


# Human disturbance as a possible cause of genetic introgression from exotic into native *Mauremys* turtles

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## Keywords

backcross; demilitarized zone (DMZ); gene flow; hybridization; wildlife trade; disturbance; *Mauremys* turtles; genetic introgression.

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## Abstract

The Kinmen Islands on the coastline of China represent a recent case of the protection of endangered wildlife in a demilitarized zone. These islands represent the last remaining habitat in southeastern China that supports a healthy natural population of Reeves' turtle *Mauremys reevesii*. However, human-mediated dispersal appears to have aided the establishment of an introduced population of the Chinese stripe-necked turtle *M. sinensis* since the late 1990s, resulting in population mixture of the two congeners. To evaluate the potential impacts of hybridization, we evaluated the magnitude of genetic introgression in ponds on Kinmen using mitochondrial cytochrome *b* sequences and 13 microsatellite loci. We further used eight environmental and biological factors of the ponds to conduct general linear regression model analysis between environmental factors and the magnitude of genetic introgression. Among the 41 sampled ponds, *M. reevesii* was recorded in 21, of which 12 ponds (57.1%) contained genetic hybrids or backcrosses, corresponding to 11.3–12.7% of the total individuals on the islands. The magnitude of genetic introgression was positively correlated with the amount of introduced *M. sinensis* occurring in a pond, and marginally negatively associated with the shortest distance from the pond to secondary roads. We conclude that this considerable genetic introgression is attributable to human-mediated dispersal of *M. sinensis*; roads and habitat modification may increase the likelihood of unexpected encounters between these two congeners.

## Introduction

Human-mediated dispersal of exotic species can negatively impact native ecosystems. The interactions between introduced and native species include competition, predation, introduction of novel pathogens and genetic introgression (Mooney & Cleland, 2001; Lockwood, Hoopes & Marchetti, 2013). Among these negative impacts, genetic introgression, that is, the movement of genetic material from one species into the gene pool of another, has received much attention in recent years. Genetic introgression is the result of hybridization between closely related species and subsequent interbreeding of fertile hybrids with one or both parental groups. Depending on the circumstances under which it occurs, Bohling (2016) categorized such hybridization as 'natural' or 'artificial' events. Artificial hybridization with exotic species may lead to a reduction in biodiversity due to loss of local uniqueness, homogenization of genetic materials, or extinction (Rhymer & Simberloff, 1996; Allendorf *et al.*, 2001; Laikre *et al.*, 2010; Crispo *et al.*, 2011).

Emerging interest in hybridization among turtles has led to an increase in reports of interspecific hybridization in

recent years (Stuart & Parham, 2007). In contrast to most other animals, Testudines are capable of hybridization among distantly related lineages (Spinks *et al.*, 2004; Buskirk, Parham & Feldman, 2005). Consequently, the potential impacts of human-mediated dispersal are far more severe for these species. Although most cases of hybridization in geoemydid turtles have occurred in captive environments (such as turtle farms and zoos), reports of hybridization in the wild are increasing (Otani, 1995; Shi *et al.*, 2005; Fritz *et al.*, 2008; Fong & Chen, 2010; Suzuki *et al.*, 2011; Fujii, Ota & Toda, 2014). However, the causes and consequences of this conservation issue have not been thoroughly explored using multiple nuclear markers or from the perspective of habitat management, and hypothesis-based research remains extremely scarce.

Reeves' turtle (or the Chinese three-keeled pond turtle, *Mauremys reevesii*) was once the most abundant freshwater turtle in East Asia (Ades, Banks & Buhlmann, 2000; Bhupathy *et al.*, 2000), with a wide distribution in central and eastern China, the Korean Peninsula, Japan and Taiwan (Iverson, 1992; Lovich, Yasukawa & Ota, 2011; Turtle Taxonomy Working Group, 2017). Despite their abundance and

large population size, exhaustive exploitation for commercial uses in food and medicine has led to a comprehensive population decline in the wild. During the past several decades, natural populations of Reeves' turtle in China have been nearly eliminated (Lovich *et al.*, 2011), and the overwhelming majority of this species are found on commercial turtle farms with large population sizes (Shi & Parham, 2001; Shi *et al.*, 2008). For these reasons, Reeves' turtle is classified as 'Endangered' on the IUCN Red List, and research on the species in its natural habitats in China is almost impossible.

Given these conditions, the Kinmen Islands provide a valuable opportunity to study Reeves' turtle in the wild. This archipelago represents a recent case of protection of endangered wildlife in a demilitarized zone (DMZ) (You *et al.*, 2013) and is one of very few sites in southeastern China sustaining a stable natural population of Reeves' turtle (Chen & Lue, 2010; Lin *et al.*, 2015). Although located only 3 km from the coastline of continental China, these islands have been under military control by the Taiwanese government since the separation of the two political regimes after World War II. To defend against the Chinese army, numerous Taiwanese soldiers were once stationed on the islands during the Cold War. However, relaxation of political tensions between continental China and Taiwan resulted in the withdrawal of the majority of the military force in the late 20th century. Recent studies have shown that several endangered species have recovered from near-extinction on these islands, such as the Eurasian otter *Lutra lutra* (Hung, Li & Lee, 2004) and the Burmese python *Python molurus* (You *et al.*, 2013). Reeves' turtle has also benefited from the DMZ; they inhabit the numerous ponds (more than 100 within a 150-km<sup>2</sup> area) on the islands, most of which were built to preserve freshwater during the Cold War but have been abandoned by the military (Lin *et al.*, 2015).

However, *M. reevesii* now faces habitat destruction and degradation in Kinmen due to increasing trade and tourism. This population is sensitive to artificial construction, human disturbance, alteration of bank substrates and clearing of vegetation around ponds and has experienced increased road mortality (Lin *et al.*, 2015). A further severe threat to the persistence of this remnant Reeves' turtle population is increase in the Chinese stripe-necked turtle *M. sinensis*, the most abundant freshwater turtle in Taiwan. This turtle is characterized by high fertility, high growth rate and high wild population abundance, is easy to keep, and is well-adapted to the local climate (Chen & Lue, 1998). Therefore, farming of this turtle is the second-largest turtle agriculture business in Taiwan (the largest is the Chinese soft-shelled turtle, *Pelodiscus sinensis*) (Chen, Lin & Chang, 2000). *M. sinensis* was first recorded on the islands in 1998 and was reported as 'very rare, with uncertain population source and status' in this region (Lue, Shiang & Jeng, 1998). In a comprehensive survey during 2004–2005, *M. sinensis* was not captured on these islands (Chen & Lue, 2010), indicating that this turtle had not yet established a stable population. However, the population size of this species seems to have increased during the last decade, possibly due to human-mediated dispersal (Lin *et al.*, 2015). Similar cases among

*Mauremys* species suggest that *M. sinensis* has the potential to breed with native *M. reevesii* and produce fertile hybrid offspring (Fong & Chen, 2010; Suzuki *et al.*, 2011; Xia *et al.*, 2011). Recent investigations have identified intermediate forms between *M. reevesii* and *M. sinensis* that are suspected hybrids in several ponds on the islands (Fig. 1).

