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# DNA evidence for the hybridization of wild turtles in Taiwan: possible genetic pollution from trade animals

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**Abstract** Field surveys in Taiwan have uncovered turtles presumed to be hybrids based on their intermediate morphology. We sequenced a mitochondrial (ND4) and nuclear (R35) gene of two putative hybrid individuals, along with representatives of the potential parental species (*Mauremys mutica*, *M. reevesii*, *M. sinensis*), to determine their genetic identity. Based on our data, both individuals are hybrids, with independent, recent origins resulting from the mating of a female *M. reevesii* and a male *M. sinensis*. Since we question whether the highly traded *M. reevesii* is endemic to Taiwan, this hybridization could represent human-mediated genetic pollution. We also discuss the implications of our findings on turtle conservation in Taiwan.

**Keywords** *Mauremys mutica* · *Mauremys sinensis* · *Mauremys reevesii* · Asian turtle crisis · Introduced species · Conservation

## Introduction

Recent Asian turtle research has uncovered the ability of distantly related species in the family Geoemydidae to hybridize (see Buskirk et al. 2005 for a review). This

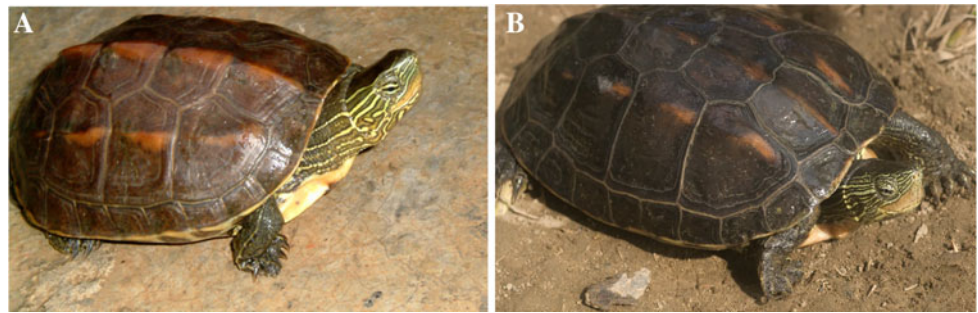
propensity for reticulation combined with a lack of detailed study of Asian geoemydids has sometimes confused herpetologists; at least five newly described species have turned out to be the result of recent, human-mediated hybridizations (Parham et al. 2001; Spinks et al. 2004; Stuart and Parham 2007). Asian turtles face extinction due to the turtle trade (van Dijk et al. 2000), and anthropogenic hybridization complicates the situation by polluting gene pools and exploiting precious research and conservation resources (Allendorf et al. 2001; Fong et al. 2007). Whereas most hybrids are thought to be formed in Chinese turtle farms (Parham and Shi 2001; Parham et al. 2001; Shi et al. 2007, 2008), there have been reports of turtle hybrids appearing in the wild (Otani 1995; Shi et al. 2005; Haramura et al. 2008). These studies reemphasize the point that understanding the origin of Chinese turtle hybrids may require multiple explanations (Parham et al. 2001).

Detailed surveys of turtle populations throughout Taiwan (Chen and Lue in press) have identified two likely hybrids (Fig. 1). Of the five freshwater turtle species found in Taiwan, the habitat and morphology of the probable hybrids narrowed the potential parental species to three: *Mauremys mutica*, *M. sinensis*, and *M. reevesii*. *Mauremys sinensis* is widely distributed in the low-elevation regions throughout Taiwan, while *M. mutica* mainly inhabits hilly areas in northern Taiwan. *Mauremys reevesii* is currently restricted to Kinmen Island (Fig. 2; ~2 km east of Fujian Province and ~270 km west of Taiwan), but was historically recorded from the Taipei Basin (Horikawa 1934; Mao 1971). The goals of this study are to genetically determine whether morphologically unidentifiable individuals are hybrids, and if so, determine their parental species. We also discuss whether *M. reevesii* is endemic to Taiwan, and the implications of our findings on Taiwanese turtle conservation.

