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Species Identification of *Kachuga tecta* Using the Cytochrome b Gene

ABSTRACT: A DNA technique has been established for the identification to species level of tortoises. The test on the shell of the animal was used to identify samples from the species *Kachuga tecta*. A total of 100 tortoise shell specimens collected from the National Council of Agriculture (COA), Taiwan, were used in this study. Primer pairs were designed to amplify partial DNA fragments of cytochrome b within the mitochondrial genome. The DNA data showed that among the 100 samples, there were four distinct haplotype DNA sequences, within which there were a total of 90 variable sites. Between haplotypes I and II, there was only 1 nucleotide difference at position 228. Between haplotypes I and III, 65 nucleotide differences were observed; haplotypes I and IV, 62 nucleotide differences; and haplotypes III and IV, 56 nucleotide differences were observed. There were 66 and 63 nucleotide differences between haplotypes II and III and haplotypes II and IV respectively. All four haplotypes were compared with the DNA sequences held at the GenBank and EMBL databases. The most similar species were *K. tecta* (haplotype I and II), *Morenia ocellata* (haplotype III) and *Geoclemys hamiltonii* (haplotype IV), and their respective mtDNA similarities were 99.5%, 99.3%, 89.9% and 99.5%. However, as haplotype III was only 89.9% homologous with *M. ocellata*, it would seem that this haplotype shows only a limited relationship with a similar species registered currently in these databases. The method established by this study is an additional method for the identification of samples protected under Convention International Trade in Endangered Species (CITES) and will improve the work for the preservation of the endangered species.

KEYWORDS: forensic science, *Kachuga tecta*, endangered species, cytochrome b gene, CITES

The tortoise, and related shelled animals, is a traditional symbol of longevity in many Far East Asian countries. Certain species of tortoise are protected under Convention International Trade in Endangered Species (CITES); however ornaments and preparations are made from the tortoise for supposed properties of producing longevity. The meat of these animals is also featured in food with supposed "tonic" properties, and the shell may be listed on menus. Normally, tortoise shells are cooked to a gelatinous consistency and mixed with other ingredients in a recipe. The products are sold in the form of gel, soup, pill, capsule, or extract, all of which are part of eastern cuisine culture. The Council of Agriculture (COA) in Taiwan currently has approximately 10,000 specimens of seized samples from tortoise species, many of which require identification.

Usually, species identification of tortoise-shell animals can be performed unambiguously using morphological characters. Often, samples seized by law enforcement organizations are processed so that visual identification is no longer possible. The species of tortoise-shell animal in this study is *Kachuga tecta* (Indian sawback turtle), which is classified as Chordata, Reptilia, Testudinata and Emydidae. This family comprises nine extant species, all of which are listed in CITES Appendix I and II as endangered species and are perilously close to extinction. *Kachuga tecta*, the most widespread and common species, was previously listed in Appendix I of CITES, indicating that no trade is possible due to the perilously low numbers of the species, and now is listed in Appendix II, where control of trade is required.

Molecular analysis is now a standard tool in taxonomic and phylogenetic studies, with most studies focusing on genes in the mitochondrial genome (1–24) or DNA loci in the nuclear genome (13,15). Within the mitochondrial genome, the cytochrome b gene (cyt b) is the most popular locus (4,8,9,11,12, 21,23), with 12S rRNA (1,4,12,13,16,19,22,24) and 16S rRNA (4,13,22) genes also being well studied. In population studies, the control region of the mtDNA (10,14), some nuclear genes (13,15), minisatellite (25) and microsatellite loci (26,27) are used. In this study, the cyt b gene of the mitochondrial genome will be used to establish a DNA test in the species identification of *K. tecta* not only because it is a standard genetic test, but also because previous reports indicated that this gene should evolve at a rate appropriate for both inter- and intrafamilial phylogenetic studies of turtles (11).

Materials and Methods

Sampling

A total number of 100 tortoise dorsal shell samples were selected randomly from more than 10,000 seized samples in the warehouse of the COA, Taiwan. These specimens were seized by officials and many of them were suspected as originating from *K. tecta*. A reference sample ventral shell of *K. tecta* was provided by the COA, which had identified this sample previously.

