

# Haplotype variation in founders of the *Mauremys annamensis* population kept in European Zoos

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**Abstract.** The critically endangered Annam leaf turtle *Mauremys annamensis* faces extinction in nature. Because of that, the conservation value of the population kept in European zoos becomes substantial for reintroduction programmes. We sampled 39 specimens of *M. annamensis* from European zoos and other collections (mainly founders, imports and putatively unrelated individuals), and also four specimens of *Mauremys mutica* for comparison. In each animal, we sequenced 817 bp of the mitochondrial ND4 gene and 940 bp of the nuclear R35 intron that were used as phylogenetic markers for *Mauremys mutica-annamensis* group by previous authors. The sequences of the R35 intron, which are characteristic for *M. annamensis* and which clearly differ from those characteristic for *M. mutica* and/or other *Mauremys* species, were mutually shared by all of the examined *M. annamensis*. They also possessed mitochondrial haplotypes belonging to the *annamensis* subclades I and II, distinctness of which was clearly confirmed by phylogenetic analyses. Thus, both nuclear and mitochondrial markers agreed in the unequivocal assignment of the examined individuals to *M. annamensis*. Although no obvious hybrids were detected within the founders of the captive population, further careful genetic evaluation using genom-wide markers is required to unequivocally confirm this result.

**Keywords.** *Mauremys*, Geoemydidae, conservation, mt gene ND4, nuclear intron R35, Vietnam, hybridization.

## INTRODUCTION

Asian turtles face an extinction crisis due to habitat destruction and high demands from the Chinese markets (van Dijk et al., 2000; Le et al., 2004; Cheung and Dudgeon, 2006; Turtle Conservation Coalition, 2011). One of the heavily exploited species is *Mauremys annamensis*, the Annam leaf turtle. (Siebenrock, 1903). This species of the family Geoemydidae has a very limited and fragmented distribution and is restricted only to central Vietnam (Le et al., 2004; Parham et al. 2006). *Mauremys annamensis* is almost extinct in the wild, with limited numbers in *ex-situ* populations in Vietnam, Europe and the USA. It is listed in the Appendix II of CITES and is globally red-listed as critically endangered by the IUCN (2013). Cap-

tive breeding seems to be one of the long-term solutions for the survival of Asian turtles (Hudson and Buhlmann, 2002; Turtle Conservation Coalition, 2011). *Mauremys annamensis* has been repeatedly bred in some European zoos, including Prague Zoo (Velenský, 2006; Raffle and Meier, 2013). Currently, these zoos have started co-ordinated *ex situ* conservation breeding of the species associated with a repatriation project. Among the programmes' top priorities at present is the repatriation of the best captive-bred specimens.

The situation of conservation breeding is complicated by hybridization among distinct species and even genera of the geoemydids (Galgon and Fritz, 2002; Fritz and Mendau, 2002; Fritz et al., 2004; Schilde et al., 2004; Spinks et al., 2004; Buskirk et al., 2005; Stuart and Par-

ham, 2006; Shi et al., 2008). Hybridization among *Mauremys annamensis*, *M. mutica*, *M. sinensis*, *M. nigricans*, *Cuora amboinensis* and *C. trifasciata* was reported both in captivity and in the wild (Parham et al., 2001; Shi and Parham, 2001; Fong and Chen, 2010). The current events of natural hybridization between *M. mutica* and *M. sinensis* on Taiwan Island (Fong and Chen, 2010) represent an especially interesting case.

