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Full Length Research Paper

Interspecific hybridization between *Mauremys reevesii* and *Mauremys sinensis*: Evidence from morphology and DNA sequence data

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Turtle hybrids have been found in some taxa, but so far, studies on interspecific hybridization between *Mauremy reevesii* and *Mauremy sinensis* have not been reported. Recently, we obtained three specimens (Hy1, Hy2, Hy3) with unusual morphological characteristics from pet trade Market, which are suspected to be hybrids rather than new species, because they were morphologies intermediate between *M. reevesii* and *M. sinensis*. In further study, we analyzed two aspects; morphological characteristics and molecular data, separately. The morphological characteristics showed that the pattern of the carapace, the plastron and neck stripes of the three specimens was between that of *M. reevesii* and *M. sinensis* (the morphological features of Hy1 and Hy2 have more resemblance with those of *M. sinensis*, and those of Hy3 have more resemblance with those of *M. reevesii*). In molecular analyses, two mitochondrial genes (12S, cytb) and two nuclear genes (RAG-1, R35) were respectively cloned from each suspected specimen. One sequence was obtained for each mitochondrial gene, while two different sequences were obtained for each nuclear gene. Phylogenetic analyses revealed that mitochondrial genes sequences from each suspected specimen clustered into the corresponding sequences of their putative female parents, while the two pairs nuclear parental alleles sequences were strongly paraphyletic, for they were included in two different genetic lineages (*M. reevesii* and *M. sinensis*). Therefore, we concluded that the three suspected specimens are hybrids (Hy1 and Hy2: *M. reevesii*♂ × *M. sinensis*♀; Hy3: *M. sinensis*♂ × *M. reevesii*♀). It is the first report that interspecies hybridization of *M. reevesii* and *M. sinensis* can cross completely.

Key words: *Mauremys reevesii*, *Mauremys sinensis*, hybridization, morphology, DNA sequence.

INTRODUCTION

Hybridization, defined as the interbreeding of individuals from genetically distinct populations, commonly occurs in various animals (Rhymer and Simberloff, 1996). In Testudines, hybridizations have also been reported in some taxa. For example, Karl et al. (1995) reported the hybridizations between marine turtle species (family Cheloniidae): *Caretta caretta* × *Lepidochelys kempii*, and *Chelonia mydas* × *Eretmochelys imbricate*. Within freshwater turtle species, hybridizations have also been found, such as *Emydoidea blandingii* × *Glyptemys insculpta* (Harding and Davis, 1999), and *Cuora mouhotii* × *Cuora bourreti* (Parham et al., 2001; Stuart and Parham, 2004). Some of the specimens matching the reported hybrids were captured in the wild, such as the hybrid (*C. mydas* × *C.caretta*) reported by James et al. (2004), which was found in St. Margarets Bay, Nova Scotia, on 2 October 2001; others were purchased from the pet trade or private breeders, such as the hybrids (*Sacalia* ×*Mauremys*) reported by Buskirk et al. (2005), which were offered by a private breeder in California, USA. These hybrids are intermediate of well known species on morphological characters, and parental species of hybrids were mainly inferred based on morphology.

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Interspecies hybrids of genera *Mauremys* (Testudines: Geoemydidae) were first reported from Japan where researchers found the propensity for related species to breed in the wild and in captivity. Since that time, seven hybridization events have been reported for this genera: *Mauremys reevesii* × *Mauremys japonica* (Aoki, 1990), *M. japonica* × *M. reevesii*, *M. japonica* × *Mauremys sinensis*, *M. reevesii* × *M. sinensis* (Otani, 1995), *Mauremys caspica* × *Mauremys rivulata* (Fritz and Wischuf, 1997), *M. reevesii*♂ × *Mauremys mutica*♀ (= "*Mauremys pritchardi*", Wink et al., 2001), *M. sinensis*♂ × *Mauremys annamensis*♀ (="*Ocadia glyphistoma*", Spinks et al., 2004). In these hybridizations, most parental lineages could not be well known, for most cases they were from captivity with several species together, and in some cases their origin were even unknown. Generally, the parental lineages could be simply inferred from morphological data. But for the specific identity of maternal versus paternal lineages, it could only be inferred from genetic data, such as mitochondrial DNA, or nuclear DNA data (Parham et al., 2001; Wink et al., 2001; Stuart and Parham, 2004, 2007). However, the hybrids reported by Aoki (1990), Otani (1995) and Fritz and Wischuf (1997) were not verified by genetic data, and the specific identity of parental lineages for the aforementioned most hybrids could not be inferred.

