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The Atlas Mountains, not the Strait of Gibraltar, as a biogeographic barrier for *Mauremys leprosa* (Reptilia: Testudines)

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Abstract. Sequence data of the mitochondrial cytochrome *b* gene of stripe-necked terrapins (*Mauremys leprosa*) have been compared from localities north and south of the Atlas Mts. (Ceuta; Morocco) and from Doñana National Park (Spain). A low maximum sequence divergence (approximately 1%) corresponds to two major clades; one is represented by localities to the north of the Atlas Mts. and in Doñana National Park and the other by localities to the south of the Atlas Mts. Differentiation between populations north and south of the Atlas Mts. is much more pronounced than that found between samples from each side of the Strait of Gibraltar. These findings suggest that the Strait of Gibraltar is, in contrast to the Atlas Mts., not a significant barrier to gene flow in stripe-necked terrapins. The major clades could reflect taxonomic segregation between populations north and south of the Atlas Mts. Sequences from Marrakech (corresponding to *M. l. marokkensis*), Ceuta, and the Doñana National Park (*M. l. leprosa*) are only weakly differentiated. South of the Atlas Mts. we found no consistent differences between samples from catchment basins of the Oued Drâa (*M. l. vanmeerhaghei*) and the Oued Noun (*M. l. saharica*). Our findings imply that taxonomic differentiation within *M. leprosa* is currently overestimated.

Key words. Reptilia: Testudines: *Mauremys leprosa*; Spain; Morocco; biogeography; genetic variation.

Introduction

The Strait of Gibraltar has been highlighted as an important biogeographic barrier that separates North African and Iberian herpetofaunas (BUSACK 1986). Recent mtDNA sequence data corroborate the barrier status of the Strait of Gibraltar for some amphibians and reptiles (*Alytes*: FROMHAGE et al. 2004; *Discoglossus*: GARCÍA-PARÍS & JOCKUSCH 1999, FROMHAGE et al. 2004; *Pelobates*: GARCÍA-PARÍS et al. 2003; *Acanthodactylus*: HARRIS et al. 2004), but not for others (*Pleurodeles waltl*: BATISTA et al. 2004, CARRANZA & ARNOLD 2004, VEITH et al. 2004; *Podarcis*: HARRIS et al. 2002). The stripe-necked terrapin (*Mauremys leprosa*), widely distributed on both sides of the Strait of Gibraltar, was long thought to be monotypic (BUSACK & ERNST 1980). SCHLEICH (1996) and BOUR & MARAN (1999), however, described seven subspecies from Morocco, suggesting considerable variation. Recent molecular investigations proposed *M. leprosa* as sister taxon to other West Palearctic *Mauremys* species (*M. caspica*, *M. rivulata*) and to several East Asiatic taxa (BARTH et al. 2004, SPINKS et al. 2004). The split between *M. leprosa* and the other taxa possibly dates back to the Late Oligocene or Early Miocene (BARTH et al. 2004), approximately matching the age of the oldest known West Palearctic *Mauremys* fossils (LAPPARENT DE BROIN & VAN DIJK 1999, LAPPARENT DE BROIN 2001, HERVET 2004). Recent subspecies descriptions, the long independent evolutionary history of *M. leprosa*, and the presence of old *Mauremys*-like fossils in West Europe and North Africa (LAPPARENT DE BROIN & VAN DIJK 1999, LAPPARENT DE BROIN 2000, 2001, HERVET 2004) suggest that populations on each side of the Strait of Gibraltar could be old and well-differentiated. We tested this hypothesis by comparing sequences of the mitochondrial cytochrome *b* gene

