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Phylogenetic diversity of endangered and critically endangered southeast Asian softshell turtles (Trionychidae: *Chitra*)

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Abstract

The intense exploitation of turtles in Asian markets has contributed to declines in turtle populations across the continent. Three-quarters of Asia's turtles are threatened and half are endangered. A recent workshop on the Asian turtle crisis identified taxonomic studies of widespread species as a priority for research because these low risk species may include unrecognized, narrowly distributed taxa of much higher concern. *Chitra indica* is a widely exploited softshell turtle (family Trionychidae) found across southern Asia. Individuals from Thailand have been described as a separate species, *Chitra chitra*, but this has not been universally accepted, and many sources consider *Chitra* monotypic. Phylogenetic analysis of sequence data from the mitochondrial ND4 gene revealed three deeply divergent, monophyletic lineages within *Chitra*: *C. indica*, *C. chitra*, and a third unnamed form from Myanmar. This new form is probably Critically Endangered, which highlights the importance of systematic studies in determining conservation priorities.

Keywords: Southeast Asia; Turtle crisis; Phylogeny; *Chitra*; mtDNA

1. Introduction

The role of phylogenetics in conservation is becoming widely accepted, particularly in poorly known taxa (Forey et al., 1994). On a very basic level, the recognition of biotic diversity is the first necessary step towards its conservation; at a more practical level, taxonomy is the foundation of current conservation legislation, prioritization and practice (Avise, 1989). Incomplete assessments of taxonomic diversity can result in undocumented extinctions (Daugherty et al., 1990), while accurate documentation of phylogenetic diversity is an important tool in developing effective conservation strategies (Vane-Wright et al., 1991). One particularly troubling form of neglected taxonomy occurs when narrowly distributed species of high conservation concern are subsumed within widespread taxa of lower conservation priority (Lamb et al., 1994; Chippindale et al., 2000).

The turtle and tortoise fauna of Asia is the most diverse, least known, and perhaps most threatened in the world. Of the approximately 260 recognized turtle species worldwide, 85 occur in Asia (Iverson, 1992) where they are affected by intense exploitation for food, the pet trade and traditional medicinal uses (van Dijk et al., 2000). In a recent workshop held in Phnom Penh, Cambodia, an international panel of biologists and wildlife trade specialists concluded that three-quarters of the known turtle species in Asia are threatened, half are Endangered, 28% are considered Critically Endangered, and one is formally recognized as Extinct (van Dijk et al., 2000). One of this panel's key recommendations was that taxonomic studies of widespread species complexes are needed to identify cryptic, high-priority species that may not be currently recognized.

Here, we present a case study of dangerously neglected taxonomy in the softshell turtle genus *Chitra* (Testudines: Trionychidae). In Asia, all softshell turtles, including *Chitra*, are highly prized as a luxury food item and for products used in traditional Chinese medicine, leading to extensive human exploitation and population declines (van Dijk et al., 2000). The genus *Chitra* has traditionally been considered as a single species, *Chitra*

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indica, which is distributed across southern Asia from Pakistan through India and Bangladesh, with an apparently disjunct population in peninsular Thailand (Iverson, 1992; Fig. 1). Recently, Wirot (1986; cited in Thirakhupt and van Dijk, 1994) recognized individuals from Thailand as a separate species, *C. chitra*, although no formal species description has been published. The recognition of *C. chitra* has come into widespread use by some authors familiar with the species (Thirakhupt and van Dijk, 1994; van Dijk and Thirakhupt, 1995; van Dijk et al., 2001), but has not been universally accepted, particularly in the broad literature on turtle diversity (Ernst and Barbour, 1989; Iverson, 1992). *Chitra indica* has recently been reclassified from Vulnerable to Endangered across its range by IUCN Red List criteria (van Dijk et al., 2000). In addition, if *Chitra chitra* is a valid species, it is classified as Critically Endangered by the IUCN, meaning that it is in imminent danger of extinction.