This pattern provides an opportunity to study the issue of Testudines hybridization from the perspectives of both evolutionary biology and conservation biology. We hypothesize that human-mediated dispersal of exotic turtles will threaten the genetic integrity of the native species, whereas artificial factors, such as road construction or alteration of pond environments, will provide further potential opportunities for interspecific hybridization. In this study, we evaluated the magnitude of genetic introgression from exotic to native turtles in each pond using mitochondrial sequences and 13 microsatellite markers. These values were further correlated with the environmental and biological factors of the ponds, which may facilitate the elucidation of the underlying mechanism of genetic introgression between exotic and native turtles.

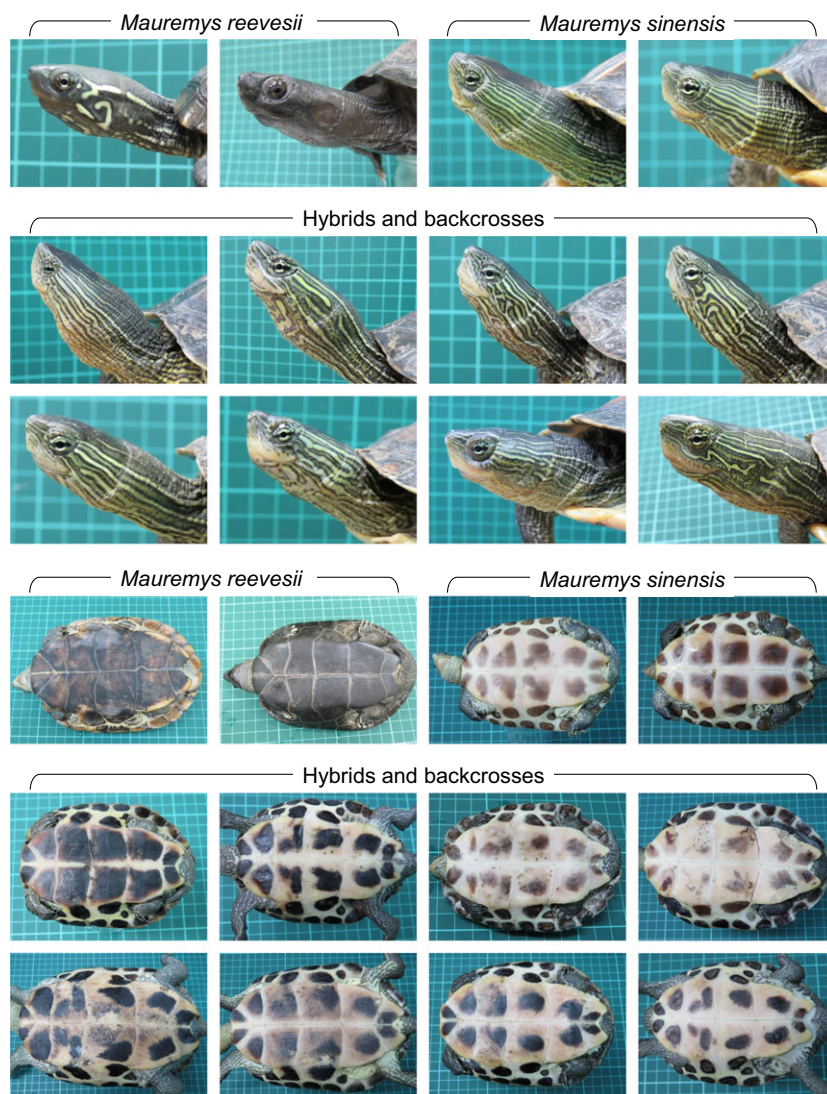
## Materials and methods

### Sample collection and identification

We conducted our sampling on the Kinmen Islands (24.386°–24.529°N, 118.210°–118.473°E) during 2011–2013, including 35 sites on Major Kinmen (134.3 km<sup>2</sup>) and six on Lesser Kinmen island (14.9 km<sup>2</sup>; Fig. 2; Table 1). The sampling sites included various water bodies such as ponds, marshes, reservoirs and streams. We used floating hoop net traps baited with canned fish to capture turtles. Depending on the size of the water body, 2–5 traps with 50-m spacing were deployed for 4 days, and the bait was replenished daily. Further details of the field work are available in our previous study (Lin *et al.*, 2015). All handling processes followed the animal use protocols approved by the IACUC of National Taiwan Normal University (License No. 107002). The capture procedures were certified by Kinmen National Park (No. 1000001622) within the nature reserve and by the Kinmen County Government (No. 1000046074) outside the nature reserve. No turtles were drowned or injured during the experiments.

The collected turtles were first identified based on morphology as pure *M. reevesii*, pure *M. sinensis*, or an intermediate form (Table 1). Compared to pure *M. reevesii*, hybrids were classified by a smaller head, the appearance of green or yellow stripes on the neck, or faded coloration on the plastrons from the typical black or dark brown to a variety of different compositions (Fig. 1). Furthermore, the hybrids were classified from pure *M. sinensis* based on broken, nonparallel, curved, or a reduced number of stripes, a wider and larger head, or darker spots or patches on the margins of the plastrons.

During the study, captured *M. sinensis* and putative hybrids were moved from the islands and housed in long-term captivity in Taiwan. After recording the carapace length, plastron length, weight and sex (if available), *M. reevesii* were released immediately to the original site after receiving individual identifying



**Figure 1** Pure *Mauremys reevesii* (upper-left), pure *M. sinensis* (upper-right) and hybrids or backcrosses (others). Compared to pure *M. reevesii*, hybrids were classified by a smaller head, the appearance of green or yellow stripes on the neck, or faded coloration on the plastrons from the typical black or dark brown to a variety of different compositions. Furthermore, the hybrids were classified from pure *M. sinensis* based on broken, nonparallel, curved, or a reduced number of stripes, a wider and larger head, or darker spots or patches on the margins of the plastrons.

marks on their marginal scutes (modified from Cagle, 1939). To compare the wild-caught turtles to pure species, 11 wild-caught *M. sinensis* from Taiwan, 9 *M. reevesii* from turtle farms (because no other wild natural population is available in adjacent regions), and two confirmed F1 hybrids (also from a turtle farm) were included in the sampling. A piece of 2-mm<sup>2</sup> web tissue was collected from each turtle and placed in 95% ethanol.