M. reevesii and *M. sinensis* are two common species in the Southeast Asian. The habitats of the two species are similar, such as shallow ponds, marshes, streams and canals etc. (Iverson, 1992; Zhou, 2006). In addition to the hybridization events within *Mauremys*, Schilde et al. (2004) reported a hybrid from *Cyclemys shanensis*♂ × *M. sinensis*♀, while Buskirk et al. (2005) also reported a hybrid from *Sacalia quadriocellata*♂ × *M. reevesii*♀. These hybridizations suggested that *M. reevesii* and *M. sinensis* have the ability to hybridize with the other individual members of its family, Geoemydidae (Buskirk et al., 2005). However, a complete interspecific hybridization between *M. reevesii* and *M. sinensis* has not been found.

In this study, three additional specimens of "*M. reevesii*" were suspected to be hybrids because of morphologies intermediate between *M. reevesii* and *M. sinensis*. In testing the hybrid origin hypothesis for the three suspected specimens, their morphological characteristics were compared with the related species; their putative maternal lineages were inferred based on the mitochondrial genes; the phylogenetic position of their putative parental lineages within relevant geoemydid turtles were analyzed base on the nuclear parents alleles.

MATERIALS AND METHODS

Collection of specimens

Three suspected specimens (Hy1: No. ANUM26080018; Hy2: No. ANUM26080019; Hy3: No. ANUM26080024) were obtained from pet trade Market of Wuhu city of China. For facilitating direct analysis among results from morphological characteristics, mtDNA and nuDNA data sets, additional 8 extant geoemydid taxa were included based on diagnostic morphological characteristics in this study (Table 1). Two wild specimens *M. sinensis* were collected in Guangxi province, three wild specimens *M. reevesii* were captured in Anhui province, the other specimens (except *O. glyphistoma*) were from the Anhui provincial key laboratory of the conservation and exploitation research of biological resources in Anhui Normal University. A hybrid specimen of *O. glyphistoma* was included as reference. A specimen of *Cuora aurocapitata* was selected as outgroup for the nearest common ancestor with the geoemydids.

Morphological analyses

M. reevesii and *M. sinensis* are different in the appearance. We chose three distinct exterior features as distinguishing standard: keel on the carapace, blotch on the plastron and the peripheral plate, stripe of the head and neck, because they are three exterior recognition criteria of species *M. reevesii* and *M. sinensis*.

Choices of molecular markers

Increasing evidence indicates that single gene sometimes reflects idiosyncrasies of individual genes rather than trees of species (Ruvolo, 1997; Spink et al., 2004). Thus, we chose four genes from mtDNA and nuDNA: 12S ribosomal (12S rRNA) gene, protein-coding cytochrome b (cyt b) gene, intron of the RNA fingerprint protein 35 (R35) gene, and single-copy, intron-free, protein-coding recombination activating gene-1 (RAG-1) (Shaffer et al., 1997; Fujita et al., 2004; Krenz et al., 2005). Because maternal lineages could be inferred base on mtDNA of maternal inheritance in vertebrates, the parents could be inferred base on nuDNA of bi-parental inheritance (Perry et al., 2002).This four genes are not only enough to reconstruct a robust phylogeny, but can also reveal the specific identity of parental lineages of the suspected hybrids.

DNA extraction, amplification and sequencing

Total genomic DNA of all specimens (except *O. glyphistoma*) was extracted according to Gustincich et al. (1991). The amplifying primers in this study are included in Table 2. The thermal cycling procedure (PCR) consisted of an initial denaturation at 94°C for 5 to 9 min, followed by 35 to 40 cycles of denaturation at 94°C for 30 to 60 s, annealing at 50 to 56°C for 45 to 60 s, and extension at 72°C for 1 to 2 min. An additional extension at 72°C for 5 to10 min followed the last cycle. PCR products were electrophoresed on a 1.0% agarose gel in the presence of ethidium bromide and DNA fragments of intended sizes were recovered using a Gel Extract Purification Kit (Axygen, Hangzhou, China). The purified PCR products were cloned into $DH_5\alpha$ cells using the pMD18-T Vector System (Promega) following manufacturer's instructions to isolate the each pair of parental alleles. Multiple cloned colonies of each gene from each specimen were re-amplified and detected, then the vectors containing intended fragments were sequenced in both directions with an ABI-PRISM3730 automated sequencer (Applied Biosystems, Foster City, USA).

Sequences analyses

All sequences were edited and aligned using MEGA 4.0(Tamura et al., 2007). In aligned sequences of each mitochondrial gene from each specimen, all cloned sequences for each gene were completely identical, while one sequence remained in subsequent analyses. In aligned sequences of each nuclear gene, for each

Table 1. Samples and genes used in this study and the GenBank accession numbers of their chose sequences.