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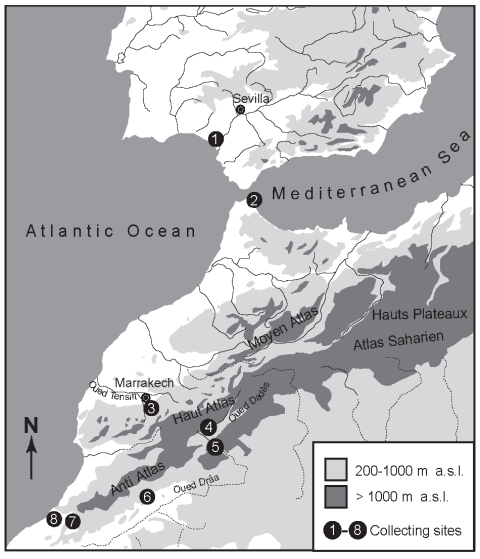


Fig. 1. Geographic origin of samples: (1) Doñana National Park; (2) Ceuta; (3) SE Marrakech; (4) Oued Dadès, NE Ouarzazate; (5) Oued Drâa, SE Ouarzazate; (6) Oued Tata, S Tata; (7) Oued Noun, E Guelmine; (8) Oued Noun, N Tiliouine. For exact localities see Table 1.

(cyt *b*) of *M. leprosa* from southwest Spain (Doñana National Park), the Spanish exclave Ceuta (North Africa), and Morocco (localities north and south of the Atlas Mts). Cyt *b* is known to be phylogenetically informative in testudinoid chelonians (CACCONE et al. 1999, LENK et al. 1999, FELDMAN & PARHAM 2002, PALKOVACS et al. 2002, STEPHENS & WIENS 2003, BARTH et al. 2004, SPINKS et al. 2004).

Materials and Methods

Sampling

We studied 18 *Mauremys leprosa* from Spain and Morocco (Fig. 1, Tab. 1). One specimen was included in a previous study (BARTH et al. 2004) and most were captured by one of us (J.-M. D.). Individuals were released after taking blood samples, measurements, and photographs. Morphological characters and habitats are described in DUCOTTERD & BOUR (2002). Blood samples were preserved in 98% pure ethanol or EDTA buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) and stored at -80 °C. Voucher photographs, blood and remaining DNA samples are permanently housed in the tissue collection of the Museum of Zoology Dresden (Museum für Tierkunde, MTD T).

Sequencing

Total genomic DNA was extracted from small quantities of blood following GUSTINCICH et al. (1991). For amplification of cyt *b* we used the primers mt-a-neu (5'-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G-3') of LENK & WINK (1997) and H-15909 (5'-AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC CAA TG-3') of LENK et al. (1999). PCR was conducted in an Eppendorf Mastercycler. Cycling procedure was: initial denaturation 5 min at 95 °C; 40 cycles, denaturation 1 min at 95 °C, primer annealing for 1 min at 50 °C, extension for 2 min at 72 °C, and stop reaction for 10 min at 72 °C. PCR products were purified using the Invisorb Spin PCRapid Kit from Invitek. Sequencing primers were Mau-F (5'-CTA GGC CTC ATC TTA ATA CT-3') and Ri-neu (5'-GTG AAG TTG TCT GGG TCT CCT AG-3'). Sequencing was performed on an ABI 3100 Genetic Analyzer (Hitachi) with the following conditions for 25 cycles: 10 s at 96 °C; 5 s at 50 °C; and 4 min at 60 °C. Obtained sequences were approximately 1,100 bp long.

Phylogenetic analysis

MEGA 2.1 (KUMAR et al. 2001) was used to estimate genetic distances and calculate sequence statistics. Gene fragments were aligned with ClustalX v. 1.83 (THOMPSON et al. 1997) and default parameters. Aligned sequences (including gaps) comprised 993 bp. *Mauremys rivulata*, retrieved from GenBank (AJ564455; BARTH et al. 2004), served as the