Recent field work has extended the range of *Chitra* in several parts of southeast Asia. The presence of *Chitra* in Myanmar (formerly Burma) has been suspected for some time (Iverson, 1992; Platt et al., 2000) but was only confirmed recently (Platt, 2001). Platt et al. (2000) suggested that *C. indica* is likely present in Myanmar based on its occurrence in neighboring India and Bangladesh. However, because Myanmar lies directly between the known range of *C. indica* and that purported for *C. chitra* in Thailand, it has never been clear if individuals from Myanmar would represent the Endangered *C. indica*, the Critically Endangered *C. chitra*, or possibly a third, currently unrecognized species. If the animals from Myanmar are *C. indica*, then they are an interesting extra-limital population of this Endangered species. If they are members of a valid *C. chitra*, then they must be considered Critically Endangered. Finally, if they represent a separate, endemic species, then the only known specimens of the taxon would be the two used in the current study and a third reported in a recent field survey in northern Myanmar (Platt, 2001). Clearly, both the correct interpretation of *C. chitra*, and the taxonomic status of the enigmatic Myanmar animals are essential components in determining conservation priorities and future management directions for these turtles.

Because of the confusion over species boundaries between *C. indica* and *C. chitra*, as well as the uncertain taxonomic assignment and undescribed diversity within newly acquired material from Myanmar, we undertook a molecular analysis of phylogenetic diversity across the genus. We have included specimens of *C. indica* from Bangladesh, "*C. chitra*" from Thailand, as well as additional material from eight animals collected from outside the previously known range in Myanmar, Malaysia, and from the Indonesian islands of Java and Sumatra. In this paper we use sequence data from the

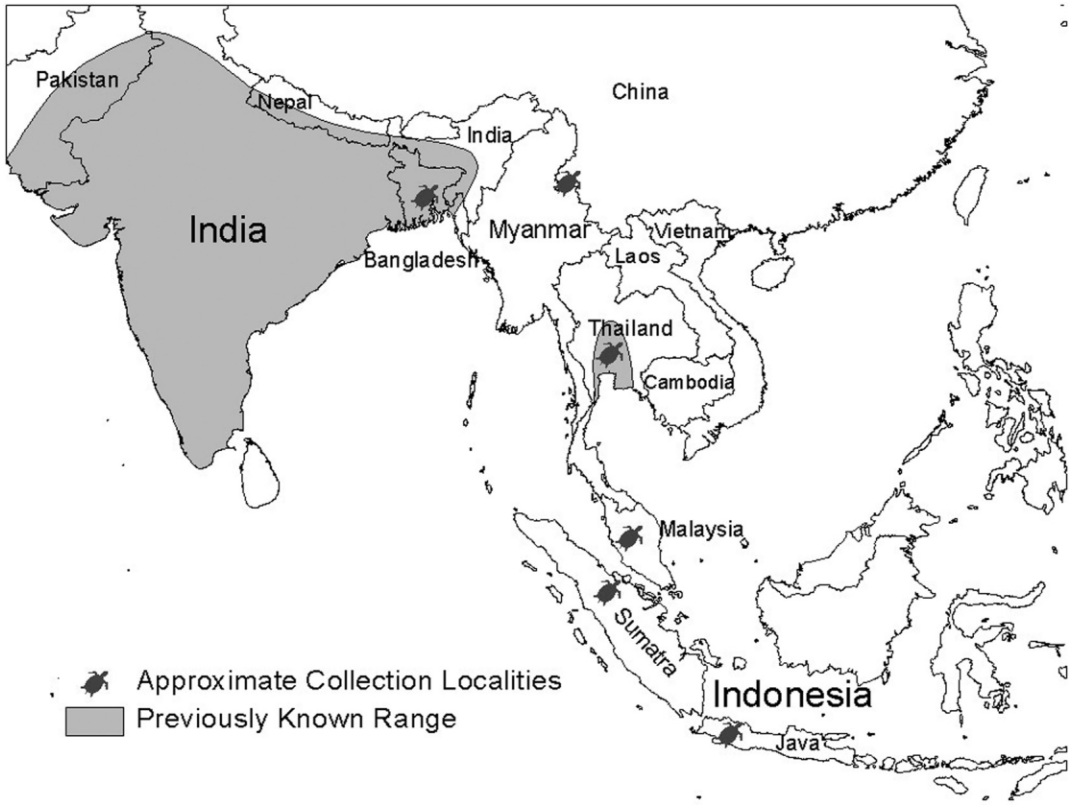

Fig. 1. Map of southeast Asia showing previously known range of *Chitra* and approximate collection localities for animals examined in this study.