ID	Species	Sample	Provenance	GenBank			
				12S	cytb	R35	RAG-1
1	The suspected hybrid (Hy1)	ANUM26080018	Pet trade market of Wuhu, Anhui	HQ442367	HQ442413	HQ442375 HQ442376	HQ442395 HQ442399
2	The suspected hybrid (Hy2)	ANUM26080019	Pet trade market of Wuhu, Anhui	HQ442368	HQ442414	HQ442377 HQ442378	HQ442396 HQ442403
3	The suspected hybrid (Hy3)	ANUM26080024	Pet trade market of Wuhu, Anhui	HQ442369	HQ442415	HQ442379 HQ442380	HQ442397 HQ442400
4	*Mauremys sinensis* 1	ANUM26080097	Guangxi	HQ425252	HQ442408	HQ442370	HQ442394
5	*Mauremys sinensis* 2	ANUM26080098	Guangxi	HQ425253	HQ442409	HQ442371	HQ442398
6	*Mauremys reevesii* 1	ANUM26080033	Anhui	HQ425249	HQ442410	HQ442372	HQ442401
7	*Mauremys reevesii* 2	ANUM26080034	Anhui	HQ425250	HQ442411	HQ442373	HQ442404
8	*Mauremys reevesii* 3	ANUM26080035	Anhui	HQ425251	HQ442412	HQ442374	HQ442402
9	*Mauremys annamensis*	ANUM26080108	Pet trade market of Guangxi	HW131942	HQ442419	HQ442387	HQ442407
10	*Cuora aurocapitata*	ANUM26080065	Anhui	AY874540	AY874540	HQ442381	HQ442389
11	*Ocadia glyphistoma*	HBS 38414	Pet dealer in Hong Kong, China		AY434596	DQ386662 DQ386663	

Table 2. Oligonucleotide primers for amplification and sequencing of 11 extant geoemydid mitochondrial and nuclearDNA.

Primer	Sequence(5' to 3')	PCR product size (kb)	Source
12S	F: TTTCATGTTTCCTTGCGGTAC R: AAAGCACGGCACTGAAGATGC	1.2	Wang (2000)
Ctyb	F: CAACATCTCAGCATGATGAAACTTCG R: CAGTTTTTGGTTTACAAGACCAATG	1.2	Barth (2004)
Rag-1	F: AAGTTTTCAGAATGGAAGTTYAAGCTNTT R: TCTTCTTTCTCAGCAAAAGCYTTNACYTG	1.2	Hugall (2007)
R35	F: ACGATTCTCGCTGATTCTTGC R: GCAGAAAACTGAATGTCTCAAAGG	1.2	Fujita (2004)

suspected hybrid, all the aligned sequences were represented as two distinct sequences, which have significant difference between them (Mann Whitney U test, $P < 0.001$); so they were respectively maintained as two separate segments in subsequent analyses. For the other each specimen, all aligned sequences of each nuclear gene were almost the same, and they were considered as a single sequence in subsequent analyses.

Thus, all segments from three suspected specimens and the

other specimens were assembled in four datasets (12S, cytb, RAG-1, R35) in MEGA 4.0.

For *O. glyphistoma*, the sequence of cyt b gene and two parental allele sequences of R35 intron were directly available from GenBank.

To assess any occurrence of incongruence among four datasets, exploratory phylogenetic analyses were initially performed for each datasets separately using the distance-based neighbour-joining (NJ) algorithm (Saitou and Nei, 1987) implemented in MEGA 4.0. As no incongruence of 12S and cyt b datasets was identified at any well-supported node, they were concatenated into a single mtDNA dataset. However, RAG-1 and R35 datasets were still maintained separately, while for the origins of two pairs, parental allele sequences could not be distinguished and could not be concatenated into a single dataset.

Phylogenetic analyses

Phylogenetic analyses of the three final datasets (mtDNA, R35, RAG-1) were performed using the maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). The ML, and MP analyses were performed using paup*4.b10 (Swofford, 2002); BI analyses was performed with mrbayes 3.1 (Huelsenbeck and Ronquist, 2001). The best-fit model of nucleotide evolution for the three datasets was estimated using the Akaike information criterion (AIC) implemented in jModelTest0.1.1 (Posada, 2008), and subsequently implemented in the ML and BI analyses.

The three suspected specimens should be new species if they do not have a recent hybrid origin. Then the mitochondrial sequence data should be very distinct from the putative female parent , and the two parental alleles of R35 or RAG-1 gene should be closely related with each other. To test these null hypotheses that these taxa do not have a recent hybrid origin, likelihood trees were reconstructed using the same model of sequence evolution, and the mtDNA data of each taxon was constrained to be very distinct from the putative female parent *M. sinensis* or *M. reevesii* in each tree, the two parental alleles of one taxon constrained to be monophyletic in each tree. The likelihood scores of each constrained trees were compared against the unconstrained tree using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) with RELL optimization implemented in PAUP* 4.0b10.

The total data sets in this study were submitted to GenBank, including those for 12S rRNA gene, cytb gene, R35 intron and rag-1 gene per individual (except *O. glyphistoma*) (Table 1).

