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MOLECULAR SUPPORT FOR THE TAXONOMIC CONCLUSIONS OF McCORD AND PRITCHARD (2002), REGARDING *CHITRA*

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(with one text-figure)

ABSTRACT.– McCord and Pritchard (2002) have presented a taxonomic revision of the trionychid turtle genus *Chitra*, in which they describe one new species (*Chitra vandijki*) from Myanmar, name two subspecies within *Chitra chitra* and consider *Chitra indica* to be monotypic across its wide range from Pakistan to Bangladesh. Here we present DNA sequence data from mitochondrial ND4 gene, which support their taxonomic conclusions. There is deep molecular divergence among the three recognized species of *Chitra* and a low level of geographically structured variation within *Chitra chitra* corresponding with mainland and island subspecies. In contrast, *Chitra indica* shows almost no molecular variation across its broad range from Pakistan to Bangladesh. This apparent genetic uniformity is an interesting biogeographic phenomenon, which merits further investigation.

KEYWORDS.– *Chitra*, mtDNA, phylogeny.

INTRODUCTION

McCord and Pritchard (2002) have presented a taxonomic revision of the softshell turtle genus *Chitra*, in which they define and explain both similarities and differences between molecular results and the "phenotypic groups" for all known forms of *Chitra*. The herpetological literature is rich with examples in which species and subspecies boundaries based on phenotypic and morphological characters are inconsistent with phylogenetic history of animals inferred from molecular markers (Burbrink et al., 2000; Rodriguez-Robles and DeJesús-Escobar, 2000), and also cases in which phenotypic and morphological uniformity belies cryptic genetic diversity (Wüster and Thorpe, 1994; Bruna et al., 1996; Roman et al., 1999; Rodriguez-Robles and DeJesús-Escobar, 2000). Therefore confidence in taxonomic conclusions is often stronger when they are supported by a detailed combination of phenotypic, morphological and molecular data. Here we present molecular evidence from mitochondrial DNA sequence data, which supports the taxonomic conclusions drawn by McCord and Pritchard (2002).

METHODS

Molecular data are included for a total of 20 *Chitra* from across the known range of the genus from Pakistan to the islands of Sumatra and Java. These represent a subset of the animals examined morphologically by McCord and Pritchard (2002). Much of these data have been presented previously (Engstrom et al., 2002), however this paper also includes new data from increased sampling of animals (WPM collection) from Bangladesh and Java and a novel geographic locality in Pakistan.

Blood and tissue samples were collected from living animals or salvaged from dead animals in the private collection of WPM. Blood samples were drawn by a lateral approach from the front