The phylogenetically closest species of *M. annamensis* is *M. mutica* (Barth et al., 2004; Feldman and Parham, 2004; Spinks et al., 2004) and these species may interbreed (Fong et al., 2007). This represents a serious problem for the efforts to build sustainable *ex-situ* breeding programs enabling the reintroduction and establishment of sustainable populations of *M. annamensis* in the wild. Hybridization events in the *annamensis-mutica* complex were demonstrated by striking incongruence among phylogenies of the individual genes, i.e., the mitochondrial and nuclear markers. Some of these incongruences may result from recent translocation and consequent hybridization; however, hybridization events that took place in the past are even more likely. Fong et al. (2007) clearly demonstrated such incongruence in the Hainan population of *M. mutica*, which differs from the “true *mutica*” of the Eastern continental China by the presence of mitochondrial haplotypes forming a clade branching within those belonging to the *M. annamensis*. In contrast, sequences of R35 intron of Hainan *M. mutica* are even less related to the corresponding sequences of the *M. annamensis* than those of the “true *mutica*”. Moreover, it was clearly demonstrated that mitochondrial haplotypes of the *M. annamensis* was split into two deeply divergent haplogroups, which are referred to as the *annamensis* subclade I and II (Fong et al., 2007; Fong, 2008). The phylogeographic pattern of these subclades is, however, unclear due to the extinction of most of the original populations in the nature.

To organize proper *ex-situ* captive breeding and to remove potential hybrids from the rescue population, it is necessary to examine the genetic variation of the founders of the *M. annamensis* population. In this study, we focused on the founders, imported and putatively unrelated individuals of the *M. annamensis* kept in European zoos and other collections. We sequenced mitochondrial (ND4 gene) and nuclear (R35 intron) parts of DNA to (1) verify the species determination of the founders, (2) assess sequence variation of the captive population, (3) assign captive specimens into the main haplogroups (subclades I and II) and to (4) exclude the discovered interspecific species hybrids from the breeding pool. For comparison, we also included a few specimens of *M. mutica* into the analyses.

## MATERIAL AND METHODS

In this paper, we examined 39 specimens of *Mauremys annamensis* from European zoos and other collections (founders, imported and putatively unrelated individuals, i.e., captive born specimens having no shared maternal ancestors in their pedigree), and also four specimens of *M. mutica* were included for comparison (Table 1). For all individuals, we sequenced a combination of mitochondrial (mtDNA) and nuclear DNA (nuDNA).

For sampling of individuals, we used a non-invasive method: we took the tip of the claw from each sampled animal and stored in Eppendorf tubes with 96% ethanol at -20°C prior DNA extraction. We isolated the total genomic DNA with DNAeasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer’s recommendations.

Using standard conditions and the primers L-ND4 and H-Leu, we amplified an 892 bp fragment of mtDNA containing the NADH dehydrogenase subunit 4 (ND4) gene and parts of tRNA (Stuart and Parham, 2004). Following the conditions in Fujita et al. (2004), and using the primers R35Ex1 and R35Ex2, we amplified the fragment of nuDNA containing 1133 bp of the RNA fingerprint protein 35 (R35) gene intron 1.

Patterns from the sequencing chromatograms indicated that at the R35 locus, some individuals were heterozygous for a length polymorphism, which usually corrupts the sequence reads downstream of the indel site (see Bhangale et al., 2005, Fig. 1B). For sequencing the R35 intron, we used internal forward and reverse primers (Spinks and Shaffer, 2007) in combination with external primers (Fujita et al., 2004) for the putative length-polymorphic individuals (Spinks and Shaffer, 2007).

Sequences of both mtDNA and nuDNA fragments were aligned and manually checked using Chromas Lite 2.01 (Technelysium Pty Ltd), BioEdit (Hall, 1999) and Clustal X 1.81 (Thompson et al., 1997).

Analyses of the estimates of evolutionary divergence between the sequences of ND4 gene and R35 intron were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The included codon positions were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Bayesian analysis (BA) was conducted with MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best-fit model (HKY+G) was selected by hLRT in Modeltest 3.7 (Posada and Crandall, 1998). Two independent runs of Bayesian analyses were conducted with a random starting tree and run for 30x10<sup>6</sup> generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 10% of trees (3,000,000 generations), which were generated before the chain reached equilibrium in the distribution of trees.