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DNA Extraction

A small fragment of approximately 5 cm^2 of shell for each specimen was cut into a number of small fragments and processed by washing, drying, and pulverizing. Approximately 100 mg of the pulverized sample was suspended in extraction buffer (0.1 M Tris-HCl pH 7.5, 3% SDS, 60 mM NaCl) with 10 μg/μL of proteinase K and kept for 56°C overnight. After digestion, a NaCl solution was added to a final concentration of 1.5 M and mixed with an equal volume of chloroform. Then, after gently shaking for half an hour, the solution was centrifuged with a high G-force (approximately 5000 × *g*). The supernatant was purified using a DNA column (Blood and Tissue Genomic Mini Kit, Viogene, Taipei, Taiwan). The resulting DNA was dissolved in 30 μL of ddH_2O.

Amplification and Sequencing of Cyt b Gene

Nested PCR amplification was used in this study. Two primer pairs were designed, as in Fig. 1 and Table 1. The universal primers of L14724 and H15149 were designed according to the report by Irwin et al. (28). H15197 was designed according to our previous study (unpublished), and L14735t was designed according to the sequence of first PCR products in this study. The outer primer pair was used first in the first PCR amplification. If the resulting PCR products were insufficient for DNA sequencing, a secondary PCR was performed using the inner primer pair and 1 μL of the first PCR products as the template. PCR amplification was performed in 50 μL of a reaction mixture, which contained 5 μL of genomic DNA, reaction buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (w/v) gelatin and 0.1% TritonX-100), 1.25 U of VioTaq DNA polymerase and 0.15 μM each of primers. Amplification was conducted in a 480 Applied Biosystems (Foster City, CA) thermal cycler under the following conditions: 40 cycles of 94°C for 45 sec, 50°C for 45 sec and 72°C for 1 min. Cycle sequencing of PCR products was conducted in a 2400 Perkin-Elmer thermal cycler with the following conditions: 25 cycles of 95°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Sequencing was performed using the primers L14735t and H15149 and the BigDye™ Terminator Kit (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit). The cycle sequencing products were separated using a 5% denatured Long Ranger™ gel (FMC BioProducts, Rockland, ME), and detected using an Applied Biosystems 373A DNA sequencer.

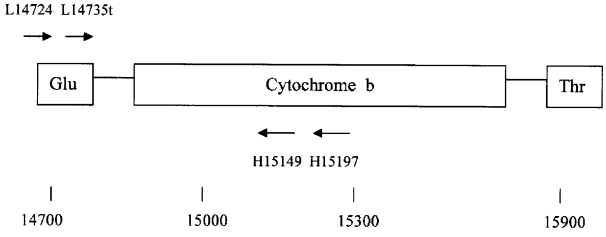

FIG. 1—*Positions of primers for the amplification of part of the cytochrome b gene of mitochondria in this study. The numbering is according to the human mtDNA sequence (31).*

Sequence Analysis

DNA sequences were aligned using the PileUp program of the GCG computer package (Wisconsin Package Version 10.3, Genetics Computer Group (GCG), Madison, WI). The consensus sequences were deduced by the Pretty program, and the genetic distance analysis was generated by Kimura's 2-parameter distance program of the GCG computer package. The phylogenetic tree was constructed by the neighbor-joining method of Phylip computer package.

Results

The extracted DNA from the 100 samples was highly degraded, most likely because of the age and storage conditions of the sample. DNA could not be visualized by agarose gel electrophoresis. The nested PCR amplification was adopted for some samples, since clear PCR products could not be obtained by the first PCR amplification. The primer pairs, L14724/H15197 and L14735t/H15149, were used to amplify part of the cyt b in two sequential reactions, producing products approximately 470 bp in size. The second set of primers was used to sequence the nested PCR products. The signals of the sequence data were without ambiguities and with a signal sufficiently strong to allow base designation with confidence.

After excluding the primer sequences and sequences upstream of the ATG position, which is the first codon of the cyt b gene, 405 bp selected for further comparison. The DNA sequences were aligned by the PileUp program of the GCG computer package, and consensus sequences were deduced by the Pretty program (Fig. 2). There were four haplotypes within the 100 samples tested, within which there were 90 variable sites in the 405 bp of analyzed sequences. Between haplotypes I and II, there was only 1 nucleotide difference at position 228. Between haplotypes I and III, 65 nucleotide differences were observed; haplotypes I and IV, 62 nucleotide differences; and haplotype III and IV, 56 nucleotide differences. There were 66 and 63 nucleotide differences between haplotypes II and III and haplotypes II and IV, respectively. The control sample provided by the COA, a ventral shell of *K. tecta*, was found to have the same sequence as haplotype I.

