Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae)

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Accepted: 11 November 2005
doi:10.1111/j.1463-6409.2005.00218.x

Fritz, U., Barata, M., Busack, S. D., Fritzsch, G. & Castilho, R. (2006). Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). — *Zoologica Scripta*, *35*, 97–108.

Mauremys leprosa, distributed in Iberia and North-west Africa, contains two major clades of mtDNA haplotypes. Clade A occurs in Portugal, Spain and Morocco north of the Atlas Mountains. Clade B occurs south of the Atlas Mountains in Morocco and north of the Atlas Mountains in eastern Algeria and Tunisia. However, we recorded a single individual containing a clade B haplotype in Morocco from north of the Atlas Mountains. This could indicate gene flow between both clades. The phylogenetically most distinct clade A haplotypes are confined to Morocco, suggesting both clades originated in North Africa. Extensive diversity within clade A in south-western Iberia argues for a glacial refuge located there. Other regions of the Iberian Peninsula, displaying distinctly lower haplotype diversities, were recolonized from within south-western Iberia. Most populations in Portugal, Spain and northern Morocco contain the most common clade A haplotype, indicating dispersal from the south-western Iberian refuge, gene flow across the Strait of Gibraltar, and reinvasion of Morocco by terrapins originating in south-western Iberia. This hypothesis is consistent with demographic analyses, suggesting rapid clade A population increase while clade B is represented by stationary, fragmented populations. We recommend the eight, morphologically weakly diagnosable, subspecies of *M. leprosa* be reduced to two, reflecting major mtDNA clades: *Mauremys l. leprosa* (Iberian Peninsula and northern Morocco) and *M. l. saharica* (southern Morocco, eastern Algeria and Tunisia). Peripheral populations could play an important role in evolution of *M. leprosa* because we found endemic haplotypes in populations along the northern and southern range borders. Previous investigations in another western Palearctic freshwater turtle (*Emys orbicularis*) discovered similar differentiation of peripheral populations, and phylogeographies of *Emys orbicularis* and *Mauremys rivulata* underline the barrier status of mountain chains, in contrast to sea straits, suggesting common patterns for western Palearctic freshwater turtles.

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Introduction

The stripe-necked terrapin, *Mauremys leprosa* (Schweigger, 1812), is one of the seven native western Palearctic freshwater turtle species (Ernst *et al*. 2000; Fritz *et al*. 2005a). Besides *M. leprosa*, two other *Mauremys* species occur in this region (*M. caspica*, *M. rivulata*); further *Mauremys* species are distributed in East Asia (Ernst *et al*. 2000; Fritz 2001; Barth *et al*. 2004; Spinks *et al*. 2004). *Mauremys caspica* is confined to

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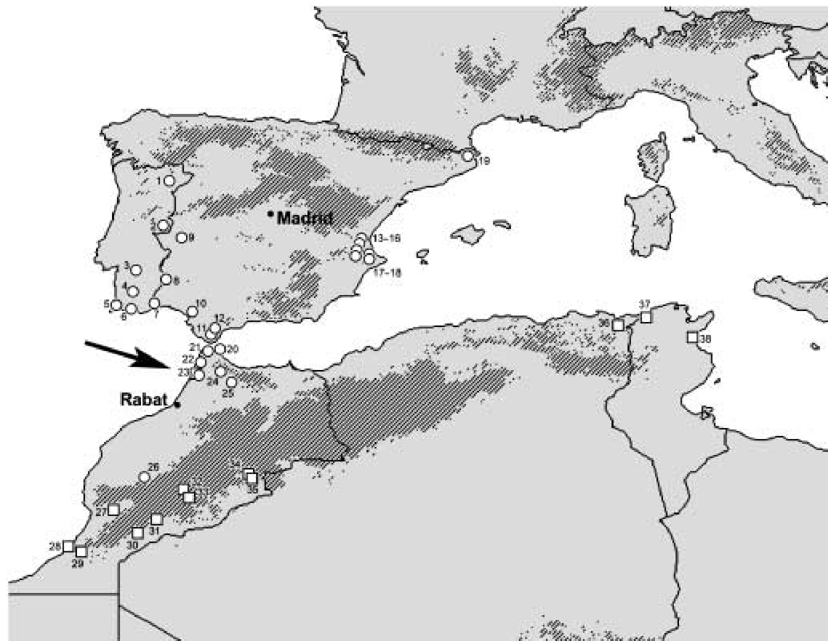

Fig. 1 Geographic origins of *Mauremys leprosa* samples, adjacent localities combined. Numbers refer to Table 1, circles indicate clade A haplotypes, squares, clade B. Arrow indicates sole locality (23) where haplotypes of both clades were found together. Areas exceeding 1000 m a.s.l. are hatched.

central and eastern Turkey, the eastern Caucasus Region, and vast parts of the Near and Middle East with a continental climate (Wischuf & Fritz 2001), while *M. rivulata* occurs along the Mediterranean coasts of the southern Balkans, Turkey, and the Near East southwards to Israel (Wischuf & Busack 2001). *Mauremys leprosa* is widely distributed on both sides of the Strait of Gibraltar, over most of Iberia and the Maghreb Region of north-western Africa (Keller & Busack 2001). Its range in North Africa is influenced by the Atlas Mountain chain, with elevations up to 4165 m. Thus, *M. leprosa* is ideally suited for studying the impact of sea straits and mountain chains on the genetic structure in a freshwater turtle.

Descriptions of seven subspecies from Morocco (Schleich 1996; Bour & Maran 1999) suggest extensive genetic variation caused by these barriers. To test their influence on genetic structure within *M. leprosa*, we analysed sequence variation of the mitochondrial cytochrome *b* gene across the entire species' range. The cytochrome *b* gene has been shown to be phylogenetically informative and to be a powerful tool for revealing phylogeography in many testudinoid chelonians (e.g. Caccone *et al*. 1999; Lenk *et al*. 1999; Feldman & Parham 2002; Palkovacs *et al*. 2002; Austin *et al*. 2003; Stephens & Wiens 2003; Barth *et al*. 2004; Mantziou *et al*. 2004; Spinks *et al*. 2004; Fritz *et al*. 2004, 2005a,b,c). Further, we compare our results to previously published data for other western Palearctic turtles (*Emys orbicularis*, *E. trinacris*: Lenk *et al*. 1999; Fritz *et al*. 2004, 2005a; *Mauremys rivulata*: Mantziou *et al*. 2004) to determine whether general phylogeographical patterns exist in this region.