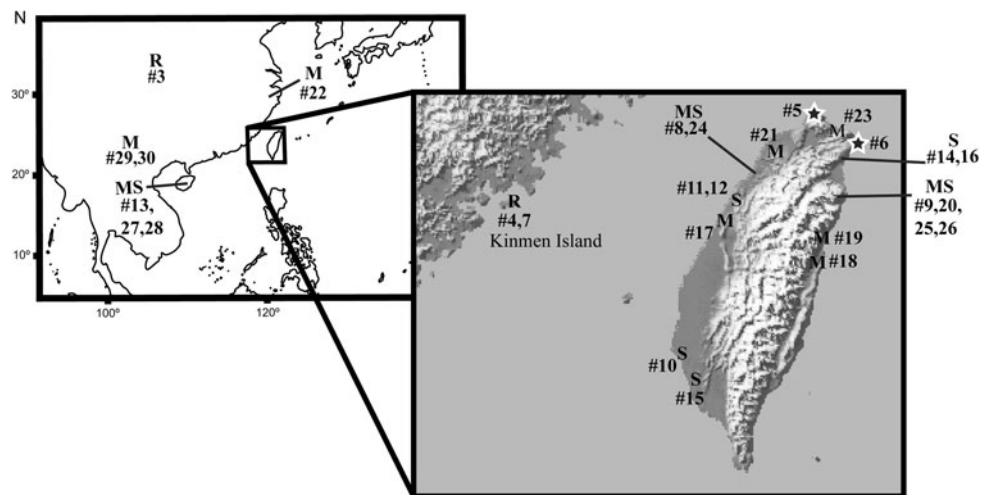
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**Fig. 1** Photographs of the two probable hybrid individuals found in the wild of Taiwan



**Fig. 2** Map showing locality of wild specimens used in this study. M = *Mauremys mutica*, S = *Mauremys sinensis*, R = *Mauremys reevesii*, ★ hybrid. Numbers of individuals correspond to specimen numbers found in Appendix 1



## Materials and methods

Twenty-one specimens were collected throughout Taiwan (Fig. 2 and Appendix 1) and identified based on diagnostic morphological characters. Included were two suspected hybrids identified by their unusual/intermediate morphology. All individuals were genotyped for a combination of mitochondrial (mtDNA) and/or nuclear DNA (nuDNA) sequences, and combined with nine Genbank sequences for phylogenetic analyses (Appendix 1). Genbank sequences were used as outgroups and to expand the geographical sampling of the three species. Individuals from Taiwan were not euthanized to take blood samples, since they are involved in long-term ecological surveys. DNA was extracted using a standard salt extraction protocol (Sambrook and Russell 2001). Amplification followed a standard PCR protocol to amplify an 892 bp mtDNA fragment containing the NADH dehydrogenase subunit 4 (ND4) gene and flanking tRNAs (Stuart and Parham 2004) and ~1133 bp of the RNA fingerprint protein gene intron 1 (R35) (Fujita et al. 2004). A TOPO TA cloning kit (Invitrogen) was used to recover both alleles of the R35 intron for the two putative hybrids, while the software program Phase (Stephens et al. 2001) was used to recover alleles for the parental species.

Phylogenetic analyses were run under maximum likelihood (ML) and Bayesian inference (BI). ML analyses were run using RAxML v7.0.4 (Stamatakis 2006) on the CIPRES Portal v1.15 (<http://www.phylo.org/portal>), with 1000 bootstrap replicates (Stamatakis et al. 2008). BI analyses were run in MrBayes v3.1.2. nuDNA data were run under a single model, while mtDNA data were partitioned into 1st, 2nd, 3rd codon position and tRNA, with models chosen using MrModelTest v2 (Nylander 2004) under the hLRT criterion. Two BI searches with random starting trees were run and compared using four chains, four million generations and sampling every 1000th generation. Burn-in was estimated using the online program AWTY (Wilgenbusch et al. 2004).