The DNA sequences were subjected to a similarity search using GenBank and EMBL databases. Haplotypes I and II matched *K. tecta* (accession number AY434583) with a homology of 99.5%. The closest match to Haplotype III was with *Morenia ocellata* (accession number AY434605) but at a homology of only 89.9%, indicating a low genetic similarity to any species listed to date in the DNA database. Haplotype IV matched the closest with *Geoclemys hamiltonii* (accession number AY434573) with a homology of 99.5%.

TABLE 1—*Sequences of nested primer pairs and their amplified size.*

	Primers	Sequences	Size (bp)
First PCR	L14724	5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′	523
	H15197	5′-CCGATATAAGGGATTGCTGA-3′	
Secondary PCR	L14735t	5′-CCATCGTTGTAATCAACTAC -3′	470
	H15149	5′-TAACTGTAGCCCCTCAGAATGATATTTGTCCTCA-3′	

The genetic distances among species in this study were determined by Kimura's 2-parameter distance program of GCG computer package. Between haplotype I sequence and haplotype II sequence, the genetic distance was 0.25; between haplotype I sequence and haplotype III sequence, it was 18.48; between haplotype I sequence and haplotype IV sequence, it was 17.67; between haplotype II sequence and haplotype III sequence, it was 18.82; between haplotype II sequence and haplotype IV sequence, it was 18.02; between haplotype III sequence and haplotype IV sequence, it was 15.64. The genetic distance of two tortoise samples indicated to be the most closely related was 0.25, between specimens of haplotype I sequence and haplotype II sequence, and the two samples most distant (18.82) was between specimens of haplotype II sequence and haplotype III sequence. A neighbor-joining tree was constructed from the genetic distance data by the Phylip computer package and the values of bootstrap analysis

```
                1                                                   50
Consensus       ATGACTAAAA ATTTACGAAA AACCCACCCA ATAGTAAAAA TCATCAACAA
tecta           .......... .......... .......... .......... ..........
haplotype I     .......... .......... .......... .......... ..........
haplotype II    .......... .......... .......... .......... ..........
haplotype III   ...g.c.... .cc....... ...t.....g ...a...... ..........
haplotype IV    ...g.c.c.. .cc....... ...t.....c ...a...... ..........

               51                                                  100
Consensus       TTCATTTATC GATCTCCCAA GTCCCTCCAA CATCTCTGCC TGATGAAACT
tecta           .......... .......... .......... .......... ..........
haplotype I     .......... .......... .......... .......... ..........
haplotype II    .......... .......... .......... .......... ..........
haplotype III   c......... ..c..t.... .c........ ......c..t ........t.
haplotype IV    c........t ..c..t.... ....t..... ...t..ct.. ........t.

              101                                                  150
Consensus       TCGGATCTCT ATTAGGCGCC TCCCTAATCC TACAAGTCAC CACAGGAATC
tecta           .......... .......... .......... .......... ..........
haplotype I     .......... .......... .......... .......... ..........
haplotype II    .......... .......... .......... .......... ..........
haplotype III   .t..c..... tc.c..a..t .g......a. .....a...t ...c...c.a
haplotype IV    .t..c..c.. cc........ .g.t.....t .....a.... ...c..g..a

              151                                                  200
Consensus       TTCCTGGCAA TACACTATTC ACCAGATATC TCACTAGCAT TCTCATCAGT
tecta           .......... .......... .......... .......... ..........
haplotype I     .......... .......... .......... .......... ..........
haplotype II    .......... .......... .......... .......... ..........
haplotype III   ...t.a.... .......c.. .........t ..c....... .......c..
haplotype IV    .....a.... .......... ...g..c... .....g.... ..........

              201                                                  250
Consensus       ATCCCATATC ACCCGAGATG TCCAATACGG ATGGCTTATC CGTAACATAC
tecta           .......... .......... .......... .......... ..........
haplotype I     .......... .......... .......... .......... ..........
haplotype II    .......... .......... .......t.. .......... ..........
haplotype III   ca........ ..t.....c. .a........ g..a...... ..a..t....
haplotype IV    .g........ ........c. .a........ g..a..c... ..c..t....
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FIG. 2—*Partial sequences of cytochrome b gene. The symbol "." indicates the same base as the consensus sequence. All the samples tested were 405 bp in size. Sample tecta stands for sequences generated from the standard sample offered by the Council of Agriculture.*

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                251                                                   300
Consensus          ATGCTAATGG AGCCTCCATC TTCTTTATAT GCATCTACCT CCACATTGGC
tecta              .......... .......... .......... .......... ..........
haplotype I        .......... .......... .......... .......... ..........
haplotype II       .......... .......... .......... .......... ..........
haplotype III      ....c..... .......c.t ..t..c.... ....t..t.. ......c...
haplotype IV       .......c.. ......tg.. .....c.... .t..t..... ......c..t

                301                                                   350
Consensus          CGAGGCCTTT ACTACAACTC ATACTTATAC AAAGAAACCT GAAACACAGG
tecta              .......... .......... .......... .......... ..........
haplotype I        .......... .......... .......... .......... ..........
haplotype II       .......... .......... .......... .......... ..........
haplotype III      .....a..c. ......g... ....c....t ........t. ..........
haplotype IV       .....a.... ....tgg... c......... .......... ..........

                351                                                   400
Consensus          AATCACCCTC TTATTCCTAA CCATGGCCAC CGCATTCGTA GGCTACGTCC
tecta              .......... .......... .......... .......... ..........
haplotype I        .......... .......... .......... .......... ..........
haplotype II       .......... .......... .......... .......... ..........
haplotype III      .....t.t.a c..c...... ....a..... t......... ........a.
haplotype IV       .g...t...a .....t.... ....a..... t......... .....t...t

                401
Consensus          TACCA
tecta              .....
haplotype I        .....
haplotype II       .....
haplotype III      .....
haplotype IV       .....
```

