

## First report of hybridization between *Mauremys leprosa* and *Mauremys sinensis* highlights the risk of exotic *Mauremys* spp. pet trade

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The global exponential growth in the number of exotic turtle species exploited in the pet trade market facilitates hybridization events between distantly related species. The 1997 EU trade ban on red-eared slider turtles (*Trachemys scripta elegans*), followed by the Spanish ban on all *T. scripta* subspecies in 2011, resulted in the importation of other chelonians (e.g. *Graptemys* spp., *Pseudemys* spp., *Mauremys* spp.), mainly from America and Asia to Spain. The importation of the Chinese stripe-necked pond turtle, *Mauremys sinensis*, via the pet trade market resulted in its release into natural environments across Europe, including the Iberian Peninsula where it co-occurs with the Western Mediterranean pond turtle, *Mauremys leprosa*. We maintained three *M. leprosa* females and two *M. sinensis* males in captivity, and analysed the obtained offspring (two hatchlings) with mitochondrial (cytochrome *b*) and nuclear (R35) genetic markers. Both morphological (i.e. with intermediate morphological characters) and genetic (heterozygous hatchlings) results confirmed the hybridization between these two species and raise concern about the negative effects of acclimatised *Mauremys sinensis* across the *Mauremys leprosa* range.

**Key words:** captivity; conservation; hybridization; *Mauremys leprosa*; *Mauremys sinensis*; Spain.

The family Geoemydidae is the largest family of turtles, comprising 19 genera and 69 aquatic or semi-aquatic species (van Dijk *et al.*, 2014), distributed throughout

Europe, North Africa, the Middle East to China, southern Russia, Indonesia and the Philippines, with only one genus occupying the American continent, from

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Mexico to Brazil (IVERSON, 1992; LE & McCORD, 2008). A large number of hybridization events have been reported within this family (see BUSKIRK *et al.*, 2005), even between phylogenetically distant genera such as *Cuora-Mauremys*, *Cuora-Geoemyda*, *Cyclemys-Mauremys* or *Sacalia-Mauremys* (e.g. OTANI, 1995; SCHILDE *et al.*, 2004; BUSKIRK *et al.*, 2005; ZHOU *et al.*, 2015), which has also led to incorrect descriptions of new species (PARHAM *et al.*, 2001; WINK *et al.*, 2001, SPINKS *et al.*, 2004, STUART & PARHAM, 2007). Within the genus *Mauremys*, cases of hybridization were reported in both captive conditions (e.g. *M. leprosa* and *M. reevesii*, NICKL, 2015), and natural populations (e.g. Asian *M. sinensis* and *M. reevesii*, XIA *et al.*, 2011; FONG & CHEN, 2010; and the European *M. rivulata* and *M. caspica*, FRITZ *et al.*, 2008).

The global exponential growth in the number of exotic turtle species increases the release of exotic species by pet-owners into native ecosystems, facilitating hybridization events with wild relatives, and potentially become invasive. One of the most commercially important turtle species is the red-eared slider, *Trachemys scripta elegans*, which became one of the 100 most harmful invasive species in the world (LOWE *et al.*, 2000). In 1997 it was banned from selling in the European pet trade market (EUROPEAN COMMISSION, 1997), as well as all subspecies of *T. scripta* in Spain since 2011 (MINISTERIO DE MEDIO AMBIENTE, MEDIO RURAL Y MARINO, 2011). After this ban, pet stores introduced other chelonians, both from America (e.g. *Graptemys* spp. and *Pseudemys* spp.), and Asia (e.g. *M. reevesii* and *M. sinensis*) to the pet trade market. According to a

survey in 684 of the 1499 official turtle farms in China, the production of Asian Geoemydidae has increased significantly in the last years, with ca. 1.8 million *M. sinensis* available for sale every year (HAITAO *et al.*, 2008).