mitochondrial ND4 gene to address the phylogenetic distinctiveness of these morphologically diverse forms. A formal taxonomic description of these taxa is currently underway by P.C.H. Pritchard and W. P. McCord and will be presented elsewhere.

2. Methods

2.1. Samples and laboratory procedures

Most of the samples used in this study were derived from animals obtained through the Asian pet trade and food markets. All market specimens were collected locally and obtained in the field by agents with whom one of us (W.P.M.) has had a long-term professional relationship. Thus, we feel confident in the accuracy of the locality data at the level presented here. Voucher specimens for individuals that died in captivity have been deposited in the Chelonian Research Institute collections (Oviedo, Florida, USA; Peter C. H. Pritchard, director; indicated below by PCHP numbers); all others remain alive in the private collection of WPM. *Pelochelys cantorii*—Peninsular Thailand, PCHP 4974; *Pelochelys bibroni*—Irian Jaya, east of Merauke, WPM; *C. indica* (two specimens)—markets of Dhaka, Bangladesh, WPM; *C. chitra* Thailand—from pet trade 20 years ago, WPM; *C. chitra* Malaysia—central east coast of peninsular Malaysia, PCHP 5412; *Chitra* Sumatra (three specimens): Eastern Sumatra, WPM; *Chitra* Java (four specimens), Eastern Java, WPM; *Chitra* Myanmar (two specimens PCHP 4896, PCHP 4897), from food market trade, taken from shipments coming from Myanmar into Ruili, China.

Blood and tissue samples were collected from living animals or salvaged from dead animals in the private collection of WPM. Blood samples were drawn from the front leg at the junction of the distal humerus and the proximal radius and ulna, and preserved in lysis buffer (10 mM EDTA, 100 mM Tris–HCl, and 1.0% SDS at pH 8.0) at a blood:buffer ratio of 1:10. One sample of muscle tissue salvaged from the specimen from Malaysia was preserved in vodka for shipment to the lab where it was transferred to 70% ethanol. All samples were stored at 4 °C. DNA was extracted using standard Phenol:Chloroform proteinase K methods (Hillis et al., 1996). Approximately 100 ng of purified DNA was used as template for the PCR amplification of the mitochondrial ND4 gene using primers ND4 (5′TGACTACCA AAAGCTCATGTACAAGC-3′), and Hist-ND4 (5′CCTATTTTAGAGCCACAGTCTAATG3′), which are slightly modified versions of primers described by Forstner et al. (1995). Reactions were run for 35 cycles at 94 °C (1 min), 50 °C (1 min), 72 °C (1 min) using Boeringer–Manheim *Taq* Polymerase, and all reactions were run using negative controls (all reagents except DNA) to detect possible contamination. PCR products were checked for size by electrophoresis in a 1% agarose gel, purified using 30,000 ng DNA filters (Ultrafree Millipore), and sequenced on an ABI 377 automatic sequencer at the University of California, Davis sequencing facility. All sequences were confirmed by sequencing both the forward and reverse strands. Sequences were aligned using Clustal X (Thompson et al., 1997) and are deposited in Genbank (accession numbers AF414360-AF414367).