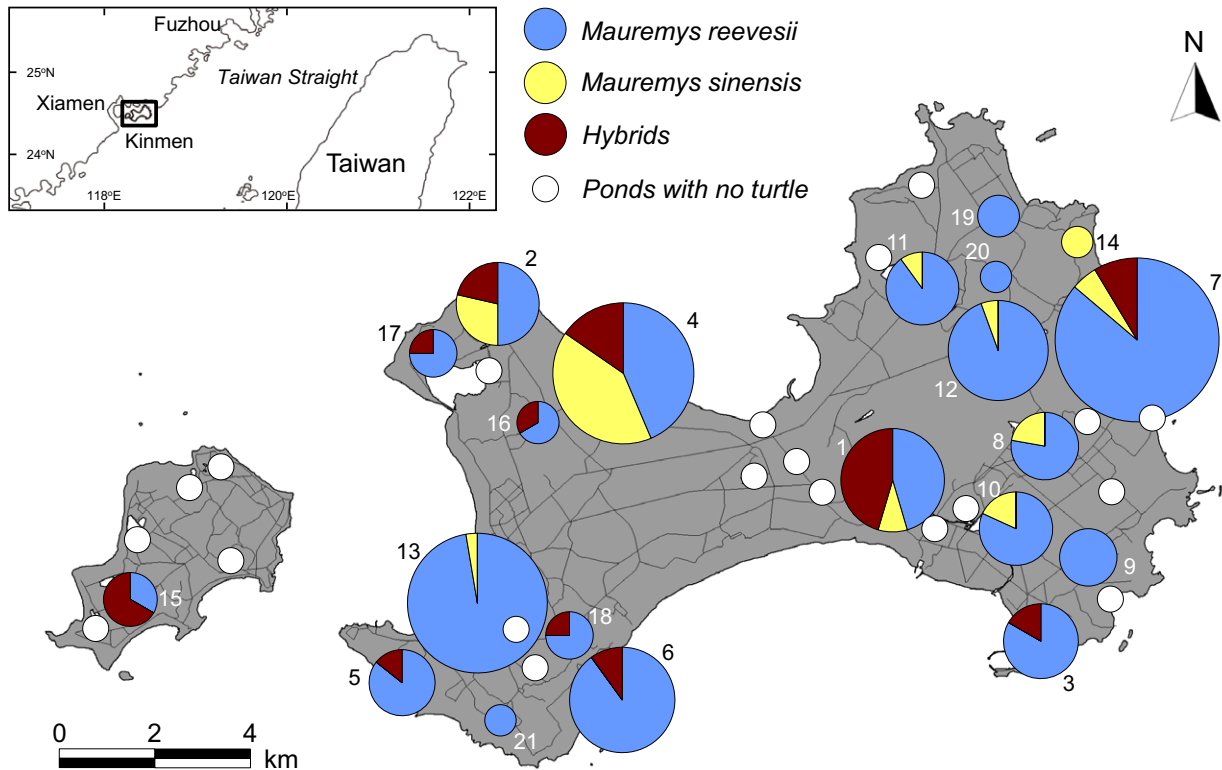
### Mitochondrial DNA sequencing and analyses

Genomic DNA was extracted using an EasyPure Genomic DNA spin kit (Bioman Scientific Co, Ltd, Taipei, Taiwan) according to the manufacturer's instructions. The mitochondrial

cytochrome *b* gene was amplified by polymerase chain reaction using the primers mt-A (5'-CAACATCTCAGC ATGATGAAACTTCG-3', Lenk & Wink, 1997) and H15909 (5'-CAGTTTTTGGTTTACAAGACCAATG-3', Lenk *et al.*, 1999) using the reaction and thermos cycling conditions provided in Appendix S1. The sequences of turtles collected from Kinmen were combined with those from confirmed *M. reevesii* and confirmed *M. sinensis*, and aligned using SEQUENCHER 4.7 software (Gene Codes Co, Ann Arbor, MI, USA). All haplotypes have been submitted to GenBank (KT763392–KT763419; Appendix S2).

Another six *Mauremys* and three *Cuora* species were included as outgroups for phylogenetic reconstruction. Maximum likelihood analysis was conducted using PHYML 3.0





**Figure 2** Sampling sites and species composition of the 41 ponds investigated in the Greater Kinmen (right) and Lesser Kinmen (left) islands. The size of each pie chart represents the comparative number of turtles captured from the pond, and the blue, yellow and dark red areas indicate the proportions of *Mauremys reevesii*, *M. sinensis* and hybrids, respectively. The species assignments in this figure are based on the population genetic structure determined by STRUCTURE 2.3.4 (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009) using 13 microsatellite loci.

(Guindon *et al.*, 2010), and Bayesian inference analyses were conducted with MRBAYES 3.2.2 (Ronquist, Huelsenbeck & Teslenko, 2011). A haplotype network was constructed using TCS (Clement, Posada & Crandall, 2000), with a 95% parsimony connection limit for the minimum number of mutations. Further details on phylogenetic analyses are provided in Appendix S1, Figs S1 and S2.