As a direct consequence of the importation of non-banned exotic species, a growing number of individuals of the Chinese stripe-necked pond turtle, *Mauremys sinensis*, were found across Europe (e.g. Italy, PANZERI *et al.*, 2014; Slovakia, JABLONSKI *et al.*, 2018), and the Iberian Peninsula (CAMPUS-SUCH *et al.*, 2016; SILVESTRE *et al.*, 2019;), where it co-occurs with wild populations of the Mediterranean Stripe-necked Terrapin (or Western Mediterranean pond turtle) *Mauremys leprosa*. While *M. sinensis* is native to China, Taiwan and Vietnam, *M. leprosa* is widely distributed in the Iberian Peninsula (with a few scattered populations in the southern France) and in the Maghreb (VERÍSSIMO *et al.*, 2016). Here, we aim to evaluate the potential hybridization between these two species by maintaining individuals in captive conditions and analyzing the obtained offspring with mitochondrial and nuclear markers.

We collected two males of *M. sinensis* (157 & 192 mm carapace length, C.L.) from Burriana marsh (39°53'29'' N, 0°2'37'' W) and three females of *M. leprosa* (224, 226 & 240 mm C.L.) from Castellón marsh (39°59'11'' N, 0°0'26'' E) in eastern Spain. We kept them in the facilities of the Centre for the Conservation of Freshwater Species of the Valencia Region (CCEDCV) in a 2x2 m outdoor pool with 2/3 of its surface filled with water (maximum depth 35 cm)

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**Figure 1.** Photo of a six-month-old hybrid between *Mauremys leprosa* and *M. sinensis*. Head and neck is typical of *M. sinensis*, with greenish and black stripes on a pale-yellow background, without the orange tones typical of *M. leprosa* juveniles (Photo: V. Sancho).

and 1/3 of loose soil to facilitate egg laying.

We fed them with semi floating Trout feed pellets (Skretting© t-6 Optiline 3P sf) until oviposition (from July 19<sup>th</sup> 2013 to October 26<sup>th</sup> 2015).

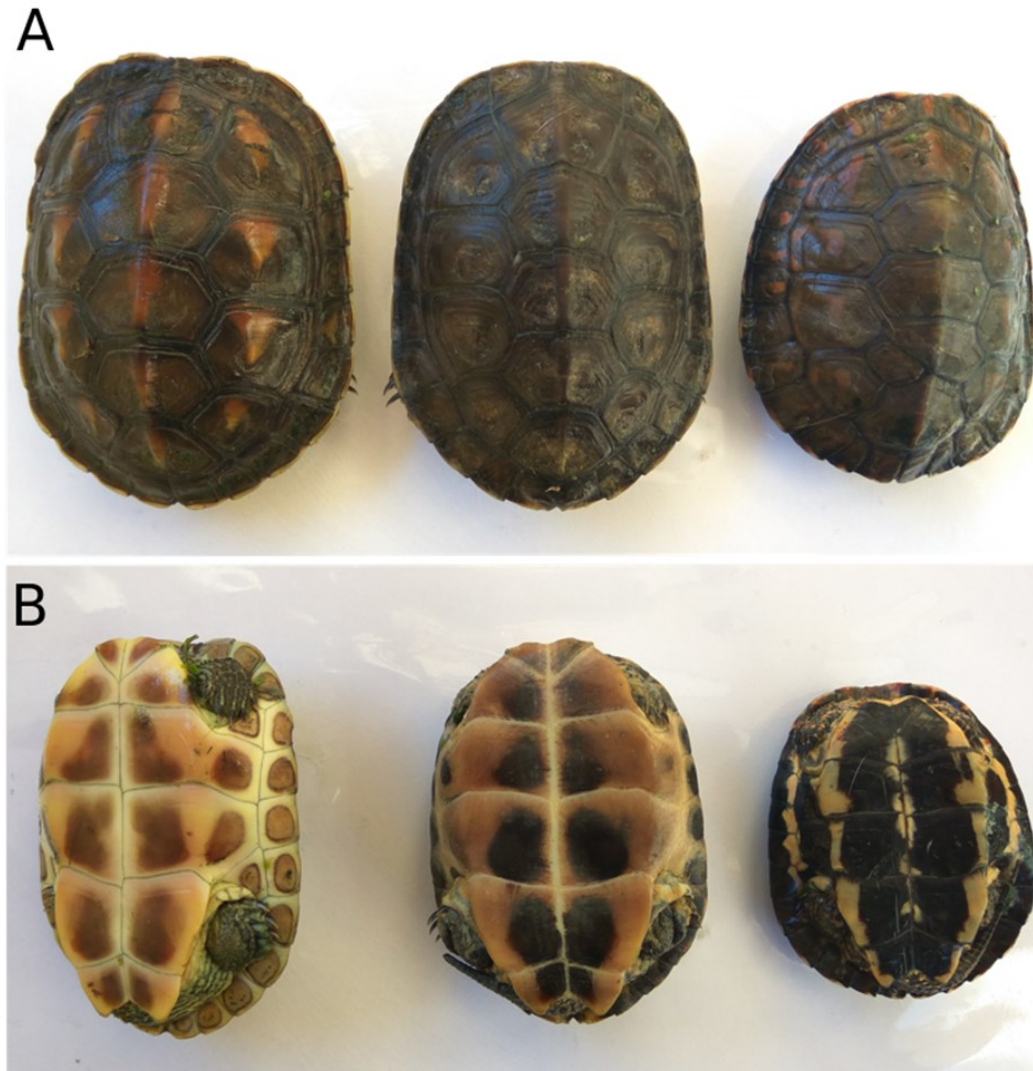
During the first breeding season, we obtained two clutches of 11 and three eggs from two *M. leprosa* females, which were incubated in moistened vermiculite (1 part of water per 1 part of vermiculite) at 28°C (ExoTerra© PT2499). After four weeks we verified that all eggs were infertile (i.e. embryos were not present upon opening them). In the second breeding season we observed two other clutches. Four eggs of the first clutch were maintained under the same conditions mentioned above, during seven weeks, after which only one egg hatched. The second female deposited nine eggs that were kept outdoors in the nest, with just one hatching after 52 days.