For these phylogenetic analyses, we also included some mtDNA and nuDNA sequence data used in intrageneric studies about the *Mauremys mutica-annamensis* complex (Fong et al., 2007; Fong and Chen, 2010) and some species from the family Geoemydidae, which were used as outgroups (GenBank numbers are listed in Appendix 1).

## RESULTS

In an alignment of the mitochondrial ND4 gene (817 bp), we detected 16 haplotypes, 25 variable sites and 17 parsimony-informative sites. All individuals of *M. annamensis* examined in this study possessed the mitochondrial ND4 gene (p-distances ranging from 0.127% to 1.826%) typical for this species (Fong et al., 2007).

Phylogenetic analyses containing our sequences in the context of those available in the GenBank confirmed haplogroups and the general topology of previously published trees (Fong et al., 2007). The BA tree (Fig. 1) suggests a principal split between the “true *mutica* clade” (BA posterior probability = 1.00) and a clade (BA = 1.00) containing both the *M. annamensis* and Hainan *M. mutica*. The latter clade further splits into three distinct clades (all BA probabilities = 1.00). These are an “*annamensis* subclade I”, “*annamensis* subclade II” and the “Hainan *mutica* clade”. Average uncorrected p-distance between the “*annamensis* subclade I” and “*annamensis* subclade II” was 1.968%. The sister relationship between the “*annamensis* subclade I” and the “Hainan *mutica* clade” is moderately supported (BA = 0.82).

ND4 sequences of our *M. annamensis* samples belong to the haplogroups previously described as the “*annamensis* subclade I” and “*annamensis* subclade II” (13 and 26 cases, respectively). Out of four examined samples of the putative *M. mutica*, ND4 sequences branch within the “true *mutica* clade” and one within the “*annamensis* subclade I”. P-distances among these four clades computed from all available sequences (including GenBank sources) suggest low mutual divergence among both the “*annamensis*” and “Hainan *mutica*” clades (Table 1).

In an alignment of nuclear R35 intron (918 bp), we detected 25 haplotypes, 20 variable sites and 7 parsimony-informative sites. All 39 specimens putatively belonging to the *M. annamensis* shared mutually similar sequences of R35 intron (p-distances from 0.132% to 0.932%). The R35 sequences in three of four *M. mutica* samples clearly differed from those of the *M. annamensis*.

Phylogenetic analysis of these sequences and those available in the GenBank (alignment of 940 bp, see

Fig. 2) confirmed the presence of the three previously described clades (Fong et al., 2007) within the *annamensis-mutica* complex: the “Hainan *mutica* clade” (BA posterior probability = 0.98) is the sister group of the true *mutica-annamensis* clade (BA = 1.00), which contains a group of *mutica* sequences corresponding to the “true *mutica* clade” (BA = 0.53) and a well-supported “*annamensis* clade” (BA = 1.00). In BA tree, the “true *mutica*” is paraphyletic with respect to the “*annamensis* clade”, however, most of the sequences of this group form a single branch with low support (BA = 0.53).

The BA analysis placed all 39 examined sequences of the *M. annamensis* into the “*annamensis* clade”. Out of four of the *M. mutica* sequences, one belongs to the “Hainan *mutica* clade”, one into the “*annamensis* clade” and the remaining two into the “true *mutica*” (Table 2).