RESULTS

Morphological compare

Based on morphologic analysis, Hy1, Hy2 and Hy3 have combinative morphological characters of both *M. sinensis* and *M. reevesii* (Figure 1). The carapaces of those with three distinct continuous keels were similar to that of *M. reevesii*, the plastrons of those with a large fanlike black blotch on each scute were similar to that of *M. sinensis*, especially the pattern of spots on peripheral plate and bridge is the same as that of *M. sinensis*, many parallel and thicker stripes of their heads and necks were also similar to that of *M. sinensis*. Furthermore, Hy1, Hy2 and Hy3 also had tiny morphological differences on the pattern of carapace keels and neck stripes, which could Hy2) that has more resemblance with *M. sinensis*, and the other (Hy3) that has more resemblance with *M. reevesii*.

Sequences analyses

The sequences of four genes (12S, cytb, RAG-1, R35) respectively consisted of 1144, 992, 1033, and 1159 bp. In sequences alignment of each mtDNA gene, all cloned sequences were completely identical. In sequences alignment of each nuDNA gene, the two pairs sequences of R35 and RAG-1 from each suspected specimen respectively contained 4 and 10 heterozygous positions.

In all mitochondrial combined sequences (12S, cytb) alignment, of the 2136 bp aligned characters, 240 were variable and 134 were parsimony-informative; among ingroup taxa, 165 sites are variable and 129 parsimony informative. In all R35 sequences alignment, of the 1033 bp aligned characters, 17 were variable and 9 were parsimony-informative; among ingroup taxa, 10 sites are variable and 8 parsimony informative. In all RAG-1sequences alignment, of the 1159 bp aligned characters, 24 were variable and 11 were parsimony-informative; among ingroup taxa, 17 sites were variable and 10 parsimony informative.

Phylogenetic relationships

Based on mtDNA dataset, all phylogenetic analyses performed with various methods showed congruent result with respect to major topological features of the mtDNA tree (Figure 2): All samples of *M. sinensis* was sister taxon of all samples of *M. reevesii*. The female parents of the three suspected specimens were clearly showed in two different genetic lineages. The sequences of Hy1 and Hy2 closely related to the lineage of *M. sinensis*, and that of Hy3 closely related to the lineage of *M. reevesii*. SH tests showed the unconstrained tree (–ln L 4243.1408; Figure 2) had a significantly better likelihood score than any of the trees constrained to be kept away from the putative female parents of Hy1 (–ln L 4261.4447, $p<0.05$; Figure 2), Hy2 (–ln L 4261.4447, $p<0.05$; Figure 2), Hy3 (–ln L 4470.0900, $p<0.05$; Figure 2).

Based on the two nuDNA datasets, all phylogenetic analyses were performed with various methods which also displayed congruent result with respect to major topological features of two nuDNA trees (Figures 3 and 4): the nuDNA datasets of the three suspected specimens were strongly polyphyletic. Based on R35 dataset (Figure 3) or RAG-1 dataset (Figure 4), the two pairs of parental alleles were closely related to *M. reevesii* and *M. sinensis*, respectively. All samples of *M. sinensis* and all samples of *M. reevesii* clearly showed two different genetic lineages. SH tests showed the two unconstrained tree ((–ln L 1533.3150, Figure 3); (–ln L 1760.4388,