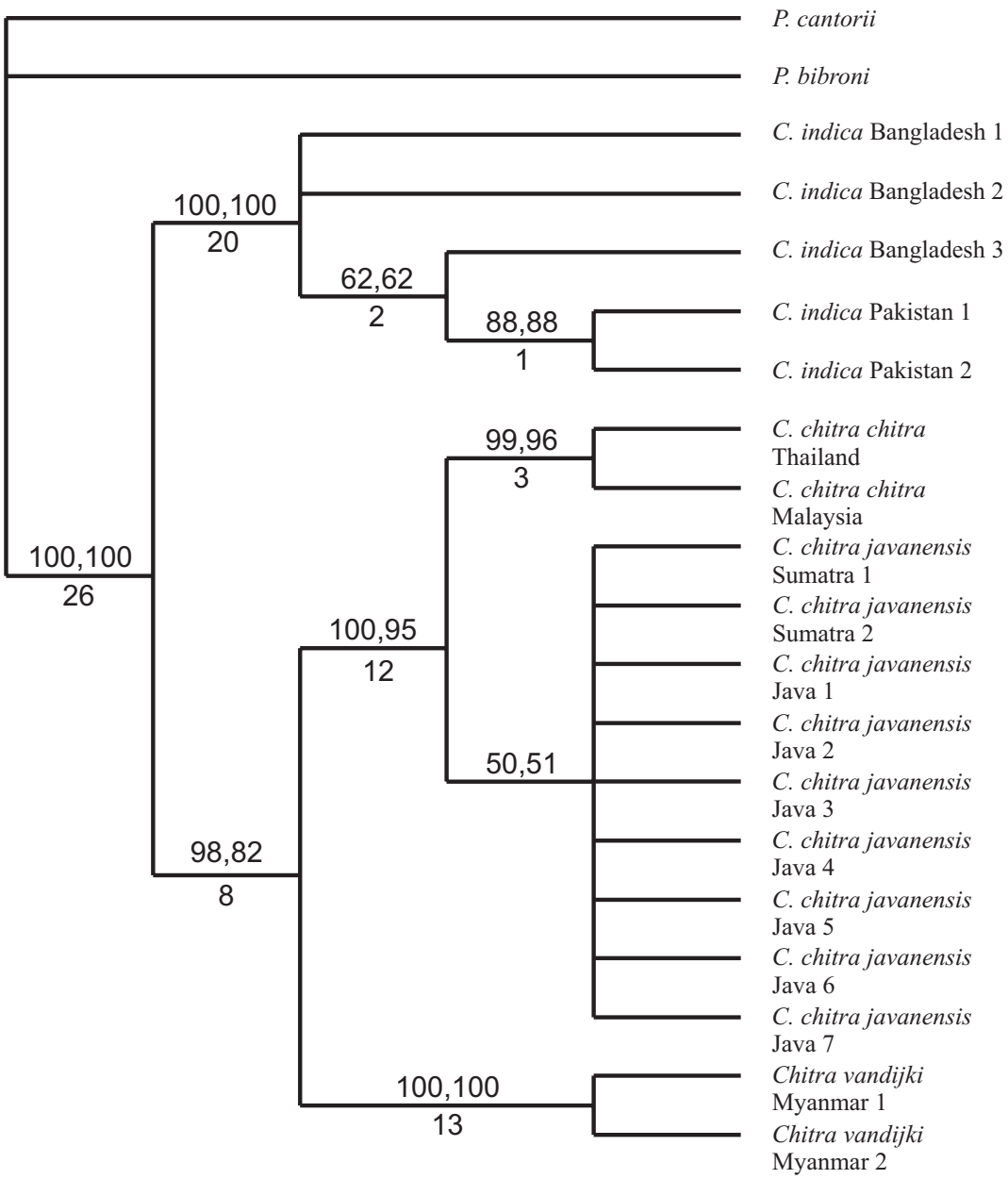

FIGURE 1: Bootstrap consensus phylogeny depicting the relationships of the genus *Chitra* based on likelihood and parsimony analysis (both indicate identical relationships) of the ND4 gene. Numbers above the node represent the bootstrap support for the node in 1000 replicates under likelihood on the left, under parsimony on the right. Numbers below the node indicate decay index for the node.

TABLE 1: Sequence divergence in ND4 among the outgroup taxa, *Pelochelys cantorii* and *Pelochelys bibroni*, and all unique *Chitra* sequences. Uncorrected ("p") distance if given below the diagonal, absolute number of differences is given above the diagonal.

	1	2	3	4	5	6	7	8	9	10	11
1. *P. bibroni*	–	53	87	88	91	90	93	92	87	88	94
2. *P. cantorii*	0.072	–	82	83	86	85	86	85	80	81	79
3. *C. indica* Bangladesh 1,2	0.119	0.112	–	1	4	3	62	61	58	57	58
4. *C. indica* Bangladesh 3	0.120	0.113	0.001	–	3	2	63	62	59	58	59
5. *C. indica* Pakistan 1	0.124	0.117	0.005	0.004	–	1	66	65	62	61	62
6. *C. indica* Pakistan 2	0.123	0.116	0.004	0.002	0.001	–	65	64	61	60	61
7. *C. chitra chitra* Thailand	0.127	0.117	0.084	0.086	0.090	0.089	–	1	8	7	39
8. *C. chitra chitra* Malaysia	0.126	0.116	0.083	0.085	0.089	0.088	0.001	–	7	6	38
9. *C. chitra javanensis* Sumatra	0.119	0.109	0.079	0.081	0.085	0.083	0.011	0.010	–	1	37
10. *C. chitra javanensis* Java	0.120	0.111	0.078	0.079	0.083	0.082	0.010	0.008	0.001	–	38
11. *C. vandijki* Myanmar	0.128	0.108	0.079	0.081	0.085	0.083	0.053	0.052	0.051	0.051	–

leg at the junction of the distal humerus and the proximal radius and ulna, and preserved in lysis buffer (10 mM EDTA, 100mM Tris-HCl, and 1.0% SDS at pH 8.0) at a blood:buffer ratio of 1:10. All samples were stored at 4°C. DNA was extracted using standard Phenol:Chloroform proteinase K methods (Hillis et al., 1996). PCR amplification of the mitochondrial ND4 gene was carried out using primers ND4 (5'TGACTACCAAAAGCTCATGTACAAGC -3'), and Hist-ND4 (5'CCTATTTTAGAGC CACAGTCTAATG3'), which are slightly modified versions of primers described by Forstner et al. (1995). Reactions were run for 35 cycles at 94º C (1 min), 50º C (1 min), 72º C (1 min). PCR products were sequenced at the Division of Biological Sciences Sequencing Facility at the University of California, Davis. All sequences were confirmed by sequencing both the forward and reverse strands. Sequences were aligned by eye using the program SeqEd (ABI) and deposited in Genbank with accession numbers (AF494489-93).