2.2. Data analysis

Phylogenetic analysis was conducted using parsimony and maximum likelihood methods as implemented in PAUP* ver 4.0b3a (Swofford, 2000). All trees were rooted using *P. cantorii* and *P. bibroni* as outgroups. In more extensive studies of softshell turtle phylogenetics these species have been shown to be the closest extant relatives of *Chitra* (Meylan, 1987; Engstrom et al., unpublished data). Parsimony searches were conducted using the branch and bound search algorithm with all characters equally weighted. Maximum likelihood analysis was conducted using empirically determined base frequencies, no enforcement of a molecular clock, unequal substitution rates across sites assuming a gamma distribution with the number of invariant sites, the shape parameter (α), and the transition/transversion ratio estimated from the data. Statistical support for topologies was assessed using non-parametric bootstrap resampling. Under parsimony, 1000 bootstrap replicates were performed using heuristic searches, while under likelihood, 100 bootstrap replicates were performed using parameter values estimated from the most likely tree based on heuristic searches. Decay indices were calculated using the program AutoDecay version 4.0.2 (Eriksson, 1998).

3. Results

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 positions of the adjacent histidine tRNA gene. 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). Of 731 aligned nucleotides, 81 were variable within *Chitra* and of these 79 were parsimony informative. First codon positions accounted for 17 of the variable sites (of which 16 were parsimony informative), while second codon positions accounted for five variable sites, all of which were parsimony

informative. The majority of the variation detected was in third position sites, which accounted for 59 variable sites (58 parsimony informative). Base frequencies showed the paucity of guanine typical of mitochondrial protein coding genes, with base frequencies A: 0.341, C: 0.334, G: 0.105, T: 0.220. Uncorrected pair-wise distances range from 0.0 to 8.8% within the *Chitra* ingroup and 10.5–12.8% between ingroup and outgroup (*Pelochelys*) taxa (Table 1).

Parsimony analysis resulted in five equally parsimonious trees (length = 179; CI = 0.89; RI = 0.91). The strict consensus of these trees was identical to the bootstrap consensus trees for both parsimony and likelihood (Fig. 2). Maximum likelihood analysis resulted in a single tree (Fig. 3), which is very similar to the bootstrap consensus trees, but provides weak support for the reciprocal monophyly of the island and mainland forms of *C. chitra*. In all molecular analyses, there is strong support for five monophyletic groups within *Chitra*: (1) a *C. indica* clade, (2) a non *C. indica* clade, (3) a clade consisting of the two individuals from Myanmar, (4) a *C. chitra* clade consisting of individuals from Thailand, Malaysia, Sumatra and Java and (5) a mainland *C. chitra* clade including Malaysia and Thailand specimens. The relationship of this mainland clade to island populations in Sumatra and Java remains unresolved in the parsimony and bootstrap analyses, while the maximum likelihood tree provides weak evidence indicating that the island forms may be a monophyletic sister group to the mainland clade.

Sequence divergence between the *C. indica* clade and the clade including *C. chitra* and the *Chitra* from Myanmar was as high as 8.6% (Table 1), confirming a relatively deep divergence of these two lineages. Divergence between the *C. chitra* clade and the Myanmar clade was also high, ranging from 5.1 to 5.3%, again indicating substantial divergence between these two lineages. In contrast, molecular divergence within the *C. chitra* clade was very low. Sequences from the two individuals sampled from Sumatra were identical, as were sequences of the three individuals sampled from Java. These two island populations differed from each other by a single transition substitution. Individuals from mainland Thailand and Malaysia also differed from one another by a single base pair change, and these mainland animals differed from island individuals by 7–8 base pair changes (1% sequence divergence).

4. Discussion

The mtDNA data presented here strongly support the presence of three divergent evolutionary lineages within the genus *Chitra*; the widely recognized *C. indica*, the recently named *C. chitra* (Wirot, 1986), and a third, previously unrecognized lineage from Myanmar. The high level of sequence divergence (8.6%) between *C. indica* and the clade including *C. chitra* and the *Chitra* from Myanmar is comparable to divergence levels evident in other well-recognized species in the family Trionychidae (Weisrock and Janzen, 2000; Engstrom et al., unpublished data). If mtDNA evolution in the genus *Chitra* proceeds at the same slow rate of about 0.125%/lineage/myr that has been observed in other turtles (Avise et al., 1992), then the molecular divergence between these two lineages suggests a divergence date of approximately 34 million years ago (mya). Similarly, the 5.3% sequence divergence between *C. chitra* and the *Chitra* from Myanmar would correspond to a divergence date of approximately 21 mya. There is considerable variation in estimates of rates of molecular evolution within turtles (Avise et al., 1992; Dutton et al., 1996; Weisrock and Janzen, 2000). In the absence of a molecular clock calibrated specifically for these taxa, these estimates should be interpreted primarily as evidence that the lineages have experienced a long period