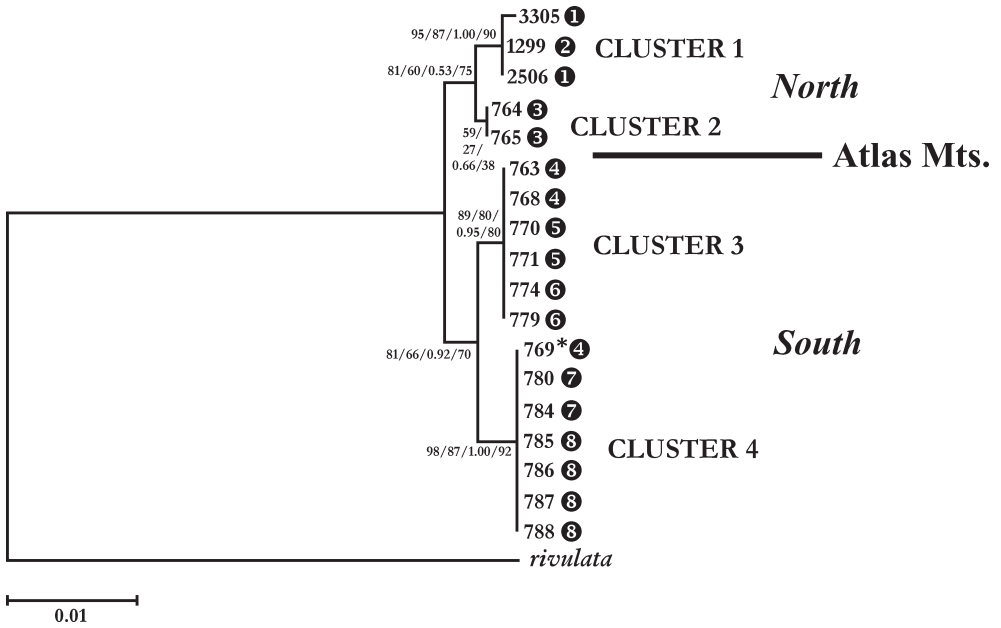


Fig. 2. Neighbour-Joining tree of the cyt *b* dataset for *Mauremys leprosa*, rooted with *M. rivulata*. Clusters 1 and 2 represent localities north of the Atlas Mts. and from both sides of the Strait of Gibraltar, clusters 3 and 4 localities south of the Atlas Mts. Large numbers at the leaves are MTD T numbers (Tab. 1); numbers in black circles denote collection sites (Fig. 1). The sample from the Oued Drâa catchment area with the haplotype occurring otherwise in the Oued Noun region is asterisked. Small numbers at the nodes are, from left to right: bootstrap values of 10,000 trees for NJ, bootstrap values for ML (TBR swapping algorithm), posterior probabilities for MrBayes, and bootstrap values for MP (1,000 resamplings). Branch lengths correspond to number of nucleotide changes.

outgroup. Different procedures were used for estimating phylogeny. To find the most appropriate model for DNA nucleotide substitution, we performed a hierarchical likelihood ratio test with Modeltest v. 3.5 (Posada & Crandall 1998). For the dataset, HKY (Hasegawa et al. 1985), a TRatio = 7.2040, and equal rates provided the best fitting of 56 tested models. These parameters were used to perform Maximum Likelihood (ML) and Bayesian analysis. The ML analysis was calculated with PAUP* 4.0b10 (Swofford 2002) using the heuristic search method with 10 random stepwise additions and TBR branch swapping for 1,000 bootstrap replicates. Bayesian analyses were performed with MrBayes V3.0b4 (Huelsenbeck & Ronquist 2001), which was used to run 1,000,000 generations, with a sampling frequency of 10 generations. From the 100,000 trees found, the first 1,000 were discarded. Neighbour-Joining (NJ) trees (Saitou & Nei 1987) were constructed with MEGA 2.1 and PAUP*. We chose the model of Kimura-2-parameter (Kimura 1980), a pairwise deletion and 10,000 bootstrap replicates. Maximum Parsimony (MP) analyses were performed with PAUP* using the heuristic search method with 10 random stepwise additions and the TBR branch swapping option. Bootstrap analyses (Felsenstein 1985) were used to examine robustness of resulting bifurcations within the trees. The MP tree was tested with 1,000 replicates.