Materials and methods

Sampling and sequencing

Blood and tissue samples of 99 *Mauremys leprosa* representing populations from 38 localities across the entire species' range (Fig. 1, Table 1) 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 until processing. Voucher blood, tissue or DNA samples are permanently housed at the Museum of Zoology (Museum für Tierkunde) Dresden, Tissue Collection, at the Museum of Vertebrate Zoology, University of California, Berkeley, or at the Biodiversity and Conservation Group, University of Algarve, Faro.

Our target sequence is the mitochondrial cytochrome *b* gene (cyt *b*). Total genomic DNA was extracted from small aliquots of blood or tissues according to 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 using the procedure: 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′). Cycle sequencing was performed for 25 cycles as follows: 10 s at

Table 1 Geographic origin of 99 *Mauremys leprosa* samples and their mtDNA haplotypes.

Locality		*n*	Haplotype	Tissue/blood samples	Vouchers
Portugal					
1 a	Vila Flôr (39°13 N, 07°15 W)	2	A2, A15	MTD T 1597	—
2 a	Castelo Branco (39°82 N, 07°50 W)	6	A16 (5), A19	MTD T 1587, 1589–90	—
3 b	Évora (38°57 N, 07°90 W)	4	A10, A16 (3)	BioCon ML 125–9	—
4 b	Castro Verde (37°42 N, 08°05 W)	4	A7, A11, A16 (2)	BioCon ML 23–4, 27–8	—
5 b	Aljezur (37°32 N, 08°80 W)	6	A6, A8, A9, A16 (2), A20	BioCon ML 142–7	—
6 b	Vilamoura (37°05 N, 08°10 W)	8	A5 (2), A12, A13, A14, A16 (3)	BioCon ML 1–5, 7–8, 17, MTD T 1584–5	—
7 b	Odeleite (37°33 N, 07°98 W)	3	A1, A3, A4	BioCon ML 96–7, 103	—
8 b	Barrancos (38°13 N, 06°98 W)	4	A16 (4)	BioCon ML 113, 115–8	—
Spain					
9 a	Extremadura Region (39°00 N, 06°00 W)	1	A16	MTD T 1432	—
10 c	Huelva: Doñana Biological Reserve (37°00 N, 06°26 W), Laguna Dulce	3	A16 (2), A21	MTD T 2506, 3305, 3408	—
11 c	Cádiz: C-440 (now A-381), 9.0 km S of intersection with CA-212	1	A17	MTD T 1216, MVZ 231989	MNCN 11099
12 c	Cádiz: Facinas, 0.3 km NE km 1 on CA-221	1	A16	MTD T 1217, MVZ 231990	MNCN 23659
12 c	Cádiz: Facinas, 10.7 km NE on CA-221	1	A16	MTD T 1218, MVZ 231991	MNCN 11527
12 c	Cádiz: Facinas, 15.7 km NE on CA-221	2	A16 (2)	MTD 1219–20, MVZ 231992–3	MNCN 11528–9
13 d	Valencia: Turía River (39°27 N, 0°19 W)	1	A16	MTD T 1437	—
14 d	Valencia: Chiva (39°28 N, 0°43 W)	2	A16 (2)	MTD T 1435–6	—
15 d	Valencia: Buñol (39°25 N, 0°47 W)	1	A23	MTD T 1433	—
16 d	Valencia: Júcar River SE Dos Aguas (39°17 N, 0°52 W)	1	A22	MTD T 1438	—
17 d	Valencia: Torrente (39°26 N, 0°27 W)	1	A16	MTD T 1439	—
18 d	Valencia: Júcar Basin: Sollana (39°18 N, 0°23 W), *c.* 20 km S Valencia	1	A16	MTD T 1441	—
19 e	Gerona: Orlina River near the village of Rabos d'Empordà (42°23 N, 02°57 W), 15 km N of Figueres	6	A18 (6)	MTD T 1571, 1577, 1792–4, 1801	—
20 f	Ceuta (35°52 N, 05°20 W)	1	A16	MTD T 1299	*
Morocco					
21 f	Tétouan: 9.9 km E Ksar-es-Srhir on S-704 (35°84.750 N, 05°45.347 W)	3	A16 (3)	MTD T 1213–5	MVZ 178065–6, 231988
22 f	Tétouan: 7 km NNE Asilah, Oued at Tanger to Larache road (35°32.560 N, 06°04.028 W)	1	A16	MTD T 1203	MVZ 162520
23 f	Tétouan: 14.7 km S Asilah on P2 (35°20.560 N, 06°04.028 W)	5	A16 (3), A24, B5	MTD T 1205–9	MVZ 178055–9
24 f	Tétouan: NW Chefchaouene (= Chechaouen; 35°17.140 N, 05°26.972 W)	1	A16	MTD T 1210	MVZ 178061
25 f	Tétouan: 11 km E Chefchaouene (= Chechaouen), on road to Bab Taza (35°04.460 N, 05°12.912 W)	2	A16 (2)	MTD T 1211–2	MVZ 178062–3
26 g	Marrakech: near Aït-Ourir (31°27.891 N, 07°46.724 W); elevation 672 m	2	A25 (2)	MTD T 764–5	*
27 h	Agadir: 3 km SW Taroudannt, Oued Souss Valley (30°26.560 N, 08°54.028 W)	1	B2	MTD T 1204	MVZ 162535
28 h	Agadir: N Tiliouine, Oued Noun Canyon (29°05.115 N, 10°15.140 W); elevation 122 m	4	B1 (4)	MTD T 785–8	*
29 h	Agadir: 17 km E Guelmine (= Goulmine), Oued Noun (28°58.433 N, 09°54.231 W); elevation 300 m	2	B1 (2)	MTD T 780, 784	*
30 i	Agadir: 18 km S Tata: Oued Tata near El-Khemis (29°35.506 N, 08°00.009 W); elevation 494 m	2	B3 (2)	MTD T 774, 779	*
31 i	Agadir: Oued Tissint SE Tissint (29°51.134 N, 07°15.323 W); elevation 438 m	1	B3	MTD T 773	*
32 i	Ouarzazate: Sidi-Flah, Oued Dadès (31°00.688 N, 06°29.931 W); elevation 1158 m	2	B3 (2)	MTD T 763, 768	*
32 i	Ouarzazate: Sidi-Flah, Oued Dadès (31°00.695 N, 06°30.808 W); elevation 1177 m	1	B1	MTD T 769	*
32 i	Ouarzazate: Sidi-Flah, Oued Dadès (31°00.858 N, 06°30.059 W)	1	B1	MTD T 1904	—