Table 1
Sequence divergence for the outgroup taxa, *Pelochelys cantorii* and *Pelochelys bibroni*, and all *Chitra* individuals[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Pelochelys cantorii*	–	53	82	83	86	85	80	80	81	81	81	77	77
2. *Pelochelys bibroni*	0.072	–	87	88	93	92	87	87	88	88	88	94	94
3. *Chitra indica* 1	0.112	0.119	–	1	62	61	58	58	57	57	57	58	58
4. *C. indica* 2	0.113	0.120	0.0014	–	63	62	59	59	58	58	58	59	59
5. *C. chitra* Thailand	0.118	0.127	0.085	0.086	–	1	8	8	7	7	7	39	39
6. *C. chitra* Malaysia	0.116	0.126	0.083	0.085	0.0014	–	7	7	6	6	6	38	38
7. *C. chitra* Sumatra 1	0.109	0.119	0.079	0.081	0.011	0.0096	–	0	1	1	1	37	37
8. *C. chitra* Sumatra 2	0.109	0.119	0.079	0.081	0.011	0.0096	0.0000	–	1	1	1	37	37
9. *C. chitra* Java 1	0.111	0.120	0.078	0.079	0.0096	0.008	0.0014	0.0014	–	0	0	38	38
10. *C. chitra* Java 2	0.111	0.120	0.078	0.079	0.0096	0.008	0.0014	0.0014	0.0000	–	0	38	38
11. *C. chitra* Java 3	0.111	0.120	0.078	0.079	0.0096	0.008	0.0014	0.0014	0.0000	0.0000	–	38	38
12. *Chitra* Myanmar 1	0.105	0.128	0.078	0.081	0.053	0.052	0.051	0.052	0.051	0.052	0.052	–	0
13. *Chitra* Myanmar 2	0.105	0.128	0.078	0.081	0.053	0.052	0.051	0.051	0.052	0.052	0.052	0.0000	–

[a] The numbers of substitutions are above the diagonal; uncorrected *P* distances are below it.

of independent evolution and not as exact dates of divergence. Given the clear phylogenetic pattern in the data, the evidence of long independent evolution of these lineages and strong concurrent morphological variation (P.C.H. Pritchard & W.P. McCord, personal communication), we argue for the recognition of three species within the genus *Chitra*.

The issue of variation within the *C. chitra* clade is less clear. Although our data clearly support a monophyletic mainland *C. chitra* clade (Fig. 2), the reciprocal monophyly of island and mainland clades is very weakly supported. The grouping of Java and Sumatra as sister to the mainland forms is preferred under the likelihood criterion, however, the difference in the likelihoods of this topology and one in which Java is sister to the Mainland is not significant ($X^2 = 0.1306$, d.f. = 1, $P > 0.50$) using a log likelihood ratio test (Cox and Hinkley, 1974). Under the assumption of island monophyly, the mainland form shows 1% sequence divergence from the two island populations. Given our paucity of sampling and the low resolving power of our data for these island samples, we suggest that more information is needed before we can make an informed decision about the evolutionary uniqueness of the Indonesian *Chitra*.