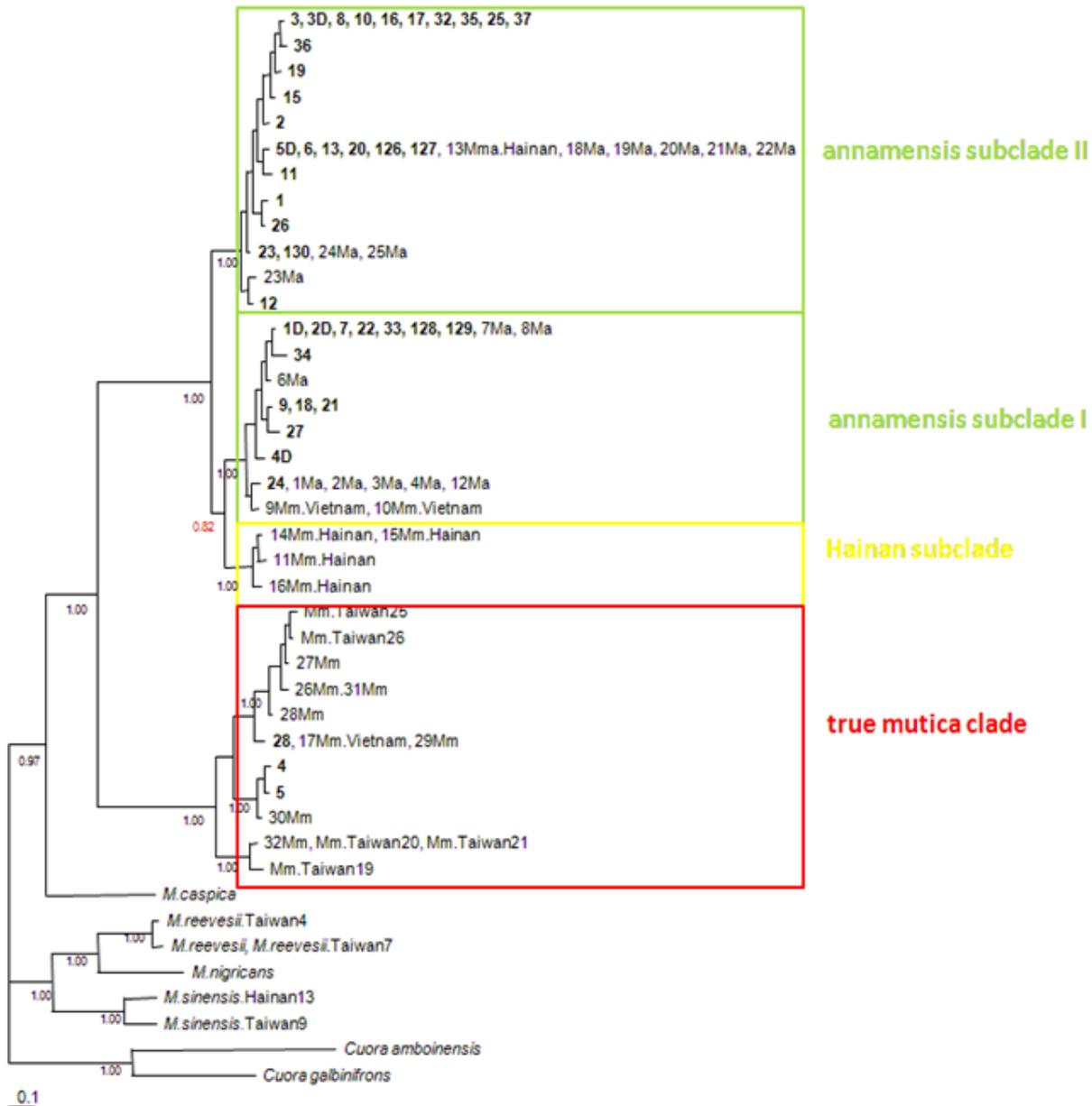
## DISCUSSION

We have no evidence suggesting the presence of the interspecific hybrids among the examined founders of the *M. annamensis* kept in European collections. Of course, without an application of expensive genome-wide markers (like SNPs, extensive number of microsatellites), it is impossible to entirely rule out partial introgression of the genomes of other related geoemydids into some founders of the European population of the *M. annamensis* (i.e., presence of hybrids of a higher order - F<sub>2</sub> and higher generations and backcrosses). Also, without cloning, we are unable to evaluate the affinity of potential heterozygotes of the R35 intron to individual mitochondrial subclades. Nevertheless, when considering other supportive evidence (age, origin), the presence of hybrids seems to be fairly unlikely.