Table 1 *Continued*

Locality		*n*	Haplotype	Tissue/blood samples	Vouchers
33 i	Ouarzazate: Tamnougalt, Oued Drâa (30°40.183 N, 06°22.881 W); elevation 917 m	2	B3 (2)	MTD T 770–1	*
34 k	Ksar es Souk: Aoufous, Oued Ziz (31°40.946 N, 04°11.008 W)	3	B4 (3)	MTD T 1908–10	—
35 k	Ksar es Souk: bridge on the Ziz River, Oued Ziz (31°31.319 N, 04°11.147 W), *c.* 10 km N Er Foud	1	B4	MTD T 1905	—
35 k	Ksar es Souk: source Aati, Oued Ziz (31°33.947 N, 04°11.617 W)	2	B4 (2)	MTD T 1906–7	—
Algeria					
36 l	Annaba: Skikda Wilayat: Hadjar Ediss, 20 km SSW Annaba (by Hwy. to Guelma; 36°43 N, 07°39 E); elevation 40 m	1	B6	MTD T 1222	MVZ 235704
Tunisia					
37 l	Suq el Ara'a: Jundubah (= Jendouba): 14 km E (by road) Tabarka (36°57 N, 08°54 E); elevation 60 m	1	B6	MTD T 1223	MVZ 235705
38 l	Al Watan al Qibli: Hammamet (Al Hammamat; 36°24 N, 10°35 E)	1	B7	MTD T 1360	—

Numbers preceding localities refer to Fig. 1, letters to locality groups in Fig. 2. If a haplotype was found more than once per locality, the recorded number is bracketed after the haplotype. Asterisks indicate samples or complete series of which voucher photographs are deposited in the MTD Tissue Sample Collection. BioCon = collection of the Biodiversity and Conservation Group at Algarve University, Faro; MNCN = Museo Nacional de Ciencias Naturales, Madrid; MTD T = Museum of Zoology (Museum für Tierkunde) Dresden Tissue Collection; MVZ = Museum of Vertebrate Zoology, Berkeley.

96 °C, 5 s at 50 °C, and 4 min at 60 °C. Products were resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Obtained sequences were approximately 1100 bp long.

Phylogenetic analysis

Sequences were aligned with ClustalW using default parameters implemented in mega 3.0 (Kumar *et al.* 2004). mega 3.0 was also used to estimate genetic distances and calculate sequence statistics. The other western Palearctic *Mauremys* species, *M. caspica* (blood sample: MTD T 747, Iran: Kerman: Bisotun = Bisitun; accession number AM110186) and *M. rivulata* (blood sample: MTD T 383, Greece: Chalkidiki Lake, near Ayios Vassilios; accession number AM110185) served as outgroups.

Phylogenetic relationships of haplotypes were estimated by neighbour joining (NJ) using program packages mega 3.0 and paup* 4.0b10 (Swofford 2002), maximum parsimony (MP) and maximum likelihood (ML) using paup* 4.0b10, and Bayesian analysis with mrbayes 3.0B4 (Huelsenbeck & Ronquist 2001). For all tree-building methods except Bayesian, the robustness of the branching pattern was tested by bootstrapping (Felsenstein 1985). For reconstruction of NJ trees (Saitou & Nei 1987), the Tamura–Nei model (Tamura & Nei 1993), pairwise deletion, and 10 000 bootstrap replicates were selected. In order to find the most appropriate model and parameters of nucleotide substitution, we performed a hierarchical likelihood ratio test with modeltest 3.6 (Posada & Crandall 1998). The transversion model (TVM) combined with a proportion of invariable sites I = 0.6745, was the best-fitting model of 56 tested models selected by AIC according to the best –ln L value. The selected TVM model and parameters were used for performing ML and Bayesian analyses.

For ML and MP analyses, we used heuristic search with 10 random stepwise additions and TBR branch swapping. For MP analysis, 1000 bootstrap replicates and a permutation test with 100 steps were conducted. For ML, only 100 bootstrap replicates were run due to the enormous computational time. We compared log-likelihood scores of ML trees constructed with and without an enforced molecular clock and found sequence evolution not clocklike ($P < 0.001$). Bayesian analyses were run for 1 000 000 generations, with a sampling frequency of 10 generations. From the 100 000 trees found, we determined a subset of trees for building our consensus tree by inspecting likelihood values of trees saved by mrbayes and set the burn-in to 1000 trees discarded to ensure stable likelihood values were achieved.