### Microsatellite genotyping and hybrid identification

Individual genotypes were assessed using 13 microsatellite loci by Ye *et al.* (2008), Zhang, Zhang & Zheng (2010) and Liu *et al.* (2012), using reaction conditions provided in Table S1. We used MICROCHECKER (Van Oosterhout *et al.*, 2004) to compute the expected and observed frequency of heterozygous in each locus to check the presence of null allele (Table S2). The microsatellite data were analyzed using three methods to detect hybrid individuals among turtles in Kinmen. First, we used the Bayesian model-based clustering software STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000; Hubisz *et al.*, 2009) to assign the genetic composition of each individual (Table S3). The number of assumed genetic clusters ( $K$ ) was set from 1 to 5, and 10 runs were performed for each  $K$  with 1 000 000 markov chain monte carlo (MCMC) iterations; the first

100 000 iterations were discarded as burn-in. The best  $K$  value was obtained based on the  $\Delta K$  (delta  $K$ ) estimated by the Evanno method (Evanno, Regnaut & Goudet, 2005) using the software STRUCTURE HARVESTER (Earl, 2012) (Fig. S3). Following the method of Godinho *et al.* (2015), we simulated pseudo populations to evaluate the effectiveness of these 13 microsatellite loci in distinguishing the two pure species and hybrids; the details are given in Appendix S1 (Fig. S4). Individuals with posterior probabilities lower than 0.875 (introgression higher than 0.125 from the other species) were defined as hybrids. According to the literature, sexual maturity of *M. reevesii* occurs at 6 years in Chinese populations and 10 years in Japanese populations (Liu & Hu, 1940; Ishihara, 1986; Lovich *et al.*, 2011). Although there are no formal studies reporting generation times at this subtropical latitude, a time span of 6 years is likely applicable to this warm-climate habitat and is also consistent with our experience in captivity. The 0.875/0.125 threshold was used to detect F1 hybridization and backcrosses within the next two generations, given the introgression history since the late 20th century.

Second, NEWHYBRIDS 1.1 Beta 3 (Anderson & Thompson, 2002) was applied to estimate the posterior probability of each individual falling into each of a set of hybrid categories. These user-defined categories included pure *M. reevesii*, pure *M. sinensis*, F1, F2 and backcrosses to either of the two

**Table 1** Morphological assignments, genetic assignments (as given by STRUCTURE and NEWHYBRIDS, respectively), genetic hybrid rates and magnitudes of genetic introgression from introduced *Mauremys sinensis* to native *M. reevesii* (as represented by the migration rate calculated using BAYESASS 1.3) for the 21 ponds in the Kinmen Islands

Sample locality	N	Morphology			STRUCTURE			NEWHYBRIDS			Hybrid rate (=Hy/N), %	Migration rate from Ms into each pond
		Mr	Ms	Int	Mr	Ms	Hy	Mr	Ms	Hy		
01 Ming Lake	22	11	1	10	10	2	10	10	0	12	45.5–54.5	0.1066
02 Shuangli Lake	15	11	4	0	8	4	3	8	4	3	26.7	0.1132
03 Liao Luo	12	12	0	0	10	0	2	11	0	1	8.3–16.7	0.0235
04 Qiong An	39	21	15	3	17	16	6	20	16	3	7.7–15.4	0.1439
05 Shui Tou	7	7	0	0	6	0	1	6	0	1	14.3	0.0362
06 Ou Cuo	20	20	0	0	18	0	2	19	0	1	5.0–10.0	0.0141
07 Nameless Pond	58	52	2	4	49	3	6	49	3	6	10.3	0.0435
08 Yangming Lake	9	7	1	1	7	2	0	7	2	0	0.0	0.0922
09 Xipu Bridge	6	6	0	0	6	0	0	6	0	0	0.0	0.0415
10 Tai Lake	11	9	2	0	9	2	0	9	2	0	0.0	0.0733
11 Guangqian Stream	10	9	1	0	9	1	0	9	1	0	0.0	0.0567
12 Longling Lake	18	17	1	0	17	1	0	17	1	0	0.0	0.0304
13 Guan Lu Bian	38	37	1	0	37	1	0	37	1	0	0.0	0.0167
14 Kai Lake	1	0	0	1	0	0	1	0	0	1	100	N/A
15 Lingshui Lake	3	3	0	0	1	0	2	1	0	2	66.7	N/A
16 Long Lake	3	3	0	0	2	0	1	2	0	1	33.3	N/A
17 Shuangli Wetland	4	4	0	0	3	0	1	3	0	1	25.0	N/A
18 Dong Sha	4	4	0	0	3	0	1	4	0	0	0.0–25.0	N/A
19 Shan Xi	2	2	0	0	2	0	0	2	0	0	0.0	N/A
20 Sha Mei	1	1	0	0	1	0	0	1	0	0	0.0	N/A
21 Gu Gang	1	1	0	0	1	0	0	1	0	0	0.0	N/A
Total	284	237	28	19	216	32	36	222	30	32	11.3–12.7	

Mr, *Mauremys reevesii*; Ms, *Mauremys sinensis*; Int, morphologically intermediate forms; Hy, genetically confirmed hybrids; N/A, migration rates were not able to be estimated due to low sample size.

species. The genotype frequency of each class was set to the default settings based on TwoGensGtypFreq.txt, a file designed by the programmer that defines the genotype frequency classes possible after two generations of mating between two species (user's guide available at [http://ib.berkeley.edu/labs/slatkin/eriq/software/new\\_hybs\\_doc.pdf](http://ib.berkeley.edu/labs/slatkin/eriq/software/new_hybs_doc.pdf)). The individuals regarded as the 'pure line' were set using the 'z' option in the input file. As recommended by the software, we set priors as Jeffreys-type to downweight the influence of rare alleles and ran the program with 1 000 000 MCMC iterations; the first 100 000 iterations were discarded as burn-in. For each individual, the probabilities of the four latter categories (F1, F2 and backcrosses) were summed to represent the hybrid probability. The results were visualized as a ternary plot constructed using the R package ggetern (Hamilton, 2017).

Discriminant Analysis of Principal Components (DAPC) (Jombart & Collins, 2015), available in the R package adegenet (Jombart, 2008), was used as the third method to investigate the population structure based on genetic data. Individuals were predefined as different categories: (1) morphological *M. reevesii*, (2) morphological *M. sinensis*, (3) morphologically intermediate forms and (4) and (5) pure individuals of both species. We applied optim.a.score to find the best number of principal components (PCs) that retained the most genetic variability while preventing model overfitting. The a-score for DAPC is a permutational approach that measures the bias of overfitting by randomly permuting

clusters and then calculates the difference between the percentage of successful reassignment in the analysis and the value obtained using randomly permuted clusters. We optimized the a-score function scan using different numbers of PCs and chose the number of PCs that yielded the highest average a-score. This analysis suggested retaining the first 28 PCs in the subsequent procedure. The first two axes in discriminant analysis were sufficient to summarize the genetic variation among the populations and produced a scatter plot illustrating the microsatellite genetic structure.