The original geographic distribution of the *Mauremys annamensis* is unknown, only few records document it. That is why it is hard to understand the significance of the two distinct mitochondrial clades, which we, as well as previous authors, detected in the *M. annamensis*. It is unclear whether these clades occur or occurred in the wild in syntopy or allopatrically. The sequence divergence

**Table 1.** Average values of estimates of evolutionary divergence between sequences (the p-values are expressed in per cents).

	<i>annamensis</i> subclade II	<i>annamensis</i> subclade I	Hainan <i>mutica</i> clade	true <i>mutica</i> clade
<i>annamensis</i> subclade II	0.083-0.167			
<i>annamensis</i> subclade I	0.844-1.193	0.167-0.420		
Hainan <i>mutica</i> clade	1.020-1.281	0.589-0.934	0.083-0.167	
true <i>mutica</i> clade	3.952-4.895	3.549-4.782	3.952-4.782	0.083-1.554



**Fig. 1.** Bayesian tree of mitochondrial DNA (ND4) of the genus *Mauremys*. Numbers at branches are support values, only values > 0.95 are shown. Samples sequenced in this study, which correspond to Table 1, in bold, remaining samples were sequenced by previous authors (Fong et al., 2007; Fong and Chen, 2010), Mm = *Mauremys mutica*, Ma = *Mauremys annamensis*. Countries of the origins of samples are shown at individuals with reliable locality.

between the two clades is only about 0.84-1.19 %. Thus, we cannot reject the possibility that retention of ancestral polymorphism is a cause of a simultaneous occurrence of these related, but still distinct clades, in the sampled population of the *M. annamensis*. Ancestral polymorphism may be irrelevant to an original population structure of the species prior to its recent decline leading to near extinction in the wild.

The distinction between the mitochondrial haplotype groups I and II has been recognized only recently and thus, the species has been treated as a single conservation unit in most zoos and collections. However, it is possible to keep the animals of the two groups apart. This would be recommended especially in the case of animals producing offspring suitable for repatriation projects. Nevertheless, such a precaution cannot sub-

**Table 2.** List of samples used in this study containing information about species, breeder, nuclear and mitochondrial haplotype subclades.