## Results

A total 29 individuals (21 new, 8 Genbank) were included for ND4 analyses and 21 individuals (15 new, 6 Genbank) for R35 analyses. Both data matrices were submitted to Treebase (#S2651; M5093-4). For ND4, uncorrected pairwise differences within the ingroup ranged from 0 to 8.2%. ML analyses resulted in a single tree ( $-\ln L = -2897.15$ ,

$\alpha = 0.172$ ). For the BI analyses, the following models were selected for the partitions: 1st pos: HKY+ $\Gamma$ ; 2nd pos: F81; 3rd pos: GTR+ $\Gamma$ ; tRNA: HKY+ $\Gamma$ . Both independent BI runs were almost identical, so after discarding a burn-in of 500 generations, data were combined. Both ML and BI analyses for ND4 gave similar results, so only the ML phylogenetic inference is shown (Fig. 3).

For R35 data, uncorrected pair-wise differences of the ingroup ranged from 0 to 1.3%. ML analyses resulted in a single tree ( $-\ln L = -2216.593$ ,  $\alpha = 0.020$ ). For BI analyses, the HKY +I+ $\Gamma$  model was selected for the entire dataset, and both independent runs were combined after removing the 500-generation burn-in due to their similar results. The ML analyses for R35 are shown in Fig. 4.

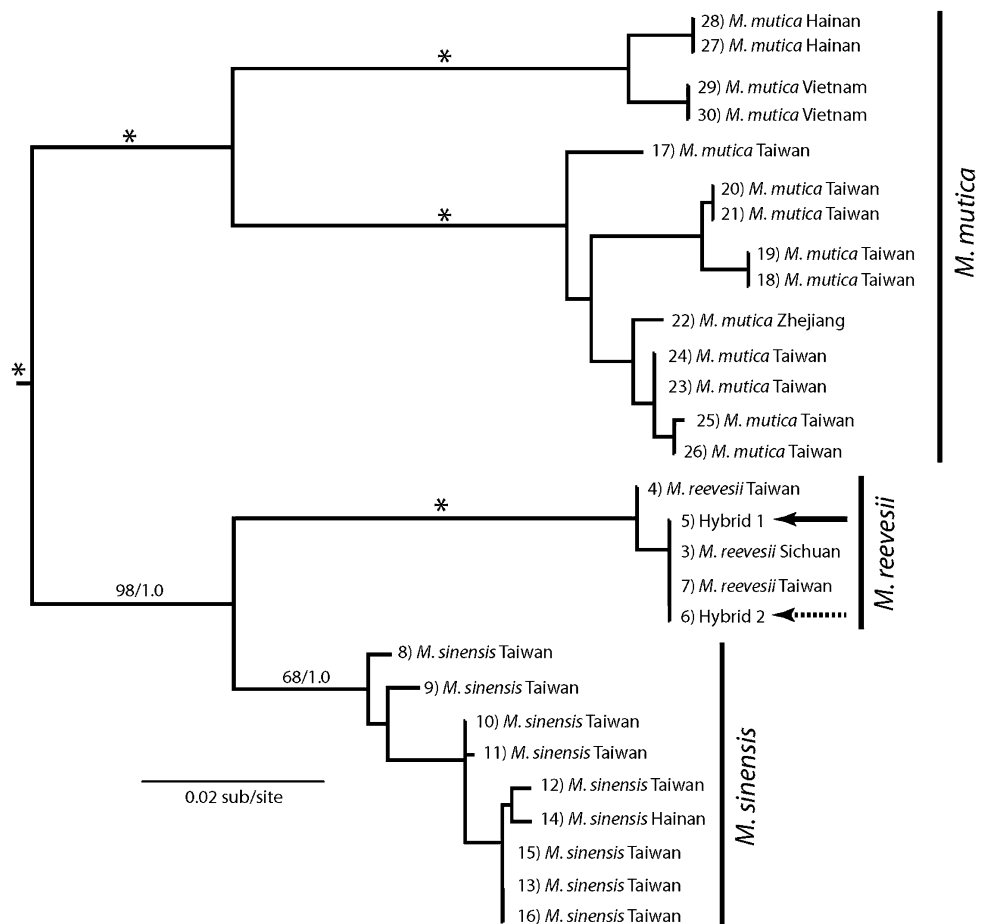
We placed the two hybrid individuals into the mitochondrial and nuclear phylogenetic frameworks. Both Hybrid 1 and 2 contained *M. reevesii* mitochondrial haplotypes (Fig. 3). In the nuclear analyses, Hybrid 1 and 2 possessed both *M. reevesii* and *M. sinensis* alleles (Fig. 4). However, the hybrids exhibited different nuclear genotypes suggesting that they were formed through independent hybridization events.