We collected buccal swabs from the two *M. sinensis* males, one of the successfully breeding *M. leprosa* females, and the two putative *M. sinensis-leprosa* hatchlings, and preserved them in ethanol for molecular analysis. We targeted DNA fragments of the cytochrome *b*

mitochondrial gene (*cytb*; ca. ~1000 base pairs) and one nuclear intron (*R35*; ca ~1000 base pairs), following a recent study unveiling the biogeographic history of *M. leprosa* (VERÍSSIMO *et al.*, 2016). For *cytb* we used the primers mt-a-neu (LENK & WINK, 1997) and H-15909 (LENK *et al.*, 1999), and primers R35 Ex1 and R35 Ex2 (FUJITA *et al.*, 2004) for *R35* (see VERÍSSIMO *et al.*, 2016 for further information on PCR and sequencing conditions for each marker). Sequences were verified and aligned by eye using Geneious R11.1.4 (<http://www.geneious.com/>). Due to an indel presence and heterozygous positions in the *R35* intron, sequences were phased in DnaSP v5.10 (LIBRADO & ROZAS, 2009) to reconstruct haplotypes. *Cytb* and *R35* sequences were blast against our own databases and BLASTn (<https://blast.ncbi.nlm.nih.gov/>) to confirm species assignment of the specimens used. This includes 68 and 28 *cytb* sequences (unique haplotypes) and 14 (unique haplotypes) and 19 *R35* sequences for *M. leprosa* and *M. sinensis*, respectively.

Heads and necks of the two hatchlings were typical of *M. sinensis* with greenish

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**Figure 2.** (A) Dorsal view comparing the carapace of *M. sinensis* (left), hybrid (center) and *M. leprosa* (right). (B) Ventral view comparing the plastron of *M. sinensis* (left), hybrid (center) and *M. leprosa* (right). The two four year-old hybrids shown brownish carapace without orange tones, and plastron with quadrangular blotches, but lacking the second blotch on pectoral and abdominal scutes. Photos: J. M. López.

and black stripes on a pale-yellow background, without the orange tones typical of *M. leprosa* juveniles (Fig. 1). The carapace showed a well marked central

keel, while the side keels were reduced. The colour was uniformly brown, without the orange tones typical of *M. sinensis*. The plastron showed an intermediate