Nr.		Species	Breeder	ND4	R35
<b><i>Mauremys annamensis</i> mtDNA subclade II</b>					
1		<i>Mauremys annamensis</i>	H. Becker	<i>annamensis</i> subclade II	<i>annamensis</i> clade
2		<i>Mauremys annamensis</i>	H. Becker	<i>annamensis</i> subclade II	<i>annamensis</i> clade
3		<i>Mauremys annamensis</i>	H. Becker	<i>annamensis</i> subclade II	<i>annamensis</i> clade
3D		<i>Mauremys annamensis</i>	D. Frynta	<i>annamensis</i> subclade II	<i>annamensis</i> clade
5D		<i>Mauremys annamensis</i>	D. Frynta	<i>annamensis</i> subclade II	<i>annamensis</i> clade
6	704774	<i>Mauremys annamensis</i>	Rotterdam	<i>annamensis</i> subclade II	<i>annamensis</i> clade
8	704525	<i>Mauremys annamensis</i>	Rotterdam	<i>annamensis</i> subclade II	<i>annamensis</i> clade
10	704524	<i>Mauremys annamensis</i>	Rotterdam	<i>annamensis</i> subclade II	<i>annamensis</i> clade
11	705067	<i>Mauremys annamensis</i>	Rotterdam	<i>annamensis</i> subclade II	<i>annamensis</i> clade
12	3	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade II	<i>annamensis</i> clade
13	4	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade II	<i>annamensis</i> clade
15	9	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade II	<i>annamensis</i> clade
16	1	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade II	<i>annamensis</i> clade
17	2	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade II	<i>annamensis</i> clade
19	6	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade II	<i>annamensis</i> clade
20		<i>Mauremys annamensis</i>	M. Schilde	<i>annamensis</i> subclade II	<i>annamensis</i> clade
23	M53	<i>Mauremys annamensis</i>	Praha	<i>annamensis</i> subclade II	<i>annamensis</i> clade
25	F21	<i>Mauremys annamensis</i>	Praha	<i>annamensis</i> subclade II	<i>annamensis</i> clade
26	F9	<i>Mauremys annamensis</i>	Praha	<i>annamensis</i> subclade II	<i>annamensis</i> clade
32	ROO718	<i>Mauremys annamensis</i>	Leipzig	<i>annamensis</i> subclade II	<i>annamensis</i> clade
35	32	<i>Mauremys annamensis</i>	Panuška	<i>annamensis</i> subclade II	<i>annamensis</i> clade
36	33	<i>Mauremys annamensis</i>	Panuška	<i>annamensis</i> subclade II	<i>annamensis</i> clade
37	34	<i>Mauremys annamensis</i>	Panuška	<i>annamensis</i> subclade II	<i>annamensis</i> clade
126	COS679	<i>Mauremys annamensis</i>	Chester	<i>annamensis</i> subclade II	<i>annamensis</i> clade
127	COS678	<i>Mauremys annamensis</i>	Chester	<i>annamensis</i> subclade II	<i>annamensis</i> clade
130	COS349	<i>Mauremys annamensis</i>	Chester	<i>annamensis</i> subclade II	<i>annamensis</i> clade
<b><i>Mauremys annamensis</i> mtDNA subclade I</b>					
1D		<i>Mauremys annamensis</i>	D. Frynta	<i>annamensis</i> subclade I	<i>annamensis</i> clade
2D		<i>Mauremys annamensis</i>	D. Frynta	<i>annamensis</i> subclade I	<i>annamensis</i> clade
4D		<i>Mauremys annamensis</i>	D. Frynta	<i>annamensis</i> subclade I	<i>annamensis</i> clade
7	704212	<i>Mauremys annamensis</i>	Rotterdam	<i>annamensis</i> subclade I	<i>annamensis</i> clade
9	704523	<i>Mauremys annamensis</i>	Rotterdam	<i>annamensis</i> subclade I	<i>annamensis</i> clade
18	8	<i>Mauremys annamensis</i>	Münster	<i>annamensis</i> subclade I	<i>annamensis</i> clade
21		<i>Mauremys annamensis</i>	M. Schilde	<i>annamensis</i> subclade I	<i>annamensis</i> clade
22		<i>Mauremys annamensis</i>	M. Schilde	<i>annamensis</i> subclade I	<i>annamensis</i> clade
24	M7	<i>Mauremys annamensis</i>	Praha	<i>annamensis</i> subclade I	<i>annamensis</i> clade
33	ROO720	<i>Mauremys annamensis</i>	Leipzig	<i>annamensis</i> subclade I	<i>annamensis</i> clade
34	ROO719	<i>Mauremys annamensis</i>	Leipzig	<i>annamensis</i> subclade I	<i>annamensis</i> clade
128	CZ/921	<i>Mauremys annamensis</i>	Chester	<i>annamensis</i> subclade I	<i>annamensis</i> clade
129	CZ/922	<i>Mauremys annamensis</i>	Chester	<i>annamensis</i> subclade I	<i>annamensis</i> clade
<b><i>Mauremys mutica</i></b>					
4		<i>Mauremys mutica</i>	H. Becker	true <i>mutica</i> clade	true <i>mutica</i> clade
5		<i>Mauremys mutica</i>	H. Becker	true <i>mutica</i> clade	true <i>mutica</i> clade
<b>Animals of hybrid origin</b>					
28	3	<i>Mauremys mutica</i>	Praha	true <i>mutica</i> clade	<i>annamensis</i> clade
27	2	<i>Mauremys mutica</i>	Praha	<i>annamensis</i> subclade I	Hainan <i>mutica</i> clade