In addition, we constructed a haplotype network with tcs 1.13 (Clement *et al.* 2000). This software is based on statistical parsimony for constructing a haplotype network in that the required number of mutational steps leading from one haplotype to another is minimized. In contrast to dichotomous phylogenetic trees, networks allow for persistent ancestral nodes and reticulations. Such a network is able to demonstrate simultaneous alternative evolutionary pathways, with occurrence of reticulations visualizing ambiguous

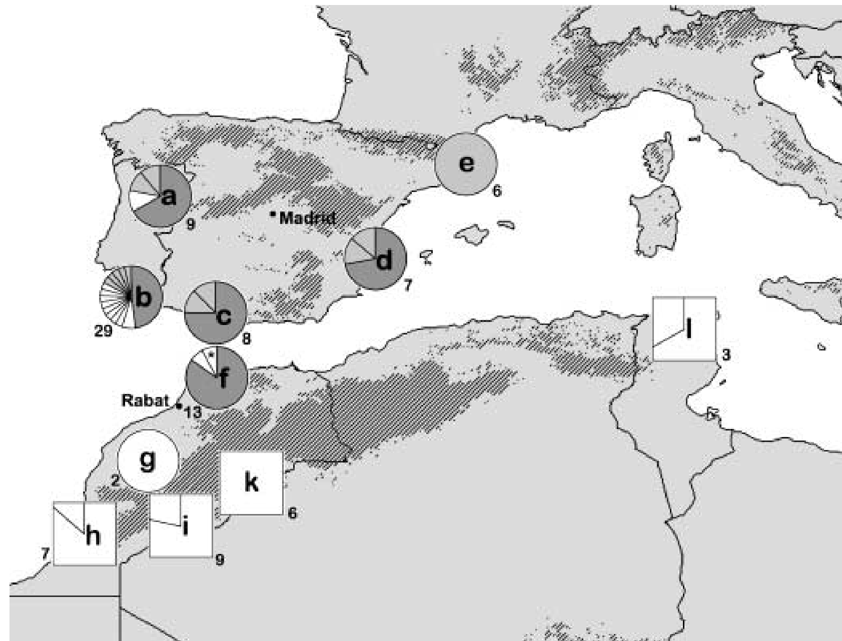

Fig. 2 Haplotype diversity in *Mauremys leprosa* (percentages), locality groups as defined in Table 1. Circles indicate clade A; squares, clade B. The most common haplotype (A16) dark grey; variants of A16, differing by only one mutation step, light grey. Haplotypes differing from A16 in more than one mutation step indicated in white. Numbers correspond to sample sizes, asterisk in f denotes haplotype B5.

or uncertain domains. In haplotypic data, loops can also indicate reverse or parallel mutations. Moreover, the position of a haplotype in a network implies information regarding its age. Older haplotypes are thought to have a greater likelihood of being located internally in the network (Posada & Crandall 2001).

Molecular diversity

Samples from individual populations were combined to locality groups as defined in Fig. 2 and Table 1. Population genetic statistics were estimated using ARLEQUIN 2.001 (Schneider *et al*. 2001) and MEGA 3.0 (Kumar *et al*. 2004). The level of polymorphism of each locality group and each clade resulting from the phylogenetic trees was estimated regarding the number of haplotypes, polymorphic sites (S), nucleotide diversity (π), haplotype diversity (h) (Nei 1987), and mean nucleotide pairwise differences. To assess nucleotide diversity between locality groups, we used the formula $D_{xy} = D - 0.5(D_x + D_y)$ (Nei 1987), accounting for ancestral polymorphism. D is the mean nucleotide diversity between the two groups, D_x and D_y are the mean nucleotide diversity of each group, and D_{xy} is the corrected nucleotide diversity between the two groups. With MEGA 3.0, D_x, D_y and D_t (mean nucleotide diversity of all individuals = D_x and D_y together) were computed directly. Then D_x, D_y and D_t were used for calculating D via the following formula: $D_tN_t(N_t - 1)/2 = DN_xN_y + D_xN_x(N_x - 1)/2 + D_yN_y(N_y - 1)/2$, where N_x, N_y and N_t are sample sizes of populations x, y and t, respectively, and where $N_t = N_x + N_y$ (Zheng *et al.* 2003). For estimating genetic distances between sequence pairs, the Tamura–Nei (TrN) model was chosen, as the more complex transversion model is not implemented in MEGA.

Historical population dynamics

Historical patterns of population structure were inferred from mismatch distribution of pairwise nucleotide differences in the entire data set, and from within clades A and B as revealed by phylogenetic analysis using DNASP 4.10.3 (Rozas *et al*. 2003). This analysis compares distribution of frequency of pairs of individuals who differ by a certain number of nucleotide differences (Slatkin & Hudson 1991; Rogers & Harpending 1992; Schneider & Excoffier 1999) between data sets. This frequency distribution is usually unimodal for lineages having undergone a recent bottleneck or rapid population expansion, and multimodal for lineages whose populations are in demographic equilibrium or subdivided into several distinct units.

The resulting distribution was tested against constant population size (Watterson 1975; Slatkin & Hudson 1991; Rogers & Harpending 1992) and sudden population expansion models using the mismatch distribution (Rogers & Harpending 1992) as implemented in DNASP 4.10.3 and ARLEQUIN 2.001. The test is based on three parameters: θ_0, θ_1 (θ_0 before, and θ_1 after, population growth), and τ (date of growth in units of mutational time; $\tau = 2\mu t$, where μ is the mutation rate for the whole sequence and t is the time). The validity of the expansion model was tested by a parametric bootstrap approach, which compares the fit to expected mismatch distribution of the observed and 100 simulated mismatch distributions. The fit to the expected mismatch distribution was

quantified by both the sum of squared deviations (*SSD*) between observed and simulated distributions on one hand and the expected distribution on the other, and the raggedness index (*rg*) (Harpending 1994), which takes larger values for multimodal distributions commonly found in a stationary population than for unimodal and smoother distributions typical of expanding populations (Schaefer *et al*. 2002).

Mismatch distributions have been found to be quite conservative (Ramos-Onsins & Rozas 2002) and, in order to have a wider view of long-term population history, we also computed Tajima's D (Tajima 1989), Fu's F_s (Fu 1997), Fu & Li's F^* and D^* (Fu & Li 1993) and the Ramos-Onsins & Rozas's R_2 (Ramos-Onsins & Rozas 2002), using DNASP 4.10.3.