In addition, we used Bio-Neighbour-Joining (Gascuel 1997) and Neighbour-Net

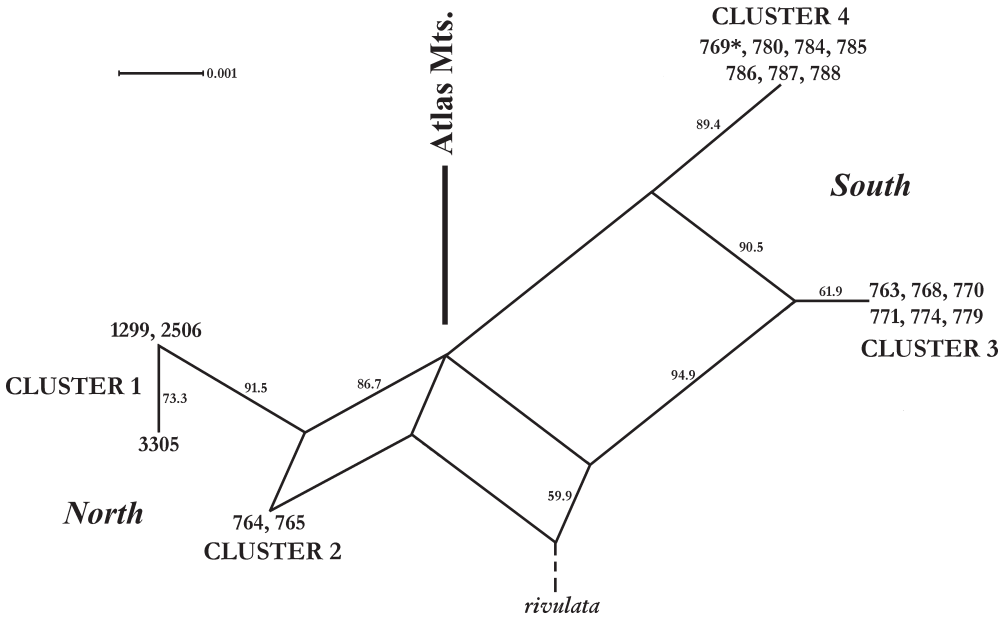


Fig. 3. Neighbour-Net of the cyt *b* dataset for *Mauremys leprosa*, rooted with *M. rivulata*. Small numbers along branches denote bootstrap values (1,000 replications), indicating probability for respective pathway. Large numbers represent MTD T numbers (Tab. 1). Branch length is proportional to number of divergent characters supporting this pathway. For further explanation, see Figure 2.

	Locality	n	Referred subspecies	MTD T	EMBL accession number
1	Spain: Doñana National Park, Laguna Dulce	2	*leprosa*	2506, 3305	AJ564457, AJ877040
2	Spain: Ceuta	1	?	1299	AJ877039
3	Morocco: SE Marrakech: near Aït-Ourir, 31°27.891N, 7°46.724W; 672 m	2	*marokkensis*	764-765	AJ877025-AJ877026
4	Morocco: NE Ouarzazate: Sidi-Flah, Oued Dadès, 31°00.688N, 6°29.931W; 1158 m (MTD T 763, 768) and 31°00.695N, 6°30.808W; 1177 m (MTD T 769)	3	*vanmeerhaghei*	763, 768-769	AJ877024, AJ877027-AJ877028
5	Morocco: SE Ouarzazate: Tamnougalt, Oued Drâa, 30°40.183, 6°22.881W, 917 m	2	*vanmeerhaghei*	770-771	AJ877029-AJ877030
6	Morocco: 18 km S Tata: El-Khemis, Oued Tata, 29°35.506, 8°00.009W, 494 m	2	*vanmeerhaghei*	774, 779	AJ877031-AJ877032
7	Morocco: 17 km E Guelmine: Oued Noun, 28°58.433N, 9°54.231W, 300 m	2	*saharica*	780, 784	AJ877033-AJ877034
8	Morocco: N Tiliouine: Oued Noun canyon, 29°05.115N, 10°15.140W, 122 m	4	*saharica*	785-788	AJ877035-AJ877038

Tab. 1. *Mauremys leprosa* samples, localities and EMBL accession numbers. MTD T: tissue collection, Museum of Zoology Dresden (Museum für Tierkunde).