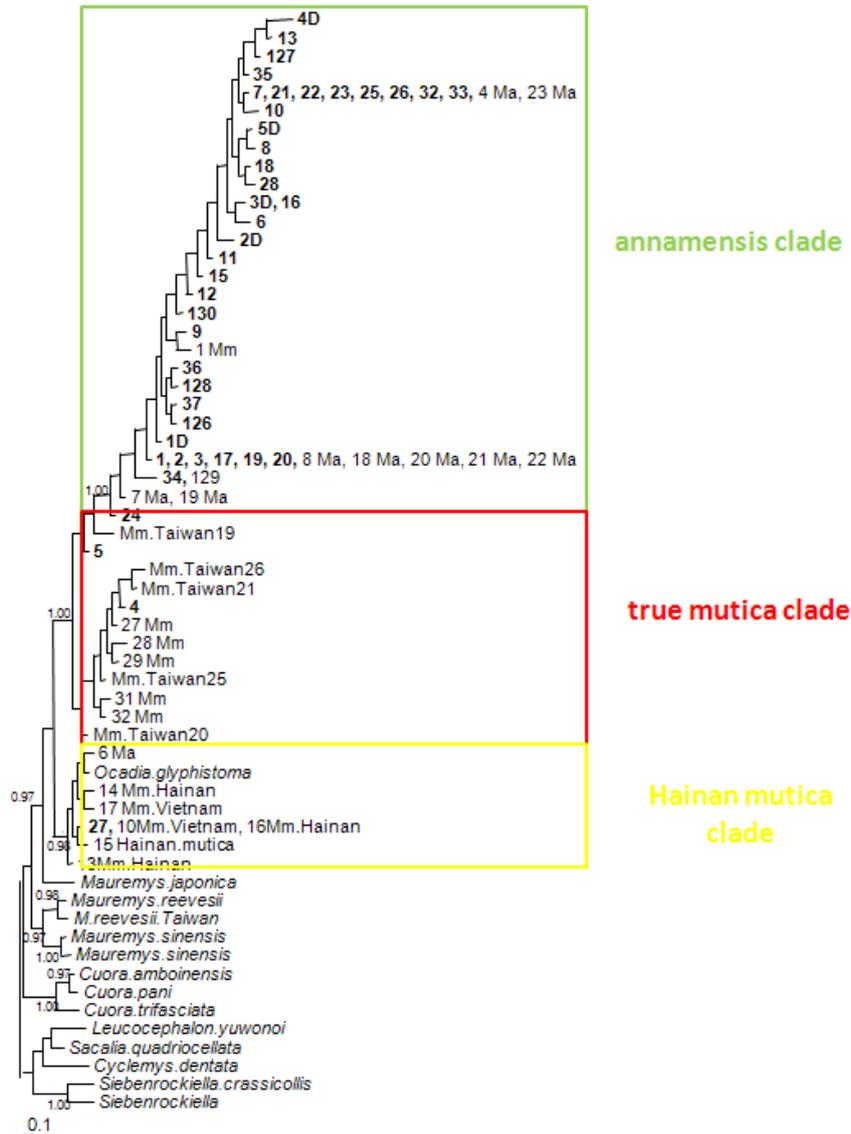


Fig. 2. Bayesian tree of nuclear DNA (R35) of the genus *Mauremys*. For further explanation, see Fig. 1

stantiate the elimination of the descendants of parents belonging to different clades from the studbook population. There is an urge call for further research of the genetic variation in the *M. annamensis* using multiple nuclear markers and/or advanced genomic methods, especially to enable a better understanding of the divergence of the two distinct subclades.

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### Appendix 1

List of sequences containing their GenBank numbers used in this study.