### Genetic introgression from *M. sinensis* to *M. reevesii*

On the basis of the genotype data for the 13 microsatellite loci and the Bayesian framework, we used BAYESASS 3.0 (Wilson & Rannala, 2003) to estimate recent migration rates. First, we picked out all *M. sinensis* individuals from Kinmen, and combined these individuals as a single dataset (population 1). In this analysis, we consistently used this *M. sinensis* dataset as 'population 1', the invader; and non-*sinensis* turtles in each pond as 'population 2', the receiver. By taking turns, we calculated the magnitude of genetic introgression from *M. sinensis* toward each pond. Since ponds less than five turtles would lead to calculation bias, only 13 ponds with a total of 265 individuals were included in this analysis (the first 13 ponds in Table 1). For each population, we conducted BAYESASS

with 1 000 000 MCMC iterations, sampling every 2000 generations, following a burn-in of 100 000 generations. The mixing parameters for migration rates and inbreeding coefficients were set at the default setting of 0.10 to ensure adequate mixing and acceptance rates following the BAYESASS v3.0 instruction manual (Wilson & Rannala, 2003).

### Relationships between genetic introgression and environmental factors

To identify the probable environmental factors leading to genetic introgression from exotic to native species, eight habitat variables were measured for each pond. These parameters were collected in a previous study (Lin *et al.*, 2015), and some are relevant to the existence or abundance of turtles. We used two parameters as dependent variables: (1) the 'migration rate' from exotic *M. sinensis* toward each pond, which was calculated using BAYESASS 3.0 (Wilson & Rannala, 2003); and the hybrid rates of each pond, which was determined by STRUCTURE 2.3.4 (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009). The environmental factors included the shortest distance to main roads (DM), the shortest distance to secondary roads (DS), the level of vegetation coverage on land (VL), the level of vegetation coverage in the water (VW), the substrate type around the pond (PS), the area of the pond (PA), the depth of the pond (PD) and the amount of genetic *M. sinensis* (defined by STRUCTURE analysis) collected in the pond during the research duration (MS) (see also Table 2 for detailed definitions of all of these variables). ArcMap (ArcGIS 9; ESRI Ltd, Redlands, CA, USA) was used to process DM and DS, whereas VL, VW, PS, PA and PD were directly evaluated at the ponds. In some of these subtropical ponds, vegetation coverage and water environmental factors present considerable seasonal fluctuations in different months. Therefore, the latter five parameters were transformed to ordinal data for subsequent analyses.

We used multiple regression analysis to examine the relationship between genetic introgression and these environmental factors. First, we checked multicollinearity, the non-independence of predictor variables that may cause statistics bias. None of the pairwise comparisons among our tested eight environmental variables showed a correlation coefficient ( $|r_{s}| > 0.7$ , suggesting absence of any significant multicollinearity among the factors in this study (Dormann *et al.*, 2013; Table S4). Therefore, all these factors were used as predictor variables to build multiple regression models to explain the magnitude of genetic introgression from *M. sinensis* into native *Mauremys*. We used forward-selection procedures (as suggested by the JMP manual; SAS Institute Inc., Cary, NC, USA) with probabilities of entering and leaving of 0.10, to choose potential habitat variables that correlated with genetic introgression of *M. reevesii*. Next, we checked the significance of different terms and interactions of selected variables. Only two factors (DS and MS) were selected in the forward-selection procedures (Table 2), and we then used multiple regression to inspect the relationships between genetic introgression and these two environmental factors. We used the Brown–Forsythe test, Shapiro–Wilk test and Durbin–Watson test to check the reliability of variance, normality and independence, respectively. All statistical analyses of introgression and environmental factors were conducted using JMP 7 (SAS Institute Inc.), setting  $\alpha = 0.05$  for all statistical tests.

## Results

### Identification of hybrids using morphology and mtDNA sequences

A total of 284 *Mauremys* turtles were captured from 21 locations among the 41 sampling sites. On the basis of morphology, we identified 237 *M. reevesii*, 28 *M. sinensis* and 19 individuals belonging to intermediate forms (Table 1). Among the 21

**Table 2** Correlations between environmental factors and the magnitude of genetic introgression ('migration rate' into each pond, evaluated based on 13 microsatellite loci using BAYESASS 3.0; see Table 1)

Abbreviation	Description	Data type	<i>P</i> value	Selected for regression of introgression
DM	Shortest distance to main road	Continuous	0.2855	No
DS	Shortest distance to secondary road	Continuous	0.0937	Yes
VL	Vegetation coverage on land <sup>a</sup>	Ordinal	0.9267	No
VW	Vegetation coverage in water <sup>b</sup>	Ordinal	0.1044	No
PS	Substrate type around of the pond <sup>c</sup>	Ordinal	0.9997	No
PA	Area of the pond <sup>d</sup>	Ordinal	0.6637	No
PD	Depth of the pond <sup>e</sup>	Ordinal	0.7079	No
MS	Amount of <i>Mauremys sinensis</i> in the pond <sup>f</sup>	Continuous	0.0032	Yes

The selection of variables was performed in a stepwise manner with an enter probability of 0.1 and a leave probability of 0.1.

<sup>a</sup>Defined by the height of vegetation within 10 m around the shore. Low: vegetation height under 50 cm; high: vegetation height above 50 cm.

<sup>b</sup>Defined by the ratio of vegetation coverage with respect to the pond area. Low: <10%; medium: 10–50%; high: >50%.

<sup>c</sup>Defined by the level of artificial modification: natural substrate and artificial substrate.

<sup>d</sup>Small: <1000 m<sup>2</sup>; medium: 1500–10 000 m<sup>2</sup>; large: >10 000 m<sup>2</sup>.

<sup>e</sup>Shallow: <0.6 m; medium: 0.6–1.5 m; deep: >1.5 m.

<sup>f</sup>Number of individuals captured during the capture process; defined by genetic assignments from STRUCTURE analysis (Table 1).

ponds in which turtles were captured, nine locations featured introduced *M. sinensis*, and 19 individuals of intermediate forms (6.7%) were recorded from five ponds (Table 1).

The mitochondrial cytochrome *b* sequences of the 284 wild turtles were pooled with those of 21 pure *Mauremys* and two hybrids from turtle farms. This dataset yielded a total of nine *M. reevesii* haplotypes (KT763392–KT763400) and 19 *M. sinensis* haplotypes (KT763401–KT763419; Appendix S2). All pure turtles could be precisely assigned to their own clades with high support from bootstrap values and Bayesian posterior probabilities (Fig. S1). Of the two confirmed hybrids from the turtle farm, one was a descendant of an *M. reevesii* mother, and the other was a descendant of an *M. sinensis* mother. However, the morphological and mitochondrial assignments revealed inconsistency for some turtles in Kinmen (Table 1). Among the 236 morphological *M. reevesii*, seven presented *M. sinensis* haplotypes. In addition, one of the 28 morphological *M. sinensis* presented *M. reevesii* sequences. Among the 19 intermediate forms, six individuals and 13 individuals presented sequences belonging to the *M. reevesii* and *M. sinensis* clades, respectively (Figs S1 and S2). These results indicate that the potential hybrids could have maternal inheritance from either species.

