doi:10.1111/j.1420-9101.2007.01485.x

Go east: phylogeographies of *Mauremys caspica* and *M. rivulata* – discordance of morphology, mitochondrial and nuclear genomic markers and rare hybridization

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Keywords:

chelonian;
gene flow;
hybridization;
phylogeography;
speciation.

Abstract

In recent years many cases of hybridization and introgression became known for chelonians, requiring a better understanding of their speciation mechanisms. Phylogeographic investigations offer basic data for this challenge. We use the sister species *Mauremys caspica* and *M. rivulata*, the most abundant terrapins in the Near and Middle East and South-east Europe, as model. Their phylogeographies provide evidence that speciation of chelonians fits the allopatric speciation model, with both species being in the parapatric phase of speciation, and that intrinsic isolation mechanisms are developed during speciation. Hybridization between *M. caspica* and *M. rivulata* is very rare, suggesting that the increasing numbers of hybrids in other species are caused by human impact on environment (breakdown of ecological isolation). Genetic differentiation within *M. caspica* and *M. rivulata* resembles the paradigm of southern genetic richness and northern purity of European biota. However, in west Asia this pattern is likely to reflect dispersal and vicariance events older than the Holocene. For *M. caspica* three distinct Pleistocene refuges are postulated (Central Anatolia, south coast of Caspian Sea, Gulf of Persia). Morphologically defined subspecies within *M. caspica* are not supported by genetic data. This is one of the few studies available about the phylogeography of west and central Asian species.

Introduction

Many investigations have analysed the phylogeography of European biota (reviews in Schmitt, 2007; Weiss & Ferrand, 2007), providing a detailed understanding of their Holocene and Pleistocene range fluctuations. By contrast, comparatively few studies focus on true central or west Asian species, so that their phylogeography remains largely unstudied. Most of the information available is largely a side-effect of studies that examine widely distributed Palaearctic species or European species whose ranges extend into Asia (e.g. for reptiles: Kalyabina *et al.*, 2001; Kalyabina-Hauf & Ananjeva, 2004; Ursenbacher *et al.*, 2006; Fritz *et al.*, 2007a,b). The few studies on reptiles endemic in central or west Asia used small sample sizes (Macey *et al.*, 1998, 1999; Guo & Wang, 2007), and hence amounted to phylogenetic rather than phylogeographic analyses. In this study, we present phylogeographies for a west Asian terrapin, *Mauremys caspica*, and the closely related south-east European and west Asian species *M. rivulata*.

Stripe-necked terrapins (genus *Mauremys*, Geoemydidae) constitute a characteristic element of the fauna of

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the Near and Middle East and the Mediterranean, being the most abundant freshwater turtles there. The genus comprises 10 omnivorous, small- to medium-sized aquatic species, occurring in a doubly disjunct range. Seven East and South-east Asian species are separated from their western Palaearctic congeners by a wide distributional gap, and the ranges of the three western species are also interrupted by a gap in the central Mediterranean region. *Mauremys leprosa* is confined to the North African Maghreb region and the Iberian Peninsula; the other two species, *M. rivulata* and *M. caspica*, occur east of the gap in the south-eastern Balkans, the Near and Middle East (Fritz & Havaš, 2007). For a long time, the three western Palaearctic species were thought to be conspecific (e.g. Loveridge & Williams, 1957; Wermuth & Mertens, 1977), their allopatric and parapatric ranges matching the model of geographically vicariant subspecies (Rensch, 1947; Mayr, 1963). On the basis of their morphological distinctness, considerable mtDNA sequence differentiation and the lack of intergradation, all three taxa are now treated as full species (Busack & Ernst, 1980; Fritz & Wischuf, 1997; Barth *et al.*, 2004; Mantziou *et al.*, 2004; Spinks *et al.*, 2004).