Name of sequence	Species	ND4	R35
1Ma	<i>M. annamensis</i>	EF034098	EF587934
10Ma	<i>M. annamensis</i>	AF348280	DQ386668
11Mm.Hainan	<i>M. annamensis</i>	EF034096	EF87929
12Ma	<i>M. annamensis</i>	EF034105	—
13Ma.Hainan	<i>M. annamensis</i>	EF034104	EF587915
14Mm.Hainan	<i>M. mutica</i>	EF034097	EF587925
15.Mm.Hainan	<i>M. mutica</i>	EF034101	EF587917
16.Mm.Hainan	<i>M. mutica</i>	EF034095	EF587930
17Mm.Vietnam	<i>M. mutica</i>	AF348278	DQ386664
18Ma	<i>M. annamensis</i>	EF034104	EF587923
19Ma	<i>M. annamensis</i>	EF034106	EF587928
20Ma	<i>M. annamensis</i>	EF034107	EF587924
21Ma	<i>M. annamensis</i>	EF034112	DQ386656
22Ma	<i>M. annamensis</i>	EF587914	EF587921
23Ma	<i>M. annamensis</i>	EF034099	EF587919
24Ma	<i>M. annamensis</i>	EF034100	—
25Ma	<i>M. annamensis</i>	EF034108	—
26Mm	<i>M. mutica</i>	EF034092	—
27Mm	<i>M. mutica</i>	EF034093	EF587931
28Mm	<i>M. mutica</i>	EF034089	EF587932
29Mm	<i>M. mutica</i>	AF348278	DQ386666
2Ma	<i>M. annamensis</i>	AY337338	EF587933
30Mm	<i>M. mutica</i>	EF034090	—
31Mm	<i>M. mutica</i>	EF034092	EF587916
32Mm	<i>M. mutica</i>	EF034094	EF587927
3Ma	<i>M. annamensis</i>	EF034103	—
4Ma	<i>M. annamensis</i>	EF034105	EF587922

Name of sequence	Species	ND4	R35
6Ma	<i>M. annamensis</i>	EF034102	EF587929
7Ma	<i>M. annamensis</i>	EF034109	EF587926
8Ma	<i>M. annamensis</i>	EF034113	DQ386655
9Mm.Vietnam	<i>M. mutica</i>	AF348279	----
Cuora amboinensis	<i>Cuora amboinensis</i>	EF011357	HQ442382
Cuora galbinifrons	<i>Cuora galbinifrons</i>	AY364617	----
Cuora pani	<i>Cuora pani</i>	—	EF011442
Cuora trifasciata	<i>Cuora trifasciata</i>	—	JQ596437
Cyclemys dentata	<i>Cyclemys dentata</i>	—	AM931697
Leucocephalon yuwonoi	<i>Leucocephalon yuwonoi</i>	—	AM931708
<i>M. nigricans</i>	<i>M. nigricans</i>	EF034111	----
<i>M. reevesii</i>	<i>M. reevesii</i>	EF034110	----
<i>M. reevesii</i> .Taiwan4	<i>M. reevesii</i>	GQ259438	GQ259459
<i>M. reevesii</i> .Taiwan7	<i>M. reevesii</i>	GQ259441	GQ259464
<i>M. sinensis</i> .Hainan13	<i>M. sinensis</i>	AY337345	DQ386678
<i>M. sinensis</i> .Taiwan9	<i>M. sinensis</i>	GQ259443	GQ259465
<i>M. caspica</i>	<i>M. caspica</i>	AY337340	----
<i>Mauremys japonica</i>	<i>Mauremys japonica</i>	—	HQ442386
Mm.Taiwan19	<i>M. mutica</i>	GQ259452	GQ259471
Mm.Taiwan20	<i>M. mutica</i>	GQ259453	GQ259472
Mm.Taiwan21	<i>M. mutica</i>	GQ259454	GQ259473
Mm.Taiwan25	<i>M. mutica</i>	GQ259457	GQ259474
Mm.Taiwan26	<i>M. mutica</i>	GQ259458	GQ259475
Ocadia glyphistoma	<i>Ocadia glyphistoma</i>	—	DQ386663
Sacalia quadriocellata	<i>Sacalia quadriocellata</i>	—	HQ442384
Siebenrockiella	<i>Siebenrockiella leytensis</i>	—	AM931708
Siebenrockiella crassicollis	<i>Siebenrockiella crassicollis</i>	—	AY954913