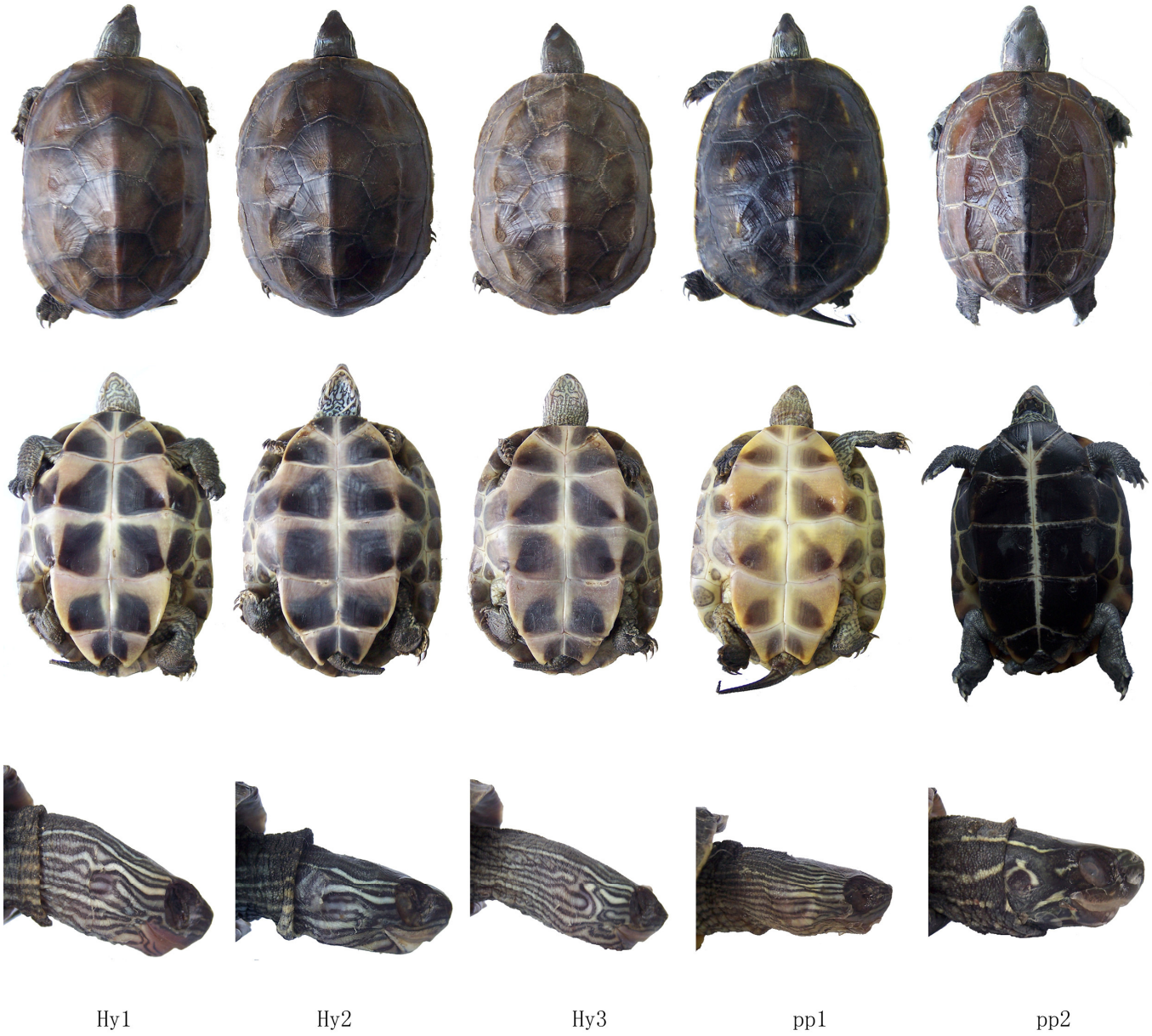

Figure 1. Specimen images of the three suspected hybrids (Hy1, Hy2, Hy3) and the putative parental species (pp1: *M. sinensis*; pp2: *M. reevesii*). Hy1, Hy2 and Hy3 have morphologies intermediate between pp1 and pp2. The carapaces of them are similar to that of pp2; whereas the plastrons and the neck stripes of them are similar to that of pp1. Especially the spots on peripheral plate and bridge of them are the same as that of pp1. Further, Hy1 and Hy2 are morphologically tiny different to Hy3 based on the pattern of carapace keels and neck stripes. Hy1 and Hy2 more resemble pp1, Hy3 more resembles pp2.

Figure 4) had respectively a significantly better likelihood score than any of the trees constrained to have monophyletic parental alleles of Hy1 (–ln L 1608.5935, $p<0.05$; Figure 3); (–ln L 1991.1773, $p<0.05$; Figure 4), Hy2 (–ln 1608.5935, $p<0.05$; Figure 3); (–ln L 1991.1773, $p<0.05$; Figure 4), Hy3 (–ln L1608.3698, $p<0.05$; Figure 3); (–ln L 1986.5969, $p<0.05$; Figure 4).

Thereby, based on our data, the three suspected specimens are hybrids with recent origins resulting from the mating of *M. reevesii*♂ × *M. sinensis*♀(Hy1, Hy2), *M. sinensis*♂ x *M. reevesii*♀(Hy3).

DISCUSSION

Three suspected specimens (Hy1, Hy2 and Hy3) have been proved as hybrids from *M. reevesii* and *M. sinensis* based on our study. Hy1 and Hy2 are the progenies of