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*Mauremys rivulata*
Cluster 1	–	3	10	9	75
Cluster 2	0.3	–	9	8	72
Cluster 3	1.0	0.9	–	5	73
Cluster 4	0.9	0.8	0.5	–	76
Mauremys rivulata	7.6	7.3	7.4	7.7	–

Tab. 2. Divergence (p-distance) between clusters of cyt *b* sequences (993 bp) of *Mauremys leprosa* and the outgroup *M. rivulata*. Number of changes above diagonal, sequence divergence (%) below diagonal. Average values are given for cluster 1. Cluster 1: Ceuta and Doñana National Park; cluster 2: Marrakech; cluster 3: catchment area of Oued Drâa; cluster 4: catchment areas of Oued Noun and Oued Drâa.

(BRYANT & MOULTON 2004) as implemented in the program SplitsTree v. 4beta10 (HUSON 1998, HUSON & BRYANT 2004). Neighbour-Net is a powerful tool for testing the consistency of phylogenetic hypotheses (BRYANT & MOULTON 2004).

Results

Nine hundred ninety-three base pairs of the *Mauremys leprosa* cytochrome *b* gene were unambiguously aligned for phylogenetic purposes. Within this alignment, 82 positions were variable and 13 were parsimony informative. These differences correspond to five weakly-differentiated haplotypes. The greatest sequence divergence of approximately 1% (p-distance) exists between localities north of the Atlas Mts. plus southwest Spain versus localities south of the Atlas Mts. (Tab. 2).

All phylogenetic methods resulted in the same topology. The Neighbour-Joining (NJ) tree (Fig. 2) and the Neighbour-Net (Fig. 3) serve as examples. In the NJ tree bootstrap and probability values for Maximum Likelihood, Bayesian and Maximum Parsimony analysis are included. In all presentations, *M. leprosa* sequences are monophyletically rooted with *M. rivulata*. A major split, supported by moderate to high bootstrap and probability values, occurs between samples from localities north of the Atlas Mts. plus southwest Spain versus localities south of the Atlas Mts.

Within the group containing the samples from the area north of the Atlas Mts. plus southwest Spain, two clades are found. These groupings correspond to Ceuta plus Doñana National Park (MTD T 1299, 2506, 3305) and two samples from the area around Marrakech (MTD T 764-765), respectively. In our sample from Ceuta we found a haplotype identical to that in one individual from Doñana National Park. Turtles from the area south of the Atlas Mts. make up the other major branch, consisting also of two clades. These clades correspond to individuals from the catchment areas of the Oued Noun or the Oued Drâa, with one exception: one sample (MTD T 769) from the Oued Dadès, a tributary of the Oued Drâa, shares its haplotype with specimens from the Oued Noun. Sequence divergence between the Ceuta plus Doñana clade and the Marrakech clade is 0.3%, while between the two clades from south of the Atlas Mts. the p-distance is 0.5% (Tab. 2).

Because we encountered few phylogenetically informative sites in the cyt *b* fragment, the branching pattern of the tree-building methods was tested by Neighbour-Net (BRYANT & MOULTON 2004), a distance-based method for constructing phylogenetic networks. Unlike a NJ tree, a Neighbour-Net (NNet) can represent conflicting phylogenetic signal in the data and demonstrate simultaneously alternative topologies. NNet allows for reticulations and multifurcations, reflecting uncertain domains and alternative pathways. If alternative pathways exist or



Fig. 4. *Mauremys leprosa saharica* with pale blue eyes (Oued Noun canyon, N Tiliouine, Morocco, 29°05.115N, 10°15.140W).

data are ambiguous, branches are represented in the graphical network by boxes. In our data ambiguities regarding the position of the *M. leprosa* clusters relative to the outgroup *M. rivulata* exist. The branching pattern of *M. leprosa* obtained with tree-building methods is confirmed with NNet however (Fig. 3).