Results

Phylogeny, haplotype and sequence diversity

Aligned sequences comprised 933 bp (including gaps at the 5′-end of a few shorter sequences). Average base frequencies of *Mauremys leprosa* were A = 0.2845, C = 0.3184, G = 0.1336, T = 0.2635. The strong bias against guanine is characteristic for mitochondrial but not for nuclear genes (Desjardins & Morais 1990). We identified 32 individual mtDNA haplotypes for which all tree-building methods resulted in an identical topology (Fig. 3). Haplotypes cluster in two major clades, of which one (A) is moderately to well-supported, and the other (B) weakly to moderately supported by bootstrap and Bayesian analysis. Clade A contains 25, clade B seven individual haplotypes (Table 2). Average sequence divergence (uncorrected *p* distance) within *M. leprosa* is 0.7% (range: 0.1–2.2%) and between clades A and B, 1.3% (range: 0.9–2.2%). Average sequence divergence within clade A is 0.4% (range: 0.1–1.4%) and within clade B 0.4% (range: 0.1–0.9%). Analysis of genetic diversity within clade A revealed 18 transitions and 19 transversions in 59 polymorphic sites, nucleotide diversity of 0.0019, haplotype diversity of 0.67, and an average pairwise differences value of 1.8 ± 0.4 (range: 1–13). Within clade B, nine transitions and one transversion were found among 13 polymorphic sites, nucleotide diversity was 0.0033, haplotype diversity was 0.80, and the average pairwise differences value was 3.1 ± 1.1 (range: 1–20).

Among all locality groups, nucleotide diversity ranged from 0 to 0.0079, with a mean value for the entire data set of 0.0057; haplotype diversity ranged from 0.29 to 1.00 (in locality groups e, g, and k) and the overall value for the entire data set was 0.81.

In the TCS network, the phylogenetically most distinct clade A haplotypes (A24 and A25) are connecting both clades. Alternative pathways occur between haplotypes A7, A10, A11, A12, A13, A24, and A25. When these ambiguities are resolved according to the assumptions that (i) rare haplotypes are more likely to be found at the tip and common haplotypes at interior nodes of the net, and (ii) a haplotype corresponding to a single individual is more likely to be connected to haplotypes of the same population than to haplotypes of other populations (Posada & Crandall 2001), the network presented in Fig. 4 is obtained.

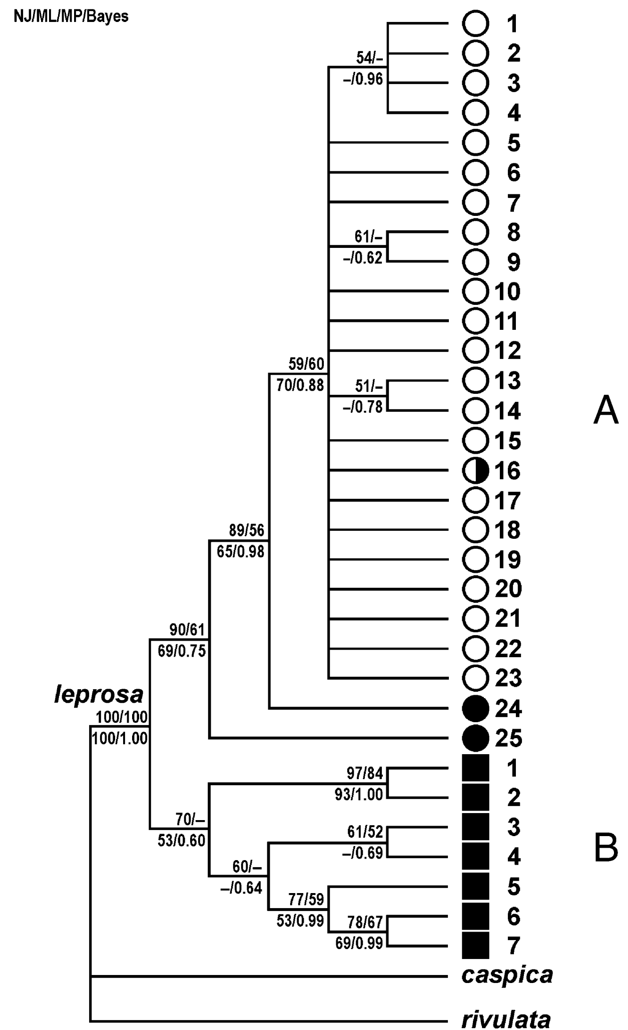

Fig. 3 *Mauremys leprosa* NJ tree of mtDNA haplotypes. Clade A haplotypes symbolized by circles, clade B by squares followed by numbers that indicate individual haplotypes. Black symbols indicate haplotypes occurring in North Africa, white symbols, Europe; semifilled circle indicates occurrence of haplotype in both Europe and Africa. Numbers above nodes are bootstrap values greater than 50 obtained under NJ and ML, numbers below nodes MP bootstrap values greater than 50 and posterior probabilities from Bayesian analysis. Dashes indicate bootstrap values below 50. Branching patterns below 50% bootstrap support under NJ, ML and MP shown as combs.

Most haplotypes were recorded in only a few individuals (Table 2); A16 is by far the most common haplotype, detected in 42 of 99 samples. Many other haplotypes in clade A are

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Table 2 Frequencies of *Mauremys leprosa* mtDNA haplotypes and their accession numbers (AM 110153–110184).

Haplotype	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Frequency	1	1	1	1	2	1	1	1	1	1	1
Accession no.	110153	110154	110155	110156	110157	110158	110159	110160	110161	110162	110163
Haplotype	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22
Frequency	1	1	1	1	42	1	6	1	1	1	1
Accession no.	110164	110165	110166	110167	110168	110169	110170	110171	110172	110173	110174
Haplotype	A23	A24	A25	B1	B2	B3	B4	B5	B6	B7	
Frequency	1	1	2	8	1	7	6	1	2	1	
Accession no.	110175	110176	110177	110178	110179	110180	110181	110182	110183	110184	