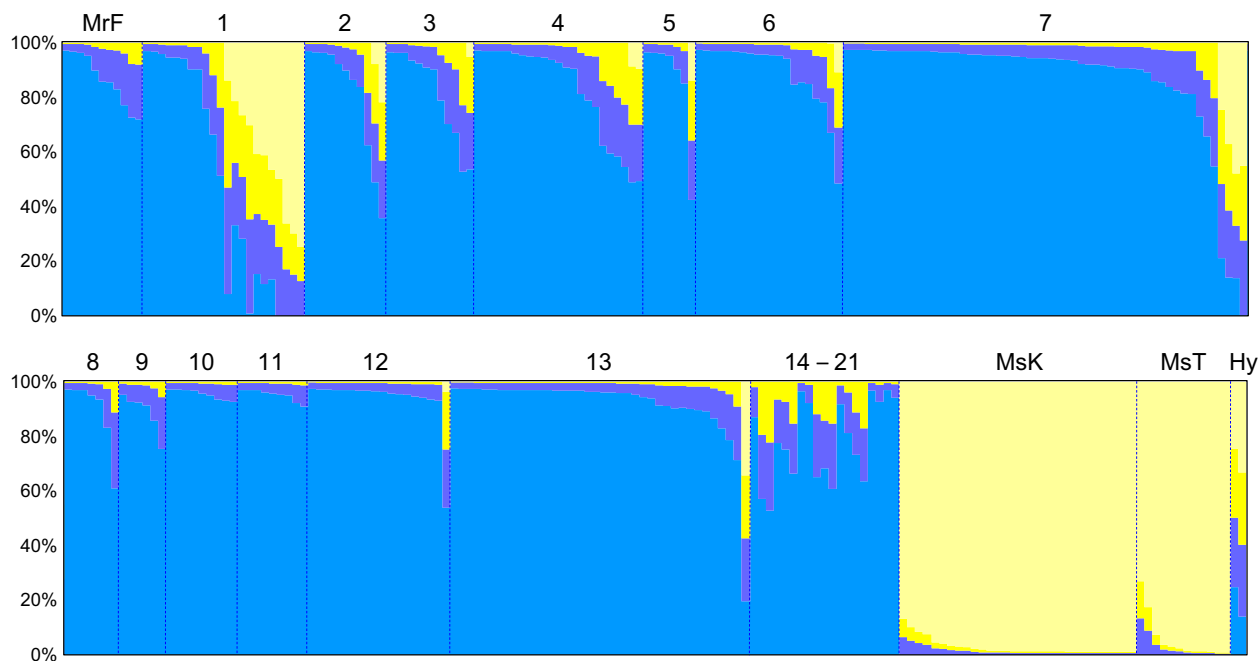
### Identification of hybrids using microsatellite data

STRUCTURE HARVESTER indicated that the best cluster number was  $K = 2$  ( $\Delta K = 5920.33$ ; see also Figs S3 and S5, Table S3

and Appendix S2 for details and raw data). Pure *M. reevesii* ( $N = 11$ ) belonged to cluster 1 (blue in Fig. 3) with probabilities ranging from 0.919 to 0.995 (mean  $\pm$  SD =  $0.969 \pm 0.026$ ), whereas pure *M. sinensis* ( $N = 11$ ) belonged to cluster 2 (yellow) with probabilities ranging from 0.913 to 0.996 ( $0.982 \pm 0.024$ ). The two confirmed F1 hybrids from the turtle farm had probabilities of 0.499/0.501 and 0.403/0.597, respectively. These results confirmed that the resolution of the 13 microsatellite loci could effectively detect hybrids, and the 0.875/0.125 threshold was subsequently used to define hybrids and backcrosses within three generations. Based on this threshold, 36 of the 284 (12.7%) wild *Mauremys* were hybrids or backcrosses (Table 1).

Calculations using NEWHYBRIDS 1.1 Beta 3 (Anderson & Thompson, 2002) yielded a similar result. All pure individuals and confirmed hybrids were allocated to the correct categories, and 32 of the 284 wild-caught *Mauremys* (11.3%) were hybrids or backcrosses (Table 1). The ternary plot of all individuals is presented in Fig. 4a.

The results of DAPC using the first 28 PCs are presented in Fig. 4b. *Mauremys reevesii* and *M. sinensis* could be clearly divided into distantly separated clusters, whereas the intermediate forms were positioned in the middle between the two. Comparing the morphology and DAPC results revealed that one morphological *M. reevesii* and two intermediate forms clustered close to *M. sinensis* and that at least eight additional morphological *M. reevesii* should be assigned as hybrids.



**Figure 3** Genetic assignments of pure *Mauremys reevesii* from turtle farms (MrF), pure *M. sinensis* from Kinmen (MsK) and Taiwan (MsT), confirmed F1 hybrids between the two (Hy) and samples collected from all the 21 ponds in Kinmen (1–21). The population genetic structure was determined using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009) with the optimal grouping number  $K = 2$ . The blue and yellow zones represent the probability of each individual being assigned as *M. reevesii* or *M. sinensis*, respectively. The zones with darker coloration represent the range of confidence intervals; their values are shown in Table S3.

Overall, the Structure, NEWHYBRIDS and DAPC analyses yielded congruent results based on the microsatellite data. All analyses indicated higher ratios (approximately twofold) of hybridization compared to the preliminary observations based solely on morphology. Based on genetic inference, the number of ponds with hybrids increased to 12 (57.1% of the 21 ponds with turtles), obviously higher than the number obtained based on the preliminary observation by morphology (5 ponds). Excluding the ponds with small sample sizes ( $N < 5$ ), the hybrid rate reached 54.5% in Ming Lake and 26.7% in Shungli Lake (Table 1). The total hybrid rate was estimated to range between 11.3% (NEWHYBRIDS) and 12.7% (STRUCTURE).