### Discussion

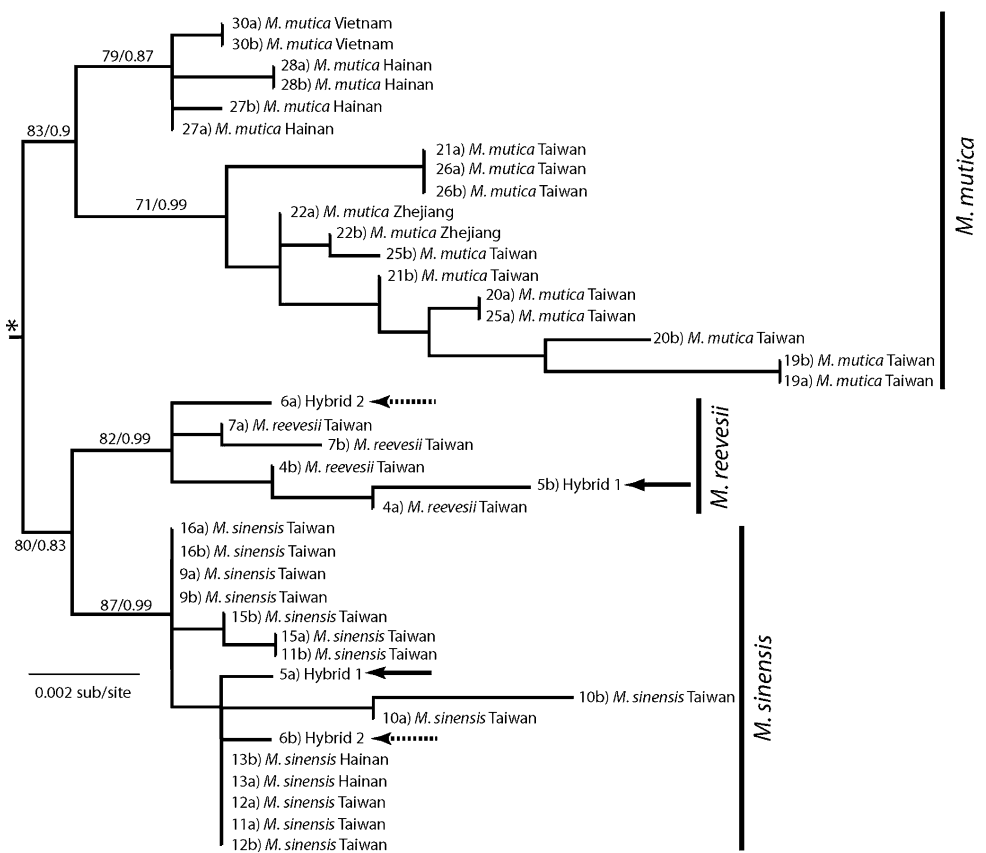
The hybrid origin of two wild-caught individuals from Taiwan was confirmed with genetic data. Both individuals had a *M. reevesii* mother (Fig. 3), and a *M. sinensis* father (Fig. 4). The nuclear data demonstrate that both hybrid individuals are not each other’s closest relatives, indicating multiple hybridization events.