Phylogenetic analysis was conducted using parsimony and maximum likelihood methods as implemented in PAUP* ver 4.0b10 (Swofford, 2000). All trees were rooted using *Pelochelys cantorii* and *P. bibroni* as outgroups. Parsimony searches were conducted using the branch and bound search algorithm with all characters equally weighted. Maximum likelihood analysis was conducted using parameter estimates provided by Modeltest Vers. 3.0 (Posada and Crandall, 2001). Statistical support for topologies was assessed using non-parametric bootstrap resampling with 1000 bootstrap replicates.

RESULTS AND DISCUSSIONS

Our primer set consistently amplified a 731 bp fragment consisting of 708 nucleotide positions coding for 236 amino acids at the 3' end of the mitochondrial ND4 gene and 23 nucleotide posi-

tions of the adjacent histidine tRNA gene. The sequences contained no indels and were unambiguously aligned. The $tRNA^{hist}$ secondary structure is consistent with other published $tRNA^{hist}$ and all ND4 sequences successfully translated into protein products similar to published turtle ND4 proteins (Starkey, 1997; Zardoya and Meyer, 1998; Kumazawa and Nishida, 1999; Mindell et al., 1999).

Parsimony analysis resulted in five equally parsimonious trees (length = 182; CI = 0.896; RI = 0.951). The strict consensus of these trees was identical to the bootstrap consensus trees for both parsimony and likelihood (Fig. 1). Maximum likelihood analysis resulted in a single tree, which is very similar to the bootstrap consensus trees, but also provides weak support for the reciprocal monophyly of the mainland and island forms of *Chitra chitra*.

The inclusion of these new data do little to change the results presented in Engstrom et al. (2002) and are completely consistent with the taxonomic conclusions presented by McCord and Pritchard (2002). In all molecular analyses there is strong support for five monophyletic groups within *Chitra*: 1) a *Chitra indica* clade including new Pakistani animals, 2) a non *Chitra indica* clade, 3) a clade consisting of the two individuals from Myanmar, 4) a *Chitra chitra* clade consisting of individuals from Thailand, Malaysia, Sumatra and Java and 5) a mainland *Chitra chitra* clade including Malaysia and Thailand specimens. Sequence divergence between the *Chitra indica* clade and the clade including *Chitra chitra* and the *Chitra* from Myanmar was as high as 8.6% (Table 1) which is comparable to divergence levels among other well-recognized species in the family Trionychidae (Weisrock and Janzen, 2000; Engstrom et al., unpublished data). The 5.3% sequence divergence between *Chitra chitra* and *Chitra* from Myanmar is greater than that reported within other trionychid species (Weisrock and Janzen, 2000), and is evidence that these two lineages have experienced a long period of independent evolution. The clear phylogenetic pattern in the molecular data, and the evidence of long independent evolution of these lineages offer strong support for McCord and Pritchard's (2002) interpretation of the *Chitra* from Myanmar as a new species, *Chitra vandijki*.

Molecular divergence within the *Chitra chitra* clades was low but showed consistent geographic structure. Sequences from the four newly sequenced individuals from Java were identical to the three previously reported sequences and together these seven animals from Java differed by a single transition substitution from the two animals from Sumatra. The two island populations show 1% sequence divergence from the mainland form represented here by individuals from Thailand and Malaysia. This level of sequence divergence is similar to levels of divergence seen among subspecies of North American softshell turtles (Weisrock and Janzen, 2000) and is consistent with McCord and Pritchard's (2002) subspecies delineations.

In contrast, within *Chitra indica,* we observed a maximum of 0.5% divergence among the five animals sampled from two localities. One at the western edge of its range in Pakistan and the other at the eastern edge of the range in Bangladesh. Two of the individuals from Bangladesh were identical while the third was more closely related to the two from Pakistan. Our sampling of *C. indica* is far from comprehensive, and should be interpreted as a suggestion of genetic uniformity across the entire range of the species. This apparent genetic uniformity across such a broad geographic area is itself an interesting biogeographic phenomenon, which merits further investigation.

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