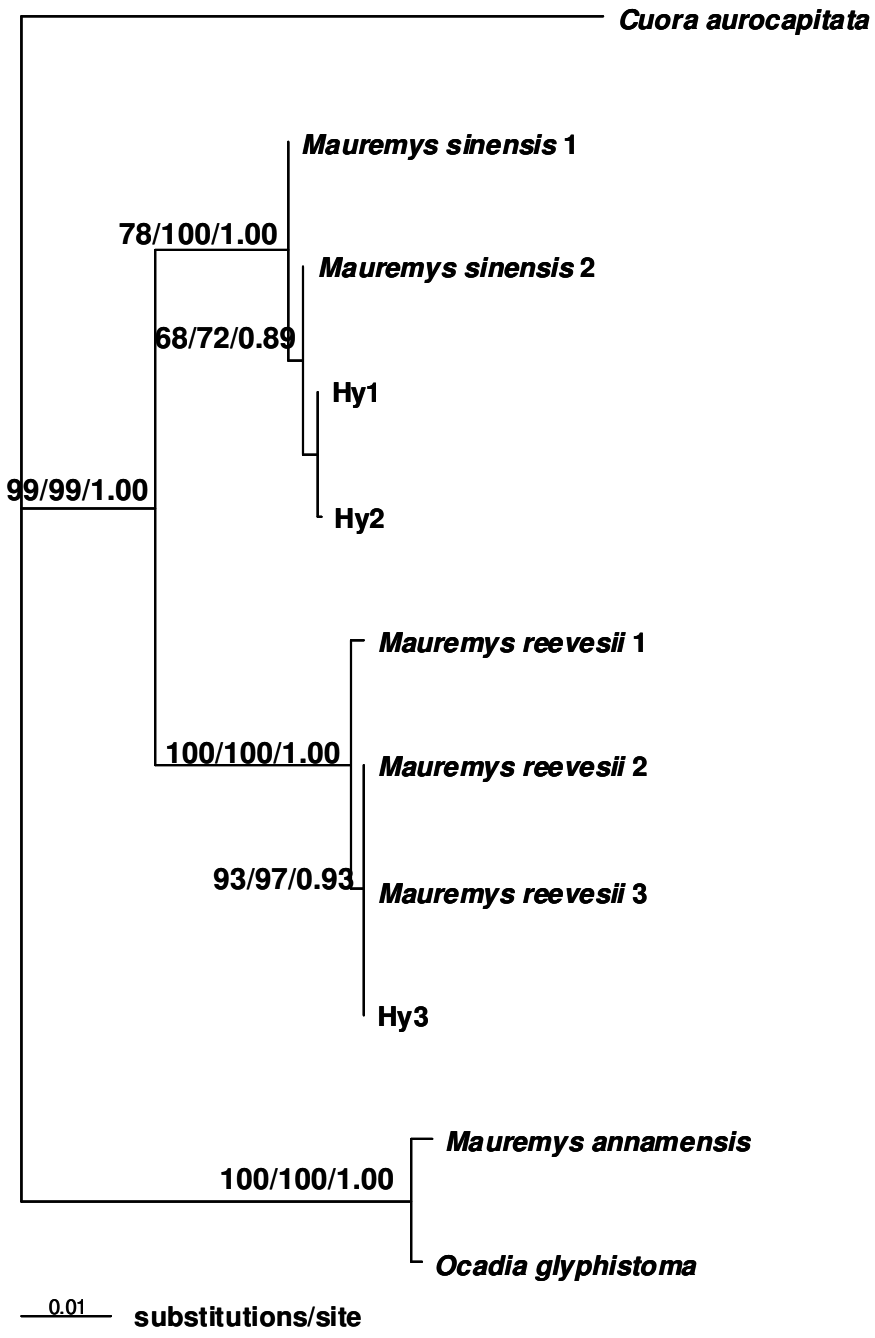

Figure 2. Maximum likelihood (ML) phylogeny of mitochondrial DNA (concatenated 12S and cytb segments, totaling 2136 bp) sampled in the eleven extant turtles. (see Table 1 for details on sample ID and collection data). Hy1, Hy2 and Hy3 stand for the individuals of the three suspected hybrids). The maximum likelihood tree (–lnL 4243.1408), using the best-fit model of sequence evolution GTR+G selected by AIC in jModeltest0.1.1, base frequencies A = 0.3370, C = 0.2844, G = 0.1427, and T = 0.2360, ti/tv ratio = 4.429. Values above branches indicate support for the subsequent node based on ML/MP/BI. The support is depicted only for nodes defining major clades relevant for our analyses.

their crosses; Hy3 is the progeny of their reciprocals crosses. It revealed that *M. reevesii* and *M. sinensis* have the ability to cross and reciprocally cross with each other. This is the first report of a complete interspecies hybridization between *M. reevesii* and *M. sinensis*.

It is known that *M. reevesii* and *M. sinensis* are closely related species, and either has small population. They have overlapping geographic distribution regions