Genetic evidence suggests *M. leprosa* as basal lineage, representing the sister taxon of a clade comprising all other species; *M. caspica* and *M. rivulata* are sister species and phylogenetically embedded within their East and South-east Asian congeners (Barth *et al.*, 2004; Spinks *et al.*, 2004). *Mauremys caspica* and *M. rivulata* occupy parapatric ranges with a long contact zone of ~1000 km in Asia Minor (Fritz & Wischuf, 1997). Comparing more than 800 specimens morphologically, Fritz & Wischuf (1997) found hybrids of *M. caspica* and *M. rivulata* at only two sites along the contact zone. This distributional situation implies that these two species diverged in allopatry and are still outcompeting one another by using the same ecological niche, not yet allowing a sympatric occurrence ('semispecies' of Mayr, 1963). While *M. rivulata* is monotypic, three subspecies are recognized within *M. caspica*, suggesting that *M. caspica* and *M. rivulata* are ideally suited models for investigating different stages of speciation of freshwater terrapins. In view of the growing number of reports of hybrids even between distantly related chelonian species (see reviews in Schilde *et al.*, 2004 and Buskirk *et al.*, 2005; see also Bowen & Karl, 2007 and Stuart & Parham, 2007) and introgressed mtDNA in several species (Guicking *et al.*, 2002; Lara-Ruiz *et al.*, 2006; Farias *et al.*, 2007; Fong *et al.*, 2007; Praschag *et al.*, 2007; Spinks & Shaffer, 2007), we hope to gain novel insights into speciation of chelonians using this model.

The present study is based on a range-wide sampling of *M. caspica* and *M. rivulata*. We use sequence variation of a mitochondrial and a nuclear genomic marker gene as well as nuclear genomic fingerprinting [Inter-Simple-Sequence-Repeat (ISSR) PCR] to address the following questions: (i) do morphological, mtDNA and nuclear genomic differentiation follow the same pattern, (ii) does interspecific hybridization represent an exceptional and localized phenomenon or does gene flow still take place on a larger scale and (iii) do the subspecies within *M. caspica* correspond to evolutionarily significant genetic lineages? Within this frame, we present genetic data from a newly discovered putative hybrid population comprising terrapins morphologically intermediate between *M. caspica* and *M. rivulata*. The other two hybrid populations in south-eastern Turkey and Syria are now extinct (Fritz & Wischuf, 1997; D. Ayaz, personal observation; Fig. 1).

Materials and methods

Sampling and chosen genes

Most samples (tail tips, salivary or blood samples of stripe-necked terrapins) were collected during fieldwork and preserved in an EDTA buffer (Fritz *et al.*, 2005) or EtOH and stored at −20 °C until processing. In addition, basic morphological data (size, coloration, pattern) were recorded and voucher photographs taken for ~150 *M. caspica* and *M. rivulata* each, representing the populations used for genetic investigations. A few ethanol-preserved samples were provided by other researchers. Remaining samples and DNA are permanently kept at −80 °C in the tissue sample collection of the Museum of Zoology, Dresden.

Our mitochondrial target sequence is the cytochrome *b* gene (cyt *b*), an ideally suited marker routinely employed for phylogeographic purposes in chelonians due to its high resolution (e.g. Lenk *et al.*, 1999; Austin & Arnold, 2001; Palkovacs *et al.*, 2002; Austin *et al.*, 2003; Fritz *et al.*, 2005, 2006a,b, 2007a,b; Praschag *et al.*, 2007). Recent investigations have shown that the nuclear genes C-mos and Rag2 are fairly variable in chelonians (Le *et al.*, 2006; Fritz & Bininda-Emonds, 2007); after sequencing and comparing both of them in several samples of stripe-necked terrapins, it was decided to use the more variable C-mos gene as nuclear genomic marker. The 13 Rag2 test sequences, obtained using the laboratory procedures outlined below and the PCR conditions and primers of Le *et al.* (2006), are available from GenBank under accession numbers AM905428–AM905440; sequences of cyt *b* and C-mos haplotypes under the accession numbers AM905559–AM905596.