It is not surprising that *M. sinensis* was one of the parental species, since it was found living syntopically with Hybrid 2. Although *M. reevesii* has been documented to readily and frequently hybridize (Buskirk et al. 2005), it was unexpected that this species was the second parental species due to its rarity on Taiwan Island. *Mauemys reevesii* is currently restricted to Kinmen Island, but historical records reported *M. reevesii* from the main island of Taiwan as long ago as 1931 (Horikawa 1934) and as recently as 1971 (Mao 1971). It is possible that *M. reevesii* is currently present on the main island of Taiwan and has gone undetected. Extensive surveys of Taiwan (Chen and Lue in press) found none, so if *M. reevesii* is present, it persists at an extremely low population density.

**Fig. 3** Maximum likelihood (ML) phylogram for mitochondrial DNA (ND4). Trees from ML and Bayesian inference resulted in almost identical trees. Nodal support is ML bootstrap/Bayesian posterior probability. Numbers of individuals correspond to specimen numbers found in Appendix 1. ←: Hybrid 1 haplotype, ←·····: Hybrid 2 haplotype



**Fig. 4** Maximum likelihood (ML) phylogram for nuclear DNA (R35). Trees from ML and Bayesian inference resulted in almost identical trees. Nodal support is ML bootstrap/Bayesian posterior probability. Numbers of individuals correspond to specimen numbers found in Appendix 1. ←: Hybrid 1 alleles, ←.....: Hybrid 2 alleles



We raise the possibility that *M. reevesii* is not endemic to Taiwan Island, but rather the result of human introduction. *Mauremys reevesii* is only known from a few historical localities on Taiwan Island, all near developed, urban areas in the Taipei Basin. Pope (1935) noted that humans commonly moved *M. reevesii* around China because it is an important ingredient of traditional Chinese medicine. Currently, *M. reevesii* is the most common hard-shell, aquatic turtle found in commercial turtle farms (Shi and Parham 2001; Shi et al. 2008) and most widely kept as a pet (Buskirk et al. 2005), providing more opportunity for escape and release into non-native habitats. Such is the case in the Ryukyu Islands of Japan, where several feral populations have been established (Masuno et al. 1998; Ota et al. 2004). Therefore, the possibility that *M. reevesii* is not endemic to Taiwan Island is high. One way to test this hypothesis is to determine the relationship of *M. reevesii* from Taiwan to other parts of its range. This is complicated by the fact that we have not found any pure specimens on Taiwan Island, and data on *M. reevesii* are limited because genetic samples from the wild are known from just Sichuan Province and Kinmen Island (Fig. 2). Despite the fact that these two localities are separated by ~1500 km, they show very limited genetic variation (0.3–1.9%, mtDNA).

The surge in occurrences of hybrid turtles appearing from the wild (Otani 1995; Shi et al. 2005; Haramura et al. 2008)

should concern turtle conservation efforts. The most likely contributing factors are habitat destruction and the turtle trade. Habitat destruction is reducing the amount of suitable area for turtles, potentially forcing species that normally did not come into contact to do so, while the turtle trade moves from their native range and farmed at industrial scales in China (Shi et al. 2007, 2008). Genetically compromised captive populations from turtle farms may escape into non-native habitats. Besides, accidental escapes (Shi et al. 2004), Buddhists, pet owners, and even customs officials are releasing turtles into the wild without considering the native ranges and genetics of these species, leading towards non-natural hybridization. Stricter regulations and law enforcement need to be undertaken to protect the remaining natural habitat, as well as the farming, trading, and transport of turtles.

*Mauremys reevesii* is currently listed as endangered on the IUCN Redlist (IUCN 2007) and protected under the Wildlife Conservation Law as a rare and valuable species in Taiwan (Wildlife Conservation Law 1989). Based on the recent turtle surveys (Chen and Lue in press), *M. reevesii* populations are either at extremely low population densities or extirpated. This makes *M. reevesii* a prime candidate to maintain their listing as endangered. However, further research should be done to determine whether *M. reevesii* populations are natural or introduced. If introduced, their conservation value would be negligible. Furthermore, they