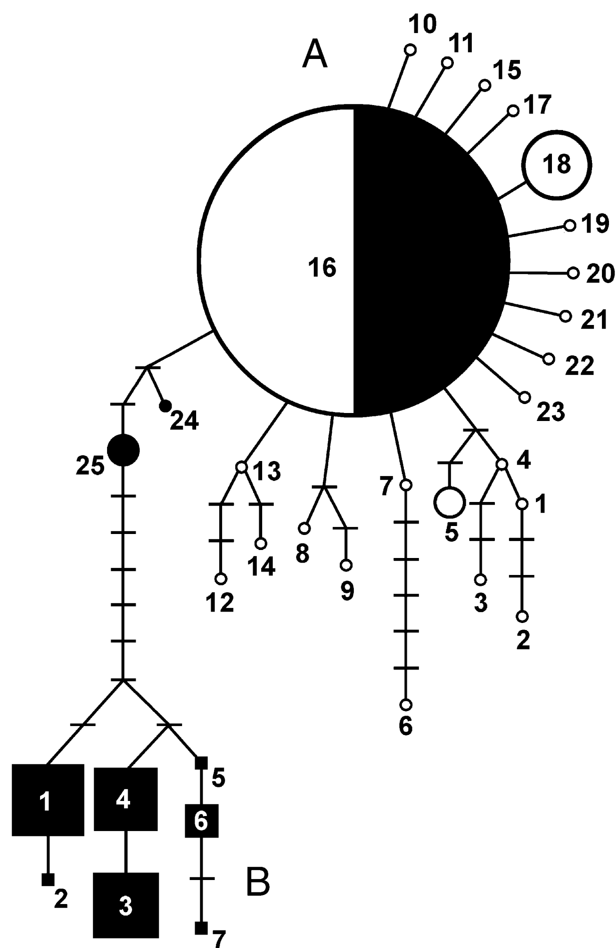

Fig. 4 Network presentation (TCS) for *Mauremys leprosa* mtDNA haplotypes. Symbol size corresponds to approximate frequency of identified haplotypes (Table 2). Circles indicate clade A haplotypes, squares, clade B. Black symbols indicate haplotypes occurring in North Africa, white symbols, Europe; semifilled circle indicates occurrence of haplotype in both Europe and Africa. Dashes indicate missing haplotypes, lines between dashes or symbols correspond to one mutation step.

rare variants of A16, differing only in one mutation step. The rareness of the distinct haplotype A25, however, could be a sampling bias because this haplotype originates in a remote region, represented by only two samples. Although only 26 among the 99 *Mauremys leprosa* we sampled contained clade B haplotypes, differentiation within clade B presents a degree of differentiation similar to that revealed within clade A.

Geographic distribution of haplotypes

Clade A haplotypes occur in North Africa and throughout Iberia, while clade B haplotypes are confined to North Africa. In North Africa, clade A haplotypes are distributed only in Morocco (and the Spanish exclave Ceuta) north of the Atlas Mountains, while clade B haplotypes occur in Morocco mainly south of the Atlas Mountains (Figs 1, 2). We recorded in a single case a turtle, syntopic with four clade A individuals (locality 23 in Table 1 and Fig. 1: 14.7 km S Asilah), harbouring a clade B haplotype (B5) in northern Morocco. Except for this record, clade B haplotypes have also been recorded in easternmost Algeria and Tunisia, and here in localities north of the Atlas Mountains (Table 1, Figs 1, 2).

Among clade A haplotypes greatest diversity occurs on the Iberian Peninsula where, particularly in southern Portugal, several clade A haplotypes may occur in one population. There is much less variation in all other studied areas. In one of the northernmost populations (Orlina River near Figueres, locality 19 in Table 1 and Fig. 1, locality group e in Fig. 2) only the endemic haplotype A18 was found among six sequenced samples, and A18 differed in one mutation step from the common haplotype A16. In northern Morocco and Ceuta we detected only three of 25 clade A haplotypes (A16, A24, and A25). A16, widespread on the Iberian Peninsula, is also the most frequently recorded haplotype in northern Morocco and Ceuta (Tables 1, 2). The phylogenetically most distinct clade A haplotypes (A24 and A25; Fig. 3) are confined to northern Morocco.

Historical demography

We evaluated two demographic parameters for all locality groups and, in more detail, the demographic history for all

samples pooled together, and for each clade separately. The Tajima *D*-value and the R_2 value are simultaneously significant only in southern Portugal (locality group b), suggesting rapid increase in population size (see below). Pooled samples departed significantly from expectation assuming population stability: mismatch distribution shape is multimodal, one mode represents pairwise differences within each clade and the other differences between clades. When clades A and B are considered separately, historical demography differs clearly between them. Clade A produced a distinctly unimodal curve consistent with sampled populations having undergone a recent bottleneck and expansion or selective sweep (Rogers & Harpending 1992), with nonsignificant but low *SSD* and *rg* values (*SSD* = 0.01, $P > 0.05$; *rg* = 0.046, $P > 0.05$), a highly significant negative *D*-value (–2.0, $P < 0.001$), a large value of F_s (–18.2, $P < 0.001$), significant negative values of Fu & Li's D^* and F^* ($D^* = -4.6$, $P < 0.05$; $F^* = -4.5$, $P < 0.05$) and a highly significant R_2 value (0.03, $P < 0.001$). Clade B shows a multimodal pairwise mismatch curve, indicative for stationary populations; none of its demographic statistics was significant.

Statistical tests of neutrality also provide strong support for distinct histories of clades A and B. In clade A, significantly more mutations at the tips of the haplotype net and tree occur than are expected with a neutral locus evolving in a population of stable size; most of the distinct haplotypes are endemic to southern Portugal (locality group b). This departure from neutrality is supported also by Tajima and Fu tests and can be interpreted in terms of historical demography (Rand 1996) as a rapid rise in population size following a very small founder population, or a bottleneck (Tajima 1989; Fu & Li 1993). Demographic analysis utilizing pairwise mismatch nucleotide differences and the R_2 test also supports this conclusion. Deviations from neutrality can be caused by selective sweeps, background selection and population growth, but these effects can be distinguished from one another by evaluating the significance of different analyses. D^* and F^* are more sensitive to background selection than is F_s (Fu 1997); F_s is mostly sensitive to population growth and selective sweeps relative to D^* and F^* (Fu & Li 1993). Therefore, a pattern of significant F_s with nonsignificant F^* and D^* indicates population expansion or selective sweep, whereas the opposite pattern would indicate background selection (Joseph *et al*. 2002). The pattern displayed by clade A is significant for F_s but also significant for F^* and D^* and it remains unclear whether the observed effect is caused by one factor, by the other, or by both acting in concert.