### Correlation between genetic introgression and environmental factors

The genetic introgression from invasive to native species was calculated and ranged between 0.0141 and 0.1439 (Table 1). Two environmental factors, the number of *M. sinensis* collected in the pond (MS) and the DS, were chosen for the forward-selection procedures (forward stepwise: MS:  $F_{1,10} = 22.20$ ,  $P = 0.0008$ ; DS:  $F_{1,10} = 5.86$ ,  $P = 0.0361$ ; VW<sub>medium</sub>:  $F_{1,10} = 3.65$ ,  $P = 0.0850$ ; VW<sub>high</sub>:  $F_{1,10} = 1.86$ ,  $P = 0.2063$ ; Table 2). These two factors were used to build a multiple regression model to explain the variance of the migration rate across the 13 populations. There was no interaction effect of MS and DS on genetic introgression ( $F_{1,9} = 0.31$ ,  $P = 0.58$ ). The final model significantly explained the variance of genetic introgression ( $F_{2,10} = 10.38$ ,  $P = 0.0037$ ,  $R^2 = 0.68$ ) without violating the assumptions of the regression analysis (Brown–Forsythe:  $F_{1,11} = 1.11$ ,  $P = 0.3154$ ; Shapiro–Wilk tests:  $W = 0.95$ ,  $P = 0.6632$ ; Durbin–Watson test:  $d = 0.34$ ,  $P = 0.6843$ ). The migration rate was positively associated with the number of *M. sinensis* ( $F_{1,10} = 14.24$ ,  $P = 0.0036$ , coefficient =  $0.0067 \pm 0.0018$ ), whereas the marginal effect of DS was negative ( $F_{1,10} = 3.44$ ,  $P = 0.0934$ , coefficient =  $-0.0007 \pm 0.0004$ ). Populations that had fewer individuals of *M. sinensis* and were farther from roads had lower genetic introgression.

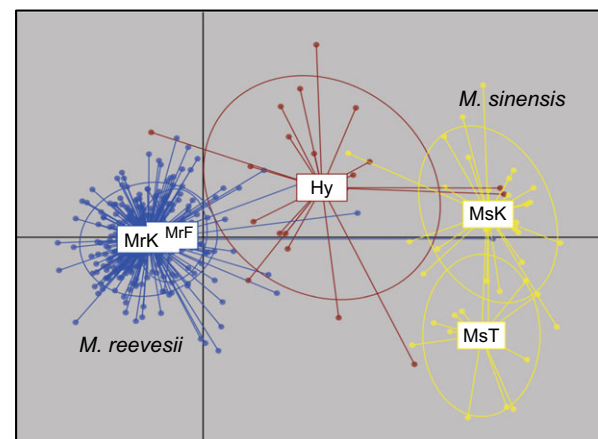
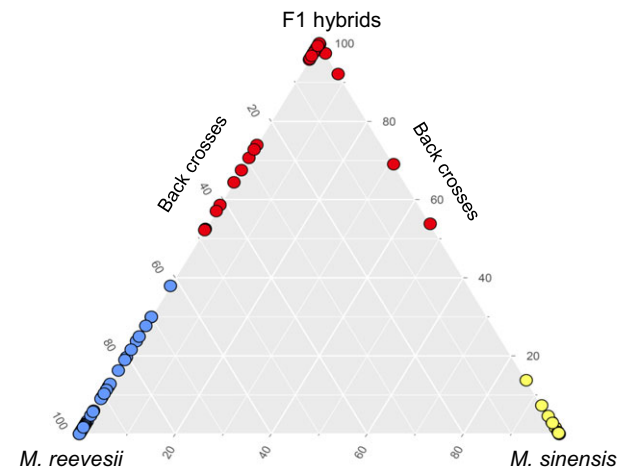
Furthermore, hybrid rate of each pond (Table 1) was applied as an alternative response variable that might be influenced by environmental factors. This tests did not show a significant correlation with levels of genetic introgression in our analysis ( $P > 0.05$ ).

## Discussion

### Quantifying hybrids and backcrosses in the wild

This research is among the first to quantitatively evaluate the magnitude of genetic introgression from introduced to native turtles in the wild. Hybridization among Testudines is not new to science; case reports are common around the world, such as well-known cases in Cheloniidae (Lara-Ruiz *et al.*, 2006; Bowen & Karl, 2007) and Emydidae (Parham *et al.*, 2013; Godwin *et al.*, 2014; Jensen *et al.*, 2014). However,

cases from the Asian family Geoemydidae were less reported until the late 20th century. At least five recently described species have been revealed to be the result of human-mediated hybridization (Parham *et al.*, 2001; Spinks *et al.*, 2004; Stuart & Parham, 2007). However, a large proportion of these hybrids were thought to have been born in Chinese



**Figure 4** (a) Assignment of each *Mauremys* turtle as *M. reevesii* (blue), *M. sinensis* (yellow), hybrids or backcrosses (dark red) as calculated using NEWHYBRIDS 1.1 Beta 3 (Anderson & Thompson, 2002). A total of 32 of the 284 wild-caught *Mauremys* (11.3%) were hybrids or backcrosses (see also Table 1 for the numbers in each pond). The posterior probabilities of pure *Mauremys* were similar, and thus most plots of the 222 *M. reevesii* and 30 *M. sinensis* are strongly overlapping. (b) Scatter plots of *M. reevesii* from Kinmen (MrK) and turtle farms (MrF), *M. sinensis* from Kinmen (MsK) and Taiwan (MsT), and suspected hybrids with intermediate morphology (Hy) on the first two axes of the Discriminant Analysis of Principal Components (DAPC). Populations are labeled in the center of the dispersion, and the large open circles indicate the 67.5% inertia ellipses for each population. Comparison of the morphology and DAPC results revealed one morphological *M. reevesii* and two intermediate forms clustered close to *M. sinensis* and at least eight additional morphological *M. reevesii* that should be assigned as hybrids.



turtle farms (Parham & Shi, 2001; Parham *et al.*, 2001; Shi *et al.*, 2008), and direct observation of hybridization among geoemydid turtles in the wild was limited to a few cases (Fong & Chen, 2010; Suzuki *et al.*, 2011). Due to the dramatic population decline of turtles in oriental Asia, the Kinmen Islands provide a valuable opportunity to evaluate the magnitude of artificial-induced genetic introgression in a natural habitat. The evidence from this study strongly supports the occurrence of hybridization when introduced turtles come into contact with native species, and this impact might be severe in highly disturbed habitats. The detection of probable backcrosses further indicates the fertility of the F1 offspring. The backcross individuals exhibited a wide variety of genetic composition, with an 'exotic proportion' (from *M. sinensis*) ranging between 0.138 (backcross to *M. reevesii*) and 0.830 (backcross to *M. sinensis*).