should be actively removed from the wild since they are genetically polluting truly endemic species such as *M. sinensis* and *M. mutica* through hybridization (Wink et al. 2001; Buskirk et al. 2005, this study). As always, multiple explanations may be necessary to explain the origin of separate *M. reevesii* populations, so the disjunct Taiwan and Kinmen Island populations should be tested independently. If future research demonstrates that *M. reevesii* in Taiwan are introduced, this species should be removed from the Wildlife Conservation Law list to free up precious conservation resources for native, endangered species in Taiwan. However, until definitive data establish its non-native status in Taiwan, *M. reevesii* should remain on the protected list. Our work highlights the possibility that the genetic integrity of turtles endemic to Taiwan has been compromised by an alien species. There is no doubt that the food and pet trade is increasing the frequency of genetic pollution of native turtle species in Asia, and provides further reason to more carefully control and monitor this trade.

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**Appendix 1**

See Table 1.

**Table 1** List of individuals used in this study, along with the location of the specimen voucher and Genbank numbers

Number	Name	Specimen #	ND4	R35
1	n/a	FMNH255695	AY364617	n/a
2	n/a	FMNH255262	n/a	DQ386654
3	<i>M. reevesii</i> Sichuan	MVZ236729	EF034110	n/a
4	<i>M. reevesii</i> Taiwan	CR	GQ259438	GQ259459
5	Hybrid 1	H1 = 12	GQ259439	GQ259460-1
6	Hybrid 2	H3 = 9	GQ259440	GQ259462-3

**Table 1** continued

Number	Name	Specimen #	ND4	R35
7	<i>M. reevesii</i> Taiwan	CR9 = 8	GQ259441	GQ259464
8	<i>M. sinensis</i> Taiwan	OS8 = 8	GQ259442	n/a
9	<i>M. sinensis</i> Taiwan	OS3 = 10	GQ259443	GQ259465
10	<i>M. sinensis</i> Taiwan	OS1 = 9	GQ259444	GQ259466
11	<i>M. sinensis</i> Taiwan	OS2 = 11	GQ259445	GQ259467
12	<i>M. sinensis</i> Taiwan	OS2 = 9	GQ259446	GQ259468
13	<i>M. sinensis</i> Hainan	MVZ230479	AY337345	DQ386678
14	<i>M. sinensis</i> Taiwan	OS8 = 2	GQ259447	n/a
15	<i>M. sinensis</i> Taiwan	OS1 = 3	GQ259448	GQ259469
16	<i>M. sinensis</i> Taiwan	OS1 = 11	GQ259449	GQ259470
17	<i>M. mutica</i> Taiwan	MM1 = 2	GQ259450	n/a
18	<i>M. mutica</i> Taiwan	MM1 = 15	GQ259451	n/a
19	<i>M. mutica</i> Taiwan	MM260	GQ259452	GQ259471
20	<i>M. mutica</i> Taiwan	MM3 = 11	GQ259453	GQ259472
21	<i>M. mutica</i> Taiwan	MM2 = 11	GQ259454	GQ259473
22	<i>M. mutica</i> Zhejiang	MVZ230487	AF034089	DQ386666
23	<i>M. mutica</i> Taiwan	MM9 = 8	GQ259455	n/a
24	<i>M. mutica</i> Taiwan	MM1 = 8	GQ259456	n/a
25	<i>M. mutica</i> Taiwan	MM1 = 11	GQ259457	GQ259474
26	<i>M. mutica</i> Taiwan	MM9 = 1	GQ259458	GQ259475
27	<i>M. mutica</i> Hainan	MVZ237108	EF034104	EF587915
28	<i>M. mutica</i> Hainan	MVZ251452	EF034101	EF587917
29	<i>M. mutica</i> Vietnam	ROM25613	AF348279	n/a
30	<i>M. mutica</i> Vietnam	ROM25614	AF348280	DQ386668

FMNH Field Museum of Natural History, Chicago, IL, USA; MVZ Museum of Vertebrate Zoology, Berkeley, California, USA; ROM Royal Ontario Museum, Toronto, Ontario, Canada; All other specimens are blood samples of marked individuals (part of ecological studies) that can be found at the National Taiwan Normal University, Taipei, Taiwan

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