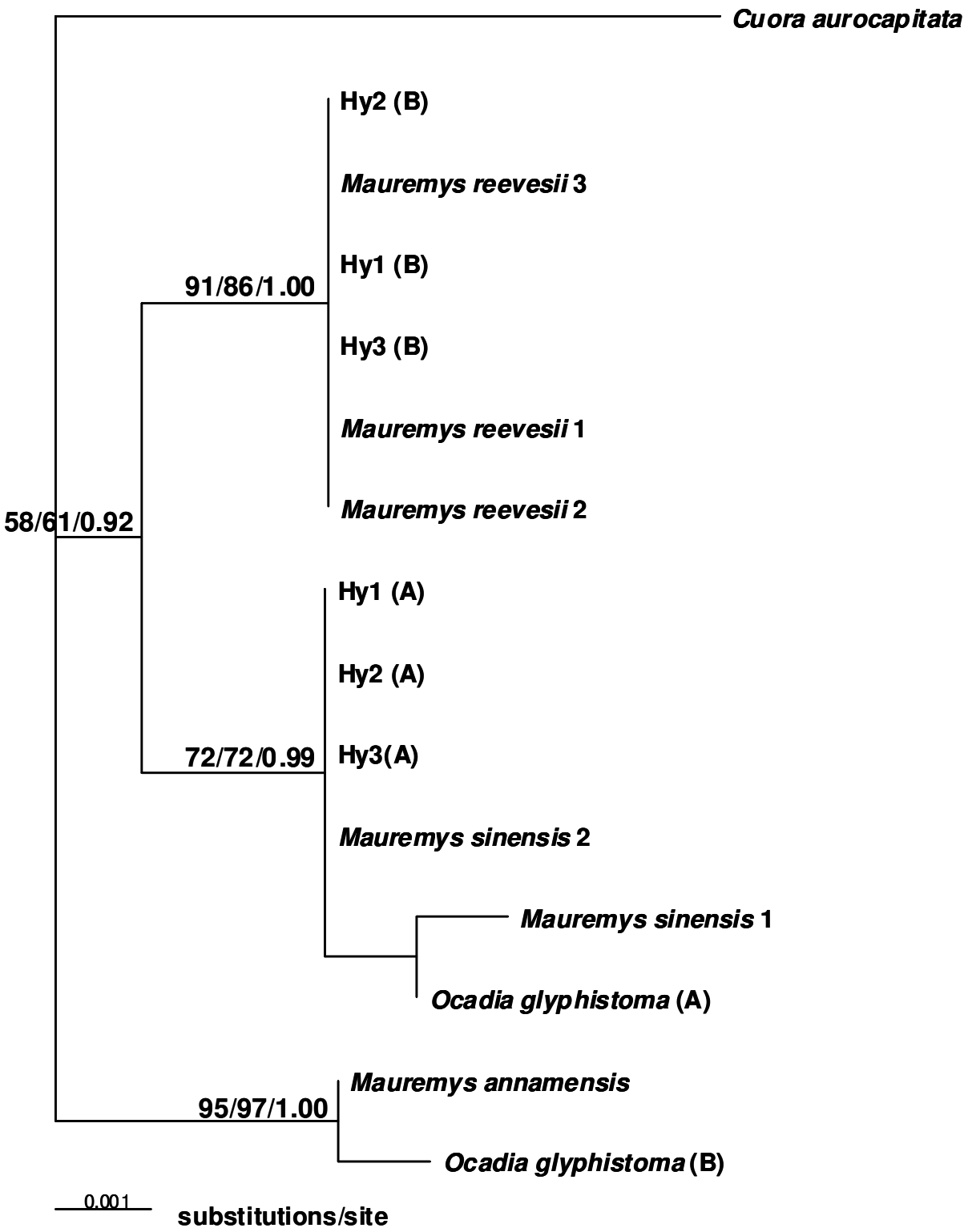

Figure 3. Maximum likelihood (ML) phylogeny of the RNA fingerprint protein 35 (R35) gene intron segments (1033bp) sampled in the eleven extant turtles, (see Table 1 for details on sample ID and collection data). Hy1, Hy2 and Hy3 stand for the individuals of the three suspected hybrids. ((A) and (B) stand for a pair parental alleles). The maximum likelihood tree (–ln L 1533.3151), using the best-fit model of sequence evolution TrN+G selected by AIC in jModeltest0.1.1, base frequencies A = 0.2794, C = 0.1736, G = 0.2133, and T = 0.3337, ti/tv ratio = 1.956. The three hybrids taxa appear twice in the tree because their two parental alleles were isolated by cloning. Values above branches indicate support for the subsequent node based on ML/MP/BI. The support is depicted only for nodes defining major clades relevant for our analyses.