FIG. 2—Continued

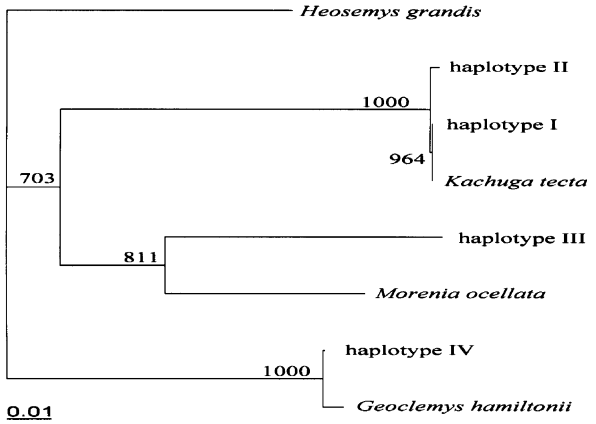

FIG. 3—*A phylogenetic tree based on the partial sequence of cytochrome b was constructed by the neighbor-joining method. Confidence values for internal lineages were assessed with the bootstrapping option. The sequence of Heosemys grandis (accession number AY434566) was extracted from GenBank for comparison. Scale bars represent branch length.*

with 1000 replicate runs were labelled on the branches of the tree (Fig. 3).

Discussion

In this study, two primer pairs were designed to amplify specifically the part of the cyt b of 100 seized samples believed to be from the tortoise species *K. tecta*. In DNA sequence analysis, haplotypes I and II matched *K. tecta* (accession number AY434583) with a homology of 99.5%, and in genetic distance analysis, between haplotype I sequence and haplotype II sequence, the genetic distance was 0.25. The results were consistent with the morphological data and our previous study, where DNA sequence diversity within the same species was from 0.25 to 2.74 (29). With respect to tortoise species, Lenk et al. (8) reported that the genetic distance within the same species (*Emys orbicularis*) was from 0.0009 to 0.0171, which is a smaller size range of variation than for many other species. From the molecular data, 88 specimens of the 100 tested had either haplotype I (82 spec-

imens) or II (6 specimens) DNA sequence and therefore were highly likely to be *K. tecta*. For the remaining 12 specimens, 11 specimens with haplotype III were the same tortoise species and the remaining sample matched the DNA sequence of *G. hamiltonii*. Both species of *K. tecta* and *G. hamiltonii* are listed in CITES II.

Spinks et al. (30) report that some turtle species may be hybrids due to the interbreeding between the closely related species and can occur in turtle farming. This interbreeding can results in the misidentification of species using mitochondrial DNA. However, if the samples analyzed are from hybrid species and were produced as a result of captive farming efforts, they are not recognized species and are therefore not candidates for protection. In such cases it may be appropriate to analyse nuclear DNA sequences because of its bi-parental inheritance.

The method established by this study can be used in the identification of species as well as for the identification of unknown samples with atypical appearances, and could be valuable for the identification of preparations made from tortoise shells. Although the method may have wide application, we have insufficient evidence, at present, to demonstrate its efficacy on processed shell materials.

Acknowledgments

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