Because native *M. reevesii* usually outnumbered introduced *M. sinensis*, the F1 offspring have a higher probability of meeting *M. reevesii*, resulting in genetic introgression. Notably, the number of hybrids or backcrosses detected by microsatellites was nearly twofold higher than that preliminarily estimated by morphology in this study, and most such detected individuals were originally identified as *M. reevesii*. During the process of backcrossing, both morphological and genetic characteristics approach those of *M. reevesii* generation by generation. Theoretically, after the F1 hybrids backcross with *M. reevesii* for two generations, the genetic composition of *M. sinensis* would decrease to 0.125; generations beyond the third generation would be indistinguishable from pure *M. reevesii* by our microsatellite markers. Therefore, the severity of genetic introgression might be greater than the level we have detected if backcrossing continues: (1) the hybrid rate may be underestimated when the decision is based solely on morphology; (2) even with the inclusion of microsatellite data, the magnitude of genetic introgression may still be underestimated, for example, due to limited genomic representation of the utilized markers (Gómez-Sánchez *et al.*, 2018).

### Human disturbance may lead to genetic introgression

In this study, we further demonstrated the impact of artificial effects on genetic introgression. The magnitude of genetic introgression was positively correlated with the amount of *M. sinensis* found in a pond and marginally increased with decreasing distance from the closest secondary roads. The consequence of the first factor is reasonable and straightforward: the release of introduced turtles increases the rate of encounter of the two congeners. In freshwater Testudines, habitat specialization has been shown to maintain partial reproductive isolation among different species (Via, 1999; De Busschere *et al.*, 2010). Based on our previous investigation, *M. reevesii* prefers ponds with dense vegetation inside and around the water body (Lin *et al.*, 2015), whereas *M. sinensis* seems to prefer open aquatic habitats at low elevations (Chen & Lue, 2010; also see the habitat description in Chen & Lue, 1998, 1999). Human-mediated dispersal

facilitates encounters between the two turtles and disruption of their ecological boundary in nature.

The second factor, distance from the pond to the closest secondary roads, had a marginal and negative association, further indicating negative impacts of human disturbance on natural habitats. In a similar study focused on hybridization among wolves and coyotes (Benson, Patterson & Wheelton, 2012), primary, secondary and tertiary roads had different impacts on the behavior and hybridization of these carnivores and the subsequent consequences. In our case, distance from main roads did not show a significant correlation with the turtle population, possibly because the main roads represent highly developed regions in which the habitats are not tolerated by any of the turtles. Most of the ponds with turtles were located far from major roads, further reducing the statistical significance of this factor. In contrast, secondary roads are found in semi-developed regions in which the conflict between habitat and human activity is still unfolding.

Our tests that relied on hybrid rate as a response variable did not yield significant results, perhaps due to insufficient sample size (i.e. the number of ponds being invaded), or due to the fact that the hybrid offspring of a pond comprises individuals which carry different ratio of invasive alleles.

There are several possible routes by which distance from roads might affect the turtle population. First, ponds located close to roads are expected to suffer high risks of artificial habitat modification, including the clearing of vegetation inside or surrounding the pond or alteration of the substrate around the pond shore. Although not statistically significant, at least three of the five ponds with the highest hybrid rates suffered from artificial modification of the pond shore; the original vegetation of these ponds was changed to vertical concrete banks. This modification increases encounters between the two *Mauremys* by trapping them in the same water body, delays the escape of females from forced copulation with males, and ultimately enhances the mating success of hetero-species pairings. Second, ponds close to the road also suffer from the risk of human-mediated release of exotic turtles because of their easy accessibility, linking to the first factor discussed above. Although the overall trend of this negative factor is only marginally significant, this association is consistent with our previous conclusion that the road effect will negatively impact the native turtle population (Lin *et al.*, 2015).

### Conclusions and suggestions

We have demonstrated high genetic introgression from introduced *M. sinensis* toward native *M. reevesii* in the wild as well as the risk of underestimation of hybrid rates based on the low ratio of morphologically intermediate forms compared to the high ratio of genetic hybrids. The abundance of hybrids and backcrosses provides strong evidence for the fertility of their offspring in the wild. We here also provide statistical evidence that human disturbance might increase the opportunity for hetero-specific encounters, resulting in higher probability of hybridization. Similar mechanisms may apply to many threatened Testudines around the world.

As possibly the last natural population in southeast China, *M. reevesii* in Kinmen is now strictly protected by the Wildlife Conservation Act of Taiwan. However, this law cannot prevent colonization of the natural habitat by exotic species and further invasion of the native gene pool. To protect this species, we propose the initiation of a program to remove exotic turtles (including *M. sinensis* and *Trachemys scripta elegans*) from ponds on Kinmen. We further appeal for the strict protection of several remote ponds that are far from human disturbance and have remained genetically uninvaded.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Maximum likelihood tree of mitochondrial cytochrome *b* haplotypes from *Mauremys* turtles.

**Figure S2.** Minimum spanning network of mitochondrial cytochrome *b* haplotypes from *Mauremys* turtles.

**Figure S3.** The distribution of delta *K* of *Mueremys* samples from 13 microsatellite loci conducted in STRUCTURE.

**Figure S4.** Population assignments of three pseudo populations from the real genotyping data: a pure *Mauremys reevesii* population (left 100 individuals in blue), a pure *M. sinensis* population (right 100 individuals in yellow), and their F1 hybrids (middle 100 individuals) assuming random mating from these parental populations.

**Figure S5.** Genetic assignments of pure *Mauremys reevesii* (MrF), pure *M. sinensis* from kinmen (MsK), pure *M. sinensis* from Taiwan (MsT), confirmed F1 hybrids (Hy), and samples collected from all the 21 ponds in Kinmen (1–21).

**Table S1.** Locus name, repeat number, primer sequences, range of allele size, annealing temperature, accession number, and references for the 13 microsatellite loci used in this study.

**Table S2.** Microsatellite genotyping of the 13 loci from the 13 populations with sample sizes higher than five.

**Table S3.** Genetic population assignment and confidence intervals of each *Mauremys* in STRUCTURE analysis.

**Table S4.** Correlations among environmental factors.

**Appendix S1.** Supplementary methods for mitochondrial DNA sequencing, phylogenetic analyses, microsatellite genotyping, effectiveness test and the correlations among environmental factors.

**Appendix S2.** Raw data for mitochondrial haplotypes and microsatellite genotyping.