Discussion

It is generally accepted that North Africa and the Iberian peninsula were last in contact during the Messinian salinity crisis (Late Miocene, approx. 5.5 Mya) when the Mediterranean basin dried up due to evaporation and the closure of previous Miocene sea straits connecting it with the Atlantic. With restoration of the connection with the Atlantic, the Mediterranean basin again filled during a catastrophic event, lasting approximately 100 years (Hsü et al. 1977, Krijgsman et al. 1999, Duggen et al. 2003). Since then, North Africa and the Iberian peninsula have been separated by shifting sea straits in the area of the current Strait of Gibraltar (de Jong 1998). If North African and Iberian *Mauremys leprosa* populations were separated by this vicariant event, genetic differentiation should be evident.

The oldest known northwest African fossils referred to *M. leprosa* (dated to the Russcinian, 5.4-3.4 Mya, the Pliocene stage immediately following the Messinian) are from Aïn Boucherit, Constantine province, Algeria. Undetermined *Mauremys* findings of the same age were also excavated in Tunisia (Lapparent de Broin 2000). The contemporary European fossil species *M. gaudryi* (Depéret, 1885), thought to be closely related to *M. leprosa* (Hervet 2004), is known from Perpignan (Department Pyrénées-Orientales, France), suggesting that ancestors of extant *M. leprosa* were present in North Africa and Europe when the sea strait between the continents re-opened during the Messinian. However, Lapparent de Broin (2001) believes that *M. leprosa* originated in North Africa and arrived in Europe not before late Pliocene or early Pleistocene.

We found identical or very similar mtDNA sequences on both sides of the Strait of Gibraltar, ranging only to 0.3% sequence divergence (Tab. 2). Similarly, Mantziou et al. (2004) reported little genetic differentiation between populations of *M. rivulata* occurring on the Greek mainland and Aegean islands, and Lenk et al. (1999) reported a Moroccan *Emys orbicularis* cyt *b* sequence resembling haplotypes occurring on the Iberian peninsula. In each case, rafting or island hopping might have prevented development of distinct mtDNA lineages. On the other hand, we detected pronounced differentiation in *M. leprosa* occurring south of the Atlas Mts. In *E. orbicularis* the Pyrenees, Alps, Dinarides, Carpathians, and the Caucasus Mts. are known to be significant biogeographic barriers, separating subspecies that harbour distinct mtDNA lineages (Fritz 1992, 1996, 2003, Lenk et al. 1999). This suggests that high mountain chains are distinctly more important barriers than sea straits for freshwater turtles.

Seven subspecies of *M. leprosa* that have recently been described from Morocco occur either north or south of the Atlas Mts. Schleich (1996) described the subspecies *M. l. atlantica*, *M. l. erhardi*, and *M. l. werner-kaestlei* from northern Morocco and *M. l. marokkensis* from the region of Marrakech.