Sites and Crandall (1997) have justifiably criticized some aspects of the current application of molecular data to define species boundaries. One of their primary critiques centers on the interpretation of molecular data without reference to an explicit species concept. In the interpretation of molecular data in this paper we have adopted an evolutionary lineage based approach to species definition (Templeton, 1994), with the assumption that long-term reproductive isolation will be reflected in the divergence of genetic markers and morphologies within those independent lineages. Sites and Crandall (1997) also expressed concern that sampling strategies are often not adequate to address the issue of boundaries between species. In fact, the most important caveat to the molecular results presented here is that they are based on an analysis of a single mitochondrial marker from a few individuals from just six localities, spread across a broad geographic range. The mtDNA haplotypes, which appear fixed in our small samples, may prove to be polymorphic in a larger sample. As limited as our sampling is, it represents our best effort to sample as much diversity as possible both within and among populations. Unfortunately, it is

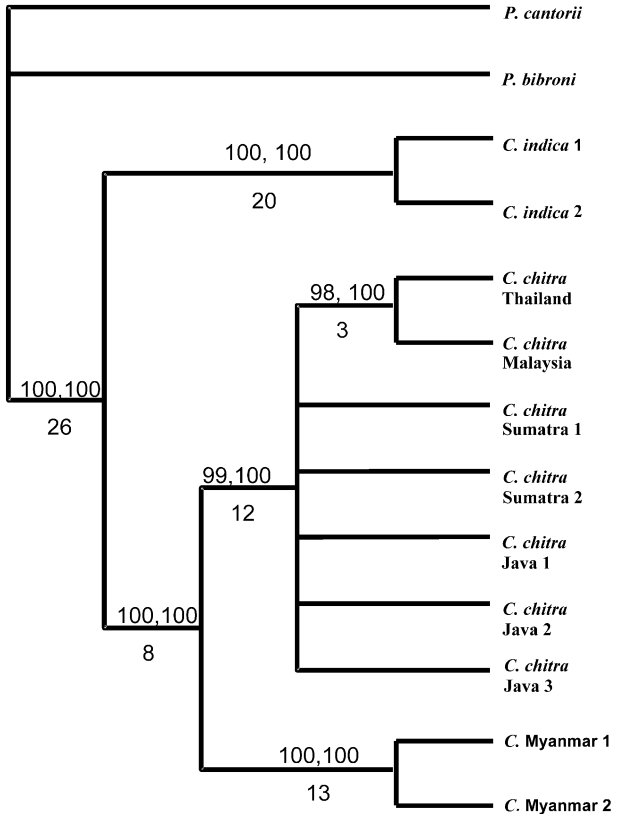

Fig. 2. Bootstrap consensus phylogeny depicting the relationships of the genus *Chitra* based on likelihood and parsimony analysis (both indicate identical relationships) of the ND4 gene. Numbers above the node represent the bootstrap support for the node in 100 replicates under likelihood on the left, and in 1000 replicates under parsimony on the right. Numbers below the node indicate decay index for the node.

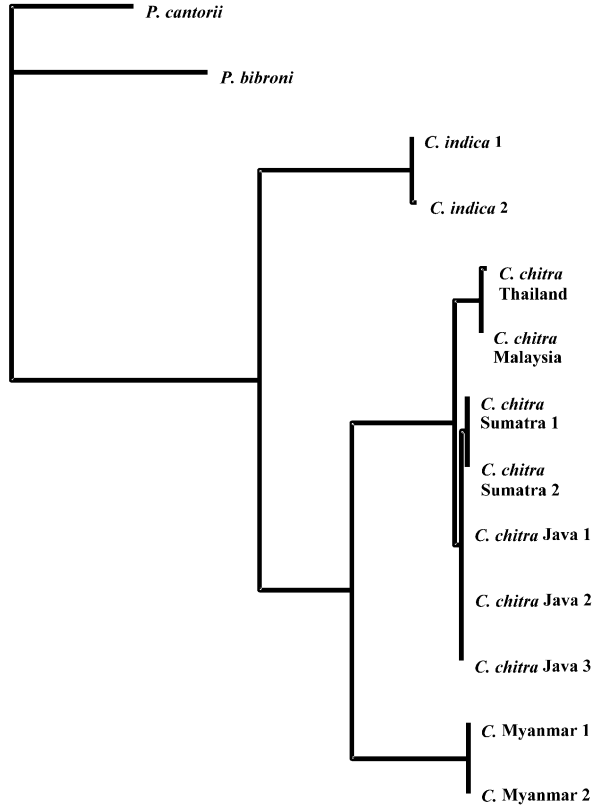

Fig. 3. The single most likely topology of the phylogenetic relationships within the genus *Chitra* based on likelihood analysis of the ND4 gene. This topology shows the monophyly of the island and mainland clades within *Chitra chitra*.