Laboratory procedures

Total genomic DNA was extracted by overnight incubation at 55 °C in lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl, 1% SDS) including 0.5 mg of proteinase K (Merck), and subsequent purification with a standard chloroform protocol. DNA was precipitated from the supernatant with 0.2 volumes of 4 M LiCl and 0.8 volumes of isopropanol, centrifuged, washed, dried and resuspended in TE buffer.

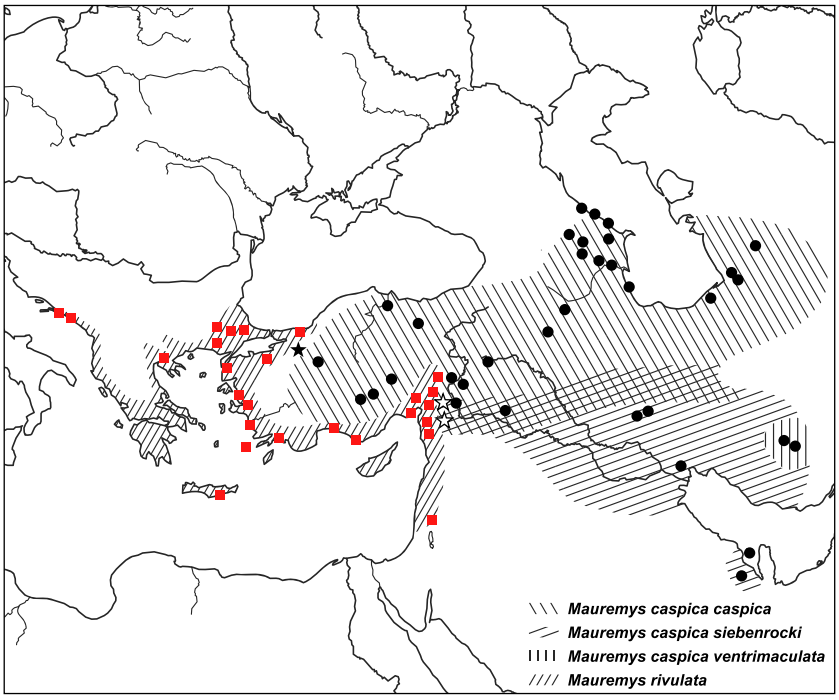

Fig. 1 Distribution of *Mauremys caspica* subspecies and *M. rivulata* according to Fritz & Wischuf (1997). Sampling sites indicated (*M. caspica*, black circles; *M. rivulata*, red squares; extinct hybrid populations, white stars; newly discovered putative hybrid population, black star); adjacent sites combined. For exact localities, see Appendix S1. Cross-hatching, intergradation zone of *M. caspica caspica* and *M. c. siebenrocki*.

Two fragments (overlapping by ~300 bp), together comprising almost the complete cyt *b* gene and the adjacent portion of the tRNA-Thr gene, were amplified for 86 samples (Appendix S1) using the primers CytbG (Spinks *et al.*, 2004), mt-E-Rev2, mt-c-For2 and mt-f-na (Fritz *et al.*, 2006b). PCR was performed in a 50 μL volume (50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of *Taq* DNA polymerase (Bioron), 10 pmol dNTPs (Eppendorf or Fermentas) and 10 pmol of each primer. After initial denaturing for 5 min at 95 °C, 35–40 cycles were performed with denaturing 1 min at 95 °C, annealing 1 min at 55–58 °C and primer extension for 2 min at 72 °C, followed by a final elongation of 10 min at 72 °C. PCR products were purified by precipitation under the following conditions: 1 volume PCR product (30 μL), 1 volume 4 M NH_4Ac (30 μL) and 12 volumes EtOH (100%; 360 μL). DNA was pelleted by centrifugation and the pellet washed with 70% ethanol. The pellet was dissolved in 20 μL H_2O. PCR products were sequenced with the primers mt-c-For2 and mt-E-Rev2 (Fritz *et al.*, 2006b) on an ABI 3130 sequencer (Applied Biosystems). Because no internal stop codons were found and nucleotide frequencies corresponded to those known for coding mtDNA, we conclude that we amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