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	1	10	20	30	40	50	60
<i>M. leprosa</i> _f_GVA6031	GTCAGACTTCTTTGCATATTTGTAATGATTTATTTACACAGGGACTGGGTTTAT						GTGATA
<i>M. sinensis</i> _m_GVA6032	GTCAGACTTCTTTGCATATTTGTAATGATTTATTTACACAGGGACTGGGTTTAT						GTGATA
<i>M. sinensis</i> _m_GVA6033	GTCAGACTTCTTTGCATATTTGTAATGATTTATTTACACAGGGACTGGGTTTAT						GTGATA
<i>M. leprosa/sinensis</i> _h_GVA6034	GTCAGACTTCTTTGCATATTTGTAATGATTTATTTACACAGGGACTGGGTTTAT						GTGATA
<i>M. leprosa/sinensis</i> _h_GVA6035	GTCAGACTTCTTTGCATATTTGTAATGATTTATTTACACAGGGACTGGGTTTAT						GTGATA
<i>M. leprosa</i> _f_GVA6031	TTAATTGAAACAGACATCCAGCAATTATGAGCAGCATAAATCATATCCATAGAATTCATT						
<i>M. sinensis</i> _m_GVA6032	TTAATTGAAACAGACATCCAGCAATTATGAGCAGCATAAATCATATCCATAGAATTCATT						
<i>M. sinensis</i> _m_GVA6033	TTAATTGAAACAGACATCCAGCAATTATGAGCAGCATAAATCATATCCATAGAATTCATT						
<i>M. leprosa/sinensis</i> _h_GVA6034	TTAATTGAAACAGACATCCAGCAATTATGAGCAGCATAAATCATATCCATAGAATTCATT						
<i>M. leprosa/sinensis</i> _h_GVA6035	TTAATTGAAACAGACATCCAGCAATTATGAGCAGCATAAATCATATCCATAGAATTCATT						
<i>M. leprosa</i> _f_GVA6031	TCAAATAAGCTGTGTTTGTGTATGTATTTATCTGGCTTTCTTCAGGATTGATACATTCA						
<i>M. sinensis</i> _m_GVA6032	TCAAATAAGCTGTGTTTGTGTATGTATTTATCTGGCTTTCTTCAGGATTGATACATTCA						
<i>M. sinensis</i> _m_GVA6033	TCAAATAAGCTGTGTTTGTGTATGTATTTATCTGGCTTTCTTCAGGATTGATACATTCA						
<i>M. leprosa/sinensis</i> _h_GVA6034	TCAAATAAGCTGTGTTTGTGTATGTATTTATCTGGCTTTCTTCAGGATTGATACATTCA						
<i>M. leprosa/sinensis</i> _h_GVA6035	TCAAATAAGCTGTGTTTGTGTATGTATTTATCTGGCTTTCTTCAGGATTGATACATTCA						
<i>M. leprosa</i> _f_GVA6031	TACAAAATCAGTGCATTTTAAAAATGAAAAGCCAGATTCATCATTGTTTTCCAGTTTTA						
<i>M. sinensis</i> _m_GVA6032	TACAAAATCAGTGCATTTTAAAAATGAAAAGCCAGATTCATCATTGTTTTCCAGTTTTA						
<i>M. sinensis</i> _m_GVA6033	TACAAAATCAGTGCATTTTAAAAATGAAAAGCCAGATTCATCATTGTTTTCCAGTTTTA						
<i>M. leprosa/sinensis</i> _h_GVA6034	TACAAAATCAGTGCATTTTAAAAATGAAAAGCCAGATTCATCATTGTTTTCCAGTTTTA						
<i>M. leprosa/sinensis</i> _h_GVA6035	TACAAAATCAGTGCATTTTAAAAATGAAAAGCCAGATTCATCATTGTTTTCCAGTTTTA						
<i>M. leprosa</i> _f_GVA6031	CATCAGTGTAACTCCACTGAACCCAGCATAGAGCTAGGCTAATGGTAGGGGTGAATCAGG						
<i>M. sinensis</i> _m_GVA6032	CATCAGTGTAACTCCACTGAACCCAGCATAGAGCTAGGCTAATGGTAGGGGTGAATCAGG						
<i>M. sinensis</i> _m_GVA6033	CATCAGTGTAACTCCACTGAACCCAGCATAGAGCTAGGCTAATGGTAGGGGTGAATCAGG						
<i>M. leprosa/sinensis</i> _h_GVA6034	CATCAGTGTAACTCCACTGAACCCAGCATAGAGCTAGGCTAATGGTAGGGGTGAATCAGG						
<i>M. leprosa/sinensis</i> _h_GVA6035	CATCAGTGTAACTCCACTGAACCCAGCATAGAGCTAGGCTAATGGTAGGGGTGAATCAGG						
<i>M. leprosa</i> _f_GVA6031	TTTGTGTTTTCAAGAGTCTGGCAGGCATTAGGTTGACTCTGAGATCTGTGAGTTTTACT						
<i>M. sinensis</i> _m_GVA6032	TTTGTGTTTTCAAGAGTCTGGCAGGCATTAGGTTGACTCTGAGATCTGTGAGTTTTACT						
<i>M. sinensis</i> _m_GVA6033	TTTGTGTTTTCAAGAGTCTGGCAGGCATTAGGTTGACTCTGAGATCTGTGAGTTTTACT						
<i>M. leprosa/sinensis</i> _h_GVA6034	TTTGTGTTTTCAAGAGTCTGGCAGGCATTAGGTTGACTCTGAGATCTGTGAGTTTTACT						
<i>M. leprosa/sinensis</i> _h_GVA6035	TTTGTGTTTTCAAGAGTCTGGCAGGCATTAGGTTGACTCTGAGATCTGTGAGTTTTACT						
<i>M. leprosa</i> _f_GVA6031	AAGGCAGA						
<i>M. sinensis</i> _m_GVA6032	AAGGCAGA						
<i>M. sinensis</i> _m_GVA6033	AAGGCAGA						
<i>M. leprosa/sinensis</i> _h_GVA6034	AAGGCAGA						
<i>M. leprosa/sinensis</i> _h_GVA6035	AAGGCAGA						