unlikely that a substantially larger sample will be available for study in the foreseeable future, given that these animals are increasingly rare due to the threats of the Asian turtle trade (van Dijk et al., 2000). Our feeling is that the interpretation of phylogenetic data for Critically Endangered taxa such as *Chitra* must constitute a balance between the precision that comes from exhaustive sampling, and the necessity of making taxonomic and management decisions in a timely manner. One of our goals has been to use the samples that are available to formally recognize the diversity of turtles in this region before that diversity disappears.

With the recognition of the limitations imposed on this study by the small sample size available, and the use of a single mitochondrial marker, we have tended to interpret our results conservatively. The minor molecular differences we observed between individuals from Java and Sumatra do not strongly support nor refute taxonomic recognition of these forms. Until further evidence supporting separation of these populations becomes available, we will continue to recognize these animals as members of single species. However, our molecular data do indicate low-level divergence between the mainland and island forms that is equal to the level of divergence seen among closely related subspecies of North American softshell turtles (Weisrock and Janzen, 2000). If this molecular differentiation is accompanied by consistent morphological or nuclear DNA differentiation, then it may be appropriate to recognize two taxa (mainland and island) within *C. chitra*. Only when mtDNA sequence divergence implies the long-term evolutionary independence of lineages, as is the case among *C. indica*, *C. chitra*, and the Myanmar *Chitra* do we feel confident in using mtDNA data to suggest taxonomic revisions. Based on these molecular data we recommend that each of these three clades be recognized as species level taxa. The formal description of the new species from Myanmar is in progress (P.C.H. Pritchard & W.P. McCord, personal communication).

It will be necessary to evaluate the potentially critical conservation status of *Chitra* in Myanmar separately from the Endangered *C. indica*. The population status and distribution of this turtle is almost completely unknown. The only known specimens of this animal are the two used in this study and one individual reported in a village along the Ayeyarwady in northern Myanmar (Platt, 2001). Platt (2001) reports that the locals are familiar with *Chitra* and use the local name "kabar leik", but regard it as rare. This assessment is confirmed by the fact that all previous surveys of the turtle fauna of Myanmar have failed to record its presence (van Dijk, 1993, 1994, 1997; Kuchling, 1995; Platt, 1999; Platt, 2000; Platt et al., 2000). The fact that all three known records of *Chitra* from Myanmar are from the food trade is discouraging, and indicates that this species is likely to be threatened by the same local cultural and global economic forces that have lead to massive declines of other Asian turtle species (van Dijk et al., 2000). It is not likely that the Myanmar *Chitra* will be protected by its rarity because large softshell turtles are preferred in the Asian turtle trade and draw very high prices (van Dijk et al., 2000). In addition, *Chitra* will continue to be taken opportunistically by local fishermen in pursuit of fish or turtle species such as *Nilsonnia formosa, Lissemys scutata* and *Cyclemys dentata* which are still relatively common (Platt et al., 2000).

It is clear from this study that the global turtle trade threatens not just the known Asian turtle species but additional unknown species as well. The discovery of significant genetic diversity within the eastern half of the distribution of *Chitra* indicates that phylogenetic conservation research is an essential compliment to the economic, ecological, and legislative efforts that are being rallied to slow the rising specter of extinction due to the Asian turtle trade. Confirmation of the valid species status of *C. chitra* will hopefully aid in its universal recognition as a Critically Endangered species. Recognition of the Myanmar *Chitra* as a distinct species would bring the number of endemic turtle species in Myanmar to seven, which highlights the importance of this biologically rich but economically poor nation to regional and global turtle biodiversity. Further systematic research is likely to discover additional hidden diversity in other Asian turtle species, and we feel that this activity should be given high priority in conservation assessments of the endangered Asian turtle fauna. The documentation of this diversity must be seen as an activity that is done not just for posterity but for immediate action and protection.

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