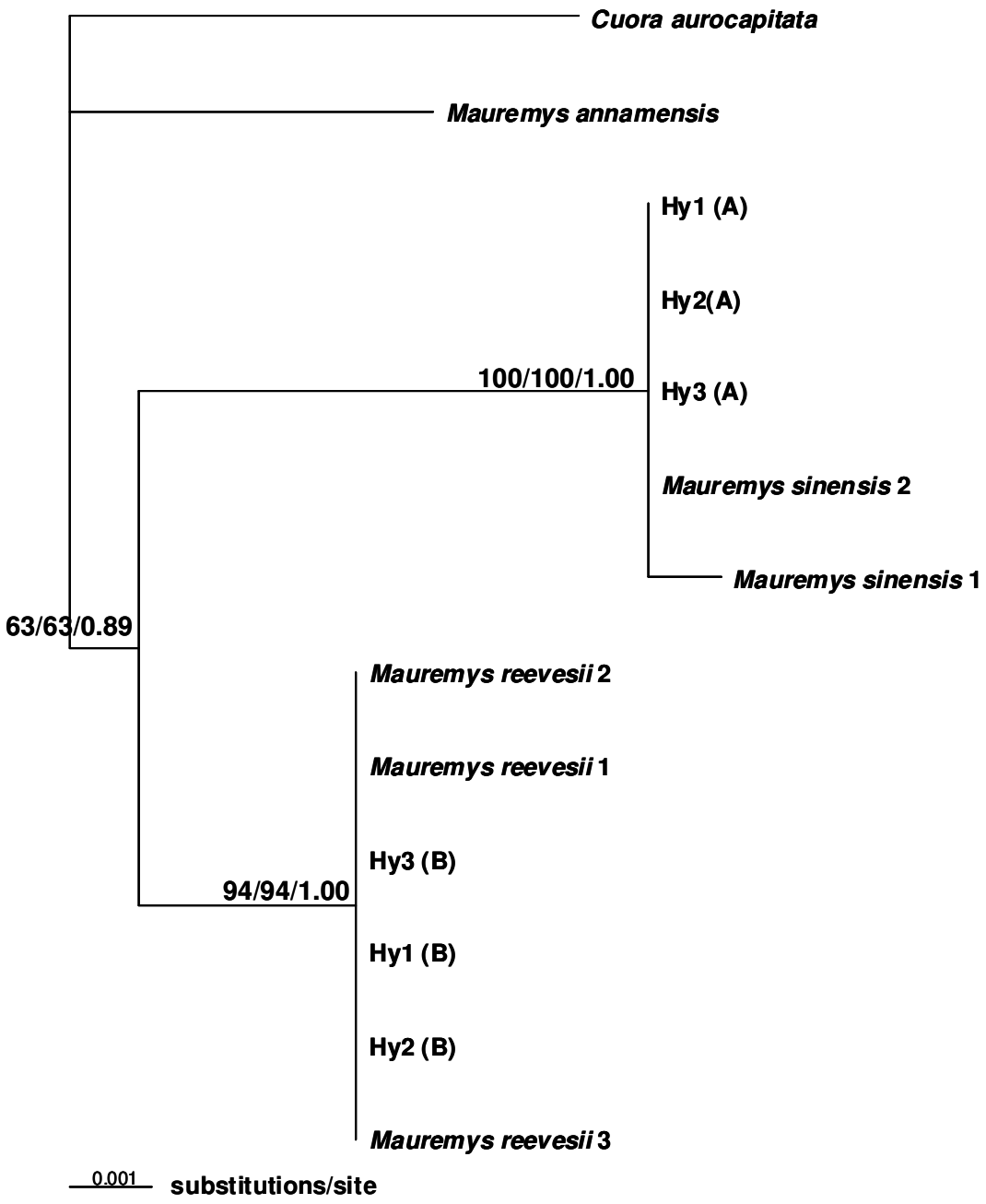

Figure 4. Maximum likelihood (ML) phylogeny of the RAG-1 gene segments (1159bp) sampled in the ten extant turtles, (see table 1 for details on sample ID and collection data. Hy1, Hy2 and Hy3 stand for the individuals of the three suspected hybrids. ((A) and (B) stand for a pair parental alleles). The maximum likelihood tree (–ln L 1760.4388), using the best-fit model of sequence evolution TrN selected by AIC in jModeltest0.1.1, base frequencies A = 0.3237, C = 0.2334, G = 0.2259, and T = 0.2170, ti/tv ratio = 5.899. The three hybrids taxa appear twice in the tree because their two parental alleles were isolated by cloning. Values above branches indicate support for the subsequent node based on ML/MP/BI. The support is depicted only for nodes defining major clades relevant for our analyses.

(Iverson, 1992; Zhou, 2006). The review of Buskirk et al. (2005) has inferred that the reproductive isolating mechanisms of some batagurid turtles should be relatively weak. To sum up the aforementioned, hybridizations can occur between some geoemydids under certain condition (Buskirk et al., 2005; Stuart and Parham, 2007). The hybrids reported here supported this hypothesis, though, we cannot judge whether our hybrids specimens from the

local pet market are natural source or human activities source.

If these hybrids were natural source, the interspecies hybridization may have considerable importance for future adaptation and even speciation; if they are human activities source, these turtles would face extinction due to polluting gene pools (Allendorf et al., 2001; Fong et al., 2007).

In recent years, for the demands of food, pets and traditional medicine, wild populations of turtles have been declining severely; almost instantly, many large-scale turtle farms come forth in the world. Breeding of small population and various species together greatly increased the potential for inbreeding depression and hybridization of turtles. In southern Asia, individual farm operators acknowledged producing and selling the hybrids for excessive profits (Parham and Shi, 2001; Parham et al., 2001; Shi et al., 2007).

Conclusion

We suggest that conservation resources are better directed toward seeking for and protecting populations of the extant turtle taxa with the relatively poor reproductive isolating mechanisms that do represent distinct evolutionary lineages. Unreasonable raising method should be cancelled in turtle farms, such as various species of turtles housed in a pool. Products of artificial breeding should serve as substitutes for wild individuals of legitimate taxa in the food, traditional medicine, and pet trade, but should be prohibited to influx natural ecosystem.

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