**Figure 3.** R35 sequence alignment for *M. Leprosa*, *M. sinensis* and their hybrids, highlighting with different colours the position of the four diagnostic nucleotide bases. Female (f), males (m), hybrids (h), and voucher codes (GVA) are shown in the specimens' ID code.

pattern between *M. sinensis* and *M. leprosa*, with the characteristic dark quadrangular blotches of *M. sinensis*, with a brownish background, although they both lack the second blotch on the bridge side of pectoral and abdominal scutes (Figs. 1 and 2).

The obtained *cytb* sequences for the female and the two males were identified as *M. leprosa* and *M. sinensis*, respectively. However, all the *cytb*

sequences differ in a few positions from the sequences retrieved from GenBank, and thus were deposited with the following codes: MT629907- MT629911. The two *M. sinensis* males were differentiated in one mutation.

The R35 nuclear sequences were trimmed to ca. 368 bp due to the impossibility of unambiguous base calling when *M. sinensis* and *M. leprosa* haplotypes overlapped after one detected



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intron, and were confirmed as *M. leprosa* (GenBank Acc. No.: KX374284.1) and *M. sinensis* (GenBank Acc. No.: GQ259470.1). There are four diagnostic positions between *M. leprosa* and *M. sinensis* (Fig. 3).

The two hatchlings showed *M. leprosa* mitochondrial haplotype (as mitochondrial DNA is inherited in the maternal line) and heterozygous positions in each of the four variable positions observed in R35 sequences (Fig. 3).

Our results unambiguously confirm F1 hybrids between *M. leprosa* and *M. sinensis*. Despite these hybrids were obtained in captivity, and the low levels of fertile eggs during the experiment, we confirm the hybridization between the exotic *Mauremys sinensis* and the native *Mauremys leprosa* and highlight the particular risk of *Mauremys* spp. pet trade in the Iberian Peninsula and northern Maghreb, which would have important implications to the long-term survival of the Mediterranean Stripe-necked Terrapin. Indeed, reproduction of *M. sinensis* was detected in semi-natural populations of the Iberian Peninsula (SILVESTRE *et al.*, 2019), which confirms the acclimatization of this Asian turtle to the Mediterranean environment typical of *M. leprosa*. It remains unknown if *M. sinensis/leprosa* F1 are fertile, or the effects on population growth and survival of the hybrids.

As *M. sinensis* is increasingly present in pet shops and owners often release them in the wild, the conservation of *M. leprosa* populations may be compromised if the sale of these exotic taxa continues.

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