Discussion

Although southern Portugal is our best-sampled region, the high diversity of clade A haplotypes encountered there (Fig. 2) cannot be explained by sample size alone. Haplotype distribution, as well as demography, suggests different histories for the two *Mauremys leprosa* clades. Clade A probably underwent rapid expansion (dispersal), as mirrored by demographic parameters and the occurrence of the widespread haplotype A16 (and closely related haplotypes differing in only one nucleotide) in nearly all studied populations. In contrast, clade B remained stationary, displaying a pattern of population fragmentation (vicariance), lacking widespread haplotypes, but with endemic haplotypes in different populations (Table 1).

The oldest known fossils referred to *M. leprosa* (Ruscinian, 5.4–3.4 Myr BP) were excavated in Algeria (Aïn Boucherit, Constantine Province); species-undetermined *Mauremys* material of the same age was also found in Tunisia (de Lapparent de Broin 2000). The oldest unambiguous *M. leprosa* fossils from Europe are distinctly younger and date to the Middle Pleistocene of Mealhada, Portugal. Older, species-undetermined *Mauremys* fossils from the Late Pliocene/Lower Pleistocene boundary at Granada, however, could represent *M. leprosa* (de Lapparent de Broin & Antunes 2000; de Lapparent de Broin 2001), supporting the assumption that *M. leprosa* evolved in north-western Africa and arrived in Europe not before Late Pliocene or Early Pleistocene (approximately 1.8 Myr BP; de Lapparent de Broin 2001). This scenario is consistent with our results in that the greatest haplotype diversity is found in North Africa (Morocco) where both clades occur, suggesting, like the fossil record, an 'Out of Africa' dispersal.

Based on the fossil record and the paraphyly of African *M. leprosa* haplotypes with respect to European haplotypes (Fig. 3), we hypothesize that both clades A and B originated in North Africa, and that clade A invaded Iberia via the Strait of Gibraltar. On the Iberian Peninsula, *M. leprosa* populations were severely reduced by repeated glacial extermination, resulting in the bottleneck reflected by demography, and the last glacial caused range restriction to what is now southern Portugal and the adjacent area in Spain. A rapid expansion in range occurred after Holocene warming, resulting in the recolonization of other portions of the Iberian Peninsula. Decreasing haplotype diversity from south-west to north and north-east on the peninsula is consistent with this hypothesis and fits Hewitt's (1996) long-distance dispersal model, well-known from many animal species (e.g. Hayes & Harrison 1992; Merilä *et al*. 1997; Taberlet *et al*. 1998; Hewitt 1999, 2001; Cruzan & Templeton 2000; Fritz *et al*. 2005a). Typical for when rapid range expansion occurs, this pattern results in decreasing genetic diversity with increasing distance from the glacial refuge acting as the radiation centre.

Clade A populations obviously remained in northern Morocco, as indicated by records of endemic haplotypes A24 and A25, representing the phylogenetically most differentiated (Fig. 3) and, according to their interior position in the

network (Fig. 4), oldest haplotypes of clade A. Their partly syntopic occurrence with haplotype A16, also widely distributed on the Iberian Peninsula, suggests reinvasion of northern Africa from the Iberian Peninsula via the Strait of Gibraltar, although incomplete lineage sorting could be an alternative. Sea straits generally present no significant biogeographical barrier to other western Palearctic turtle species (*Emys orbicularis*, *Mauremys rivulata* (Mantziou *et al*. 2004; Fritz *et al*. 2005b), although the Sicilian endemic *E. trinacris* might be an exception (Fritz *et al*. 2005a)). Moreover, as in *E. orbicularis*, the Strait of Gibraltar is no significant barrier to the ribbed newt (*Pleurodeles waltl*) or to wall lizards of the genus *Podarcis* (Harris *et al*. 2002; Carranza & Arnold 2004; Veith *et al*. 2004).

In contrast, and also as in *E. orbicularis* where mountain chains separate distinct mtDNA lineages, mountains, specifically the Atlas chain, constitute a significant biogeographical barrier to *M. leprosa* (Fritz *et al*. 2005b), separating clades A and B from one another in Morocco. With the exception of one record of a clade B haplotype (see below), all Moroccan samples north of the Atlas Mountains represent clade A haplotypes, while clade B is confined to regions south of the chain. However, additional sampling is necessary along the Algerian Mediterranean coast. Our samples from easternmost Algeria and Tunisia north of the Atlas Mountains belong to clade B. In *Pleurodeles* newts, species that occur in habitats resembling those of *M. leprosa*, there is a similar major break. *Pleurodeles waltl* is restricted to the northern part of Morocco and replaced in Algeria and Tunisia by *P. poireti* and *P. nebulosus* (Carranza & Arnold 2004; Carranza & Wade 2004; Veith *et al*. 2004), suggestive of a common pattern derived from unknown historic events causing genetic interruption in this region.

Reptile trade and turtle collection is known to have occurred for decades in Morocco. While we cannot exclude the possibility that the single specimen we discovered in northern Morocco with a clade B haplotype was introduced there by man, this record could also indicate gene flow between both clades. The similarity of its haplotype (B5) to those in eastern Algeria and Tunisia (B6, B7) suggests introgression from the east and that both *M. leprosa* clades may meet somewhere along the North African Mediterranean coast.

Six samples of one of the northernmost, isolated populations of *M. leprosa* (Orlina River near Figueres, Spain; locality 19 in Table 1 and Fig. 1, locality group e in Fig. 2) possess an endemic haplotype (A18), closely related to the common haplotype, A16. A similar phenomenon is known in the European pond turtle, *Emys o. orbicularis*, where populations near the northern range border in Germany and adjacent Poland harbour an endemic haplotype, also differing in one nucleotide from a widespread southern ancestor (Lenk *et al*. 1999; Fritz *et al*. 2004). In addition, fragmented populations along the southern range borders display in both species endemic local haplotypes, which are generally more differentiated than the northern endemics. This reflects the higher age of southern populations and indicates that peripheral populations play an important role in evolution of western Palaearctic freshwater turtles. Founder effects, inbreeding, and genetic drift of these typically small populations probably result in genetic distinctiveness within a short span of time. Conservation efforts should therefore focus not only on individual-rich populations from core regions of the range, but also on peripheral populations.