An ~600 bp long fragment of the C-mos gene of 72 stripe-necked terrapins (Appendix S1) was amplified and sequenced in the same procedure, but using the primers Cmos1 and Cmos3 (Le *et al.*, 2006) and the following PCR programme: initial denaturing at 95 °C for 5 min and 40 cycles of denaturing 30 s at 95 °C, annealing 45 s at 58 °C and primer extension for 1 min at 72 °C, followed by a final elongation for 10 min at 72 °C.

Estimation of gametic phase of C-mos

Twenty-two of the studied 72 diploid individuals were found to be heterozygous at one to four sites in the 577 bp long alignment. For individuals with more than one heterozygous position gametic phase cannot be identified from the sequences directly; gametic haplotypes (alleles) have to be determined either through cloning of the alleles from PCR products or through statistical inference maximizing the likelihood of genotype frequencies. Identification of sequences employing a cloning approach is not only time-consuming and expensive, but also has been shown to be unreliable due to frequent recombination events during the cloning procedure (Yu *et al.*, 2006). Thus, we reconstructed gametic haplotypes and individual genotypes using the expectation-maximization (EM) algorithm as implemented in ARLEQUIN 3.1 (Excoffier *et al.*, 2005). The EM algorithm has been shown to perform well under a wide range of population and dataset scenarios (Fallin & Schork, 2000; Tishkoff *et al.*, 2000; Zhang *et al.*, 2001) and is robust to violations of the underlying assumptions of Hardy–Weinberg equilibrium (Niu *et al.*, 2002; Xu *et al.*, 2002). The zipper version of the EM algorithm was started at 50 random starting points with a maximum of 5000 iterations, an epsilon value of $1e^{-7}$ and 50 random loci (polymorphic positions) orders. Of the 12 individuals with more than one heterozygous position, three

belonged to *M. caspica*, eight to *M. rivulata* and one was morphologically identified as hybrid between both taxa. No differences were found between reconstructions of haplotypes based on the total dataset including all 72 individuals and independent estimates within the two taxa. Since Hardy–Weinberg gene frequencies might be different between the two *Mauremys* taxa included in the study, gametic haplotypes were first inferred within the two taxa independently. A comparison to a reconstruction including all 72 individuals, however, produced the same haplo- and genotypes.

Phylogenetic and population genealogy analyses

Cyt *b* sequences of the two subspecies of the third western Palaearctic *Mauremys* species, *M. leprosa*, were downloaded from GenBank and included as outgroups to the alignment (*M. l. leprosa*: AJ877039; *M. l. saharica*: AJ877035) and five previously published cyt *b* sequences of *M. caspica* (AJ564453–AJ564454, AM110186) and *M. rivulata* (AJ564455, AM110185; Barth *et al.*, 2004; Fritz *et al.*, 2006a) were added. The resulting 91 sequences (*M. caspica*, 49; *M. rivulata*, 37; putative *caspica* × *rivulata* hybrids, 5) were collapsed into haplotypes and identical sequences removed in phylogenetic analyses. Of 1040 aligned sites, 924 were constant, 13 variable characters were parsimony-uninformative and 103 variable characters were parsimony-informative. For the ingroup taxa, 969 sites were constant, nine were variable but parsimony-uninformative and 62 parsimony-informative. Likewise, the 144 inferred C-mos alleles (*M. caspica*, 66; *M. rivulata*, 68; putative *caspica* × *rivulata* hybrids, 10) were collapsed into an alignment of unique haplotypes (577 bp) that included 20 variable positions, of which 11 were parsimony-informative (Appendix S1; see also 'Results').

