Fingerprinting

PCR primer $(GACA)_4$, $(GATA)_4$ or $(CA)_{10}$ produced informative fingerprints showing several polymorphic PCR products (Fig. 2). ISSR-profiles are identical for the individuals of the same taxon, but differ between taxa. When we compare the profiles of the respective parent species *Cuora trifasciata*, *Chinemys reevesii*, and *M. mutica* we can detect a number of shared bands, but also series of bands which differ between these species (Table 3). The putative hybrid *M. iversoni* has a more complex ISSR profile and approximately half of its markers are shared with *Cuora trifasciata*, the other with *M. mutica* (Table 3). If we determine band-sharing coefficients (BSC) for the polymorphic markers (Table 3), we find a value of 0.1 between the unrelated parent species and a value of 0.6 between the hybrid and either putative parent species.

A similar pattern can be seen for *M. pritchardi* and its putative parents *M. mutica* and *Chinemys reevesii* (Table 3). Respective BSC values are 0.1 between the parent species and 0.63 between *M. pritchardi* and *M. mutica*, or 0.74 between *M. pritchardi* and *Chinemys reevesii*.

4. Discussion

The cytochrome *b* sequences clearly indicate that the putative hybrid *Mauremys iversoni* is maternally closely related to *Cuora trifasciata*, and *M. pritchardi* close to *M. mutica* (Fig. 1). Since mitochondrial genes are inherited maternally, it is not possible to gain evidence for

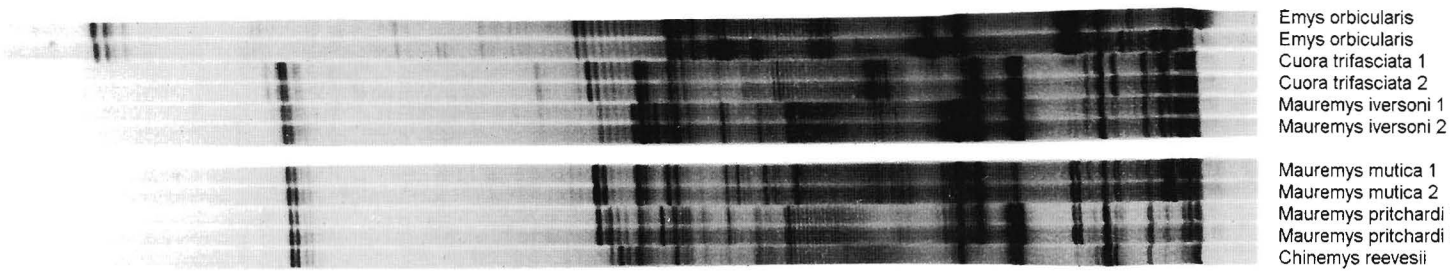

Fig. 2: Genomic fingerprints by ISSR-PCR of putative hybrid turtles and their corresponding parent species.
Illustration of the genomic fingerprint with $(GATA)_4$ primer; lengths of the PCR products vary between 100 and 2000 bp.

the respective paternal parent of *M. iversoni* or *M. pritchardi* from the cytochrome *b* analysis. However, the ISSR-analysis which detects genomic rearrangements provides good evidence that *M. iversoni* is a hybrid between *Cuora trifasciata* (maternal parent) and *M. mutica* (paternal parent) and *M. pritchardi* between *M. mutica* (maternal parent) and *Chinemys reevesii* (paternal parent) (Table 2).

It is unknown whether *M. iversoni* and *M. pritchardi* are natural hybrids or products of Asiatic turtle farms. We know that such turtle farms exist, at least for soft-shelled turtles of the family Trionychidae, but also for the most appreciated turtle species of the East Asian markets, *Cuora trifasciata* (ZHAO 1998). The high number of *M. iversoni* and *M. pritchardi* specimens appearing in the international animal trade is remarkable in any case (see PRITCHARD & MCCORD 1991, MCCORD 1997, FRITZ & OBST 1999). Hybridization between *Cuora trifasciata* and *M. mutica* is also remarkable, as these two species are morphologically very distinct taxa and thought to be only distantly related (ERNST & BARBOUR 1989).

However, genetic evidence so far does not favour a very recent hybridisation: Both hybrids have cytochrome *b* sequences which differ by 0.1 or 0.2 % nucleotide substitutions from their presumed maternal parent species. Applying a molecular clock of 0.4 % sequence divergence per million years (AVISE *et al.* 1992, BOWEN *et al.* 1993; LAMB & LYDEARD 1994, LENK *et al.* 1999), this suggests that the putative hybridisation took place 250.000 or 500.000 years ago. Also the genomic fingerprints show a few species specific PCR products for the hybrids which would not be expected in a very recent hybrid. We need more sequences from different localities to corroborate these findings, before considering a revision of the underlying taxonomy.

As turtles are currently exploited in high numbers for the Chinese food market in East and Southeast Asia, many taxa are critically endangered (FRITZ & OBST 1998, 1999; ALTHERR & FREYER 2000, RHODIN 2000). Hence it is important to decide to which taxa highest priority should be given in captive breeding programmes. As the present study suggests that *Mauremys iversoni* and *M. pritchardi* might be natural hybrids they should not be excluded from such efforts until further research is carried out.

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