Systematics

Schleich (1996) and Bour & Maran (1999) described seven *M. leprosa* subspecies from Morocco and distinguished, in addition, the nominotypical subspecies *M. l. leprosa* (Schweigger, 1812) (restricted type locality: southern Spain; Mertens & Müller 1940). Seven of the eight subspecies are represented among our samples; only *M. l. wernerkaestlei* Schleich, 1996 (type locality: Oued Oum er Rbia, south of Khenifra, northern Morocco) is lacking. Populations from many parts of the species' range have not been assigned to any subspecies by Schleich (1996) and Bour & Maran (1999); and Ernst *et al*. (2000), Keller & Busack (2001), and Fritz *et al*. (2005b) raised doubts about the validity of the *M. leprosa* subspecies.

Although taxonomic decisions should not be based on genetic data of single loci, especially haplotypic data (the 'gene tree and species tree problem' of Avise 1989; see also Wilson *et al*. 1985; Birky 1991; Funk & Omland 2003; Zhang & Hewitt 2003; Ballard & Whitlock 2004), our data provide some insights. MtDNA haplotypes of *M. l. atlantica* and *M. l. marokkensis* are polyphyletic in that haplotypes of clades A and B both occur in these taxa; *M. l. erhardii* and *M. l. saharica* possesses no unique haplotypes (Table 3). The unique haplotype (B4) of *M. l. zizi* is ancestral and closely related to B3 occurring in *M. l. vanmeerhaghei*, a geographically neighbouring subspecies (Fig. 4).

Only if species or subspecies correspond to distinct, monophyletic haplotype clades is there strong evidence for validity when haplotypic data are employed. This is not the case in most *M. leprosa* subspecies. The reverse conclusion, that these taxa are invalid, however, is not necessarily true, especially on the subspecies level. Funk & Omland (2003) demonstrated that 23% of 2319 considered animal species display either paraphyletic or polyphyletic mitochondrial gene trees, among others due to incomplete lineage sorting, introgression, or unrecognized paralogy.

Although our data set is thus inconclusive, we recommend reducing the number of *M. leprosa* subspecies for the following reasons: (1) Most diagnostic characters refer only to juvenile colour and pattern characteristics lost during ageing. Inconsistencies in assigned diagnostic characters also exist (Keller

Table 3 Subspecies allocations of studied *Mauremys leprosa* samples.

Localities	Referred subspecies	Haplotypes	Reference
Portugal, Spain			
1–9	—	A1*, A2*, A3*, A4*, A5*, A6*, A7*, A8*, A9*, A10*, A11*, A12*, A13*, A14*, A15*, A16, A19*, A20*	—
10–12	*leprosa* (Schweigger, 1812)	A16, A17*, A21*	Mertens & Müller (1940): southern Spain = restricted type locality
13–20	—	A16, A18*, A22*, A23*	—
Morocco			
21	—	A16	—
22–23	*atlantica* Schleich, 1996	A16, A24*, B5*	Schleich (1996)
24	—	A16	—
25	*erhardii* Schleich, 1996	A16	Schleich (1996)
26–27	*marokkensis* Schleich, 1996	A25*, B2*	Schleich (1996)
28–29	*saharica* Schleich, 1996	B1	Schleich (1996)
30–33	*vanmeerhaghei* Bour & Maran, 1999	B1, B3*	Bour & Maran (1999)
34–35	*zizi* Schleich, 1996	B4*	Schleich (1996)
Algeria			
36	—	B6	—
Tunisia			
37–38	—	B6, B7*	—

Unique haplotypes asterisked.

& Busack 2001; Fritz *et al*. 2005b), making subspecies determination often impossible when relying upon morphology; (2) variation of juvenile colour pattern across the entire species' range is unknown; (3) physiogeographically, there is only one long-existing major barrier in Morocco (the Atlas Mountains) that could effectively separate *M. leprosa* populations, while currently recognized taxa north (*M. l. atlantica*, *M. l. erhardii*, *M. l. marokkensis*, *M. l. wernerkaestlei*) and south (*M. l. saharica*, *M. l. vanmeerhaghei*, *M. l. zizi*) of this mountain chain occur only in tiny, patchy areas that lie, in part, in the same general hydrogeographical drainage system; (4) mtDNA data reveal only one major partitioning within *M. leprosa*, coinciding in Morocco with populations north and south of the Atlas Mountains.

We hereby propose recognition of only two subspecies, *Mauremys leprosa leprosa* (Schweigger, 1812) for turtles harbouring clade A haplotypes (Iberian Peninsula, and Morocco north of the Atlas Mountains) and *Mauremys leprosa saharica* Schleich, 1996, for turtles harbouring clade B haplotypes (Morocco south of the Atlas Mountains, easternmost Algeria and Tunisia). Taxonomic allocation of populations in western and central Algeria as well as in Libya is pending, awaiting further study.

Acknowledgements

Markus Auer, Joan Budo, Xavier Capalleras, Jean-Marc Ducotterd, Soumia Fahd, Daniel Frynta, Cati Gerique, Claudia Keller, Ignacio Lacomba, Georgia Mantziou, Ramon Mascort, Daniele Seglie, and Miguel Vences provided blood samples and Carla Cicero (University of California, Museum of Vertebrate Zoology, Berkeley) provided tissues from the MVZ tissue collection for this study. Much of the lab work was done by Anke Müller (Museum of Zoology, Dresden). Edgar Lehr assisted during earlier stages of the study, while Jonathan Raine (North Carolina State Museum of Natural Sciences) assisted with production of the maps. The late Jeanne A. Visnaw helped S. D. Busack with field collection of most MVZ tissues. Pedro Segurado instructed M. Barata in the arts of terrapin catching.

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