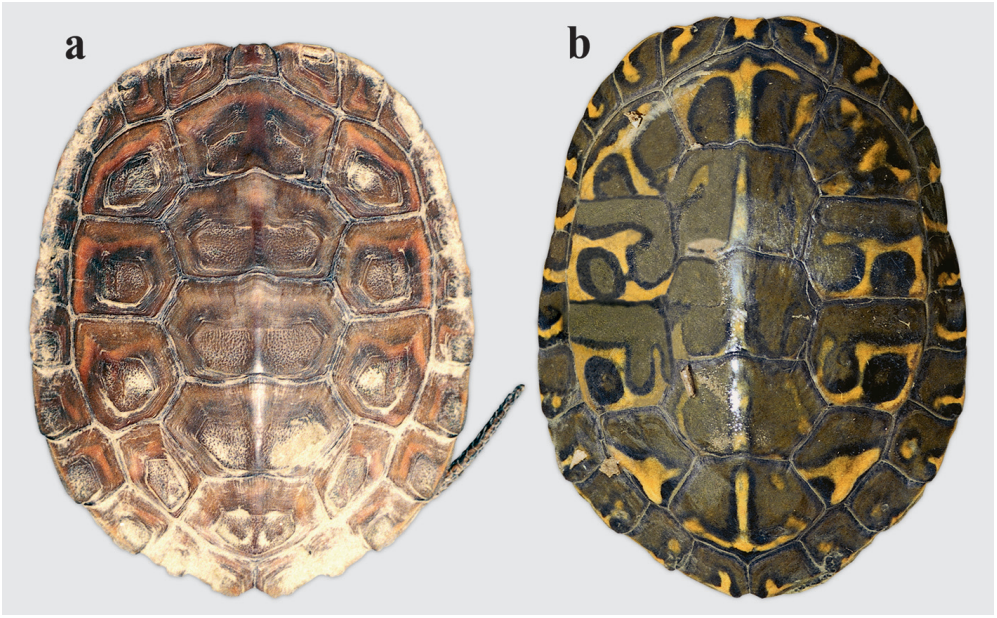


Fig. 5. Juveniles of (a) *Mauremys leprosa vanmeerhaghei* (Sidi-Flah, Oued Dadès NE Ouarzazate, 31°00.688N, 6°29.931W) and (b) *M. l. saharica* (Oued Noun canyon, N Tiliouine, Morocco, 29°05.115N, 10°15.140W). Note carapacial patterns.

From the southern slope and the area south of the Anti Atlas, SCHLEICH (1996) described *M. l. saharica* from Oued Noun and *M. l. zizi* from Oued Ziz, while BOUR & MARAN (1999) described *M. l. vanmeerhaghei* from Oued Drâa. With the exception of *M. l. vanmeerhaghei* that has a bluish iris, these subspecies differ only in juvenile colour pattern, diagnostic features lost during aging. *Mauremys leprosa vanmeerhaghei* (Oued Drâa) is the only subspecies which should be easily recognized as an adult. However, we encountered that a pale blue iris may also occur in the Oued Noun (Fig. 4), and KELLER & BUSACK (2001) highlighted additional inconsistencies regarding subspecific delineation.

Our mtDNA sequences correspond to three of the newly described taxa (*M. l. marokkensis*, *M. l. saharica*, *M. l. vanmeerhaghei*) and the nominotypical subspecies *M. l. leprosa* (SCHWEIGGER, 1812) (type locality: South Spain). The subspecific allocation of our sample from Ceuta is unclear. Neither SCHLEICH (1996) nor BOUR & MARAN (1999) attributed stripe-necked terrapins from that area to a specific subspecies. While it is beyond the scope of the present paper to discuss validity of any taxon, we wish to point out that we found the greatest sequence divergence in populations south of the Atlas Mts. This suggests some taxonomic differentiation. On the other hand, weak divergence of sequences from Ceuta, northern Morocco, and southern Spain questions the validity of subspecies described from northern Morocco. Likewise, we found south of the Atlas Mts. only weak differentiation between sequences of turtles inhabiting the catchment areas of the Oued Drâa (*M. l. vanmeerhaghei*) and the Oued Noun (*M. l. saharica*). In one sample from the Oued Dadès, a tributary of the Oued Drâa, we detected the same haplotype as in our specimens from the Oued Noun, arguing for gene flow or incomplete lineage sorting and suggesting very recent or incomplete separation of both populations.

Taking the occurrence of these populations in isolated, in part temporary water bodies into account (Ducotterd & Bour 2002), the obvious differences in coloration and pattern of juvenile *M. l. saharica* and *M. l. vanmeerhaghei* (Fig. 5) could be caused by recent genetic bottlenecks.

Our findings imply that while taxonomic differentiation within *M. leprosa* exists, it is currently overestimated with eight recognized subspecies, seven of which occur in Morocco. A complete phylogeography of *M. leprosa* will shed new light on its taxonomy and lead to a better understanding of the species' biogeography. To decide whether vicariance, dispersal or both played a role in shaping current distribution patterns and infraspecific structure, we need additional sampling from North Africa and the Iberian peninsula.

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