Cyt *b* data were analysed using the neighbour-joining (NJ) cluster algorithm and with the optimality criteria maximum parsimony (MP; equal weighting) and maximum likelihood (ML) as implemented in PAUP* 4.0b10 (Swofford, 2002; setting swap = TBR) as well as Bayesian inference of phylogeny as implemented in MRBAYES 3.1 (Ronquist & Huelsenbeck, 2003). Bayesian analysis (BA) was performed using two simultaneous runs of four chains of 10 000 000 generations sampling every 500 generations. The trees of the first generations were discarded as burn-in so that only the plateau of the most likely trees was used. The best evolutionary model for the data (ML calculation and ML distances) was selected by the Akaike information criterion using MODELTEST 3.06 (best-fit model: K81uf+I; Posada & Crandall, 1998). Under ML, the starting tree was obtained by stepwise addition. Bootstrap support was calculated with PAUP* 4.0b10 for MP (settings def hs add = cl and nreps = 10 000) and ML (nreps = 100).

Within the same species, sequence data cannot necessarily be represented by dichotomous trees because ancestral haplotypes may persist along with their descendents, and tokogenetic reticulate relationships are likely. Moreover, in organisms with sexual reproduction recombination is expected to produce reticulations in reconstructions of evolutionary relationships based on autosomal nuclear gene fragments at the population level. Thus reconstruction methods that enforce tree-like relationships might not adequately represent all the (conflicting) information on relationships present in the alignment. Therefore, we applied the median-joining (MJ) network reconstruction algorithm as implemented in NETWORK 4.2.0.1 (Bandelt *et al.*, 1999; http://www.fluxus-engineering.com) to our cyt *b* and C-mos data. This algorithm results in networks representing all equally parsimonious solutions if a sufficiently weighted genetic distance measure (epsilon) is applied. A sparse MJ network was also constructed using equal weights of characters and mutation types, as well as an epsilon value of zero. In addition, we used the Statistical Parsimony algorithm as implemented in TCS 1.21 (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. TCS calculates the outgroup weight for each haplotype and determines the ancestral haplotype according to coalescent theory.

Nuclear fingerprinting and analysis

To obtain a measure for possible gene flow between *M. caspica* and *M. rivulata*, we conducted nuclear genomic fingerprinting using ISSR PCR. This technique employs a single PCR primer that binds to di- or trinucleotide repeat motifs (microsatellites), which are abundant in eukaryotic genomes (Tautz & Renz, 1984; Condit & Hubbell, 1991). Since sequences of microsatellites are conserved over a wide range of organisms, universal primers can be used. Amplified regions correspond to the sequence between two simple sequence repeat (SSR) priming sites orientated on opposite DNA strands (Wolfe *et al.*, 1998). SSR regions appear to be scattered evenly throughout the genome (Tautz & Renz, 1984; Condit & Hubbell, 1991), resulting in a large number of polymorphic bands. The markers are inherited in a dominant or codominant Mendelian fashion but are scored as diallelic-dominant markers for data analysis (Wolfe *et al.*, 1998). The absence of a band is interpreted as primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement (Wolfe & Liston, 1998). ISSR is a powerful tool for identifying hybridization and gene flow, indicated by shared parental bands (e.g. Wolfe *et al.*, 1998; Wink *et al.*, 2001; Nagy *et al.*, 2003; Schilde *et al.*, 2004; Fritz *et al.*, 2005).

We used the nonanchored primer $(GACA)_4$ that yielded species-diagnostic banding patterns for other chelonian species in previous studies (e.g. Schilde *et al.*,

2004). PCR was performed with ~60 ng template DNA in a 30 μL volume [same PCR buffer as described above, 10 pmol primer, 10 pmol dNTPs (Fermentas) and 1 unit *Taq* polymerase (Bioron)]. Following the initial 5 min denaturing at 94 °C, the PCR program consisted of 35 cycles of 60 s at 94 °C, 120 s at the annealing temperature of 55 °C, 120 s at 72 °C and a final elongation step of 5 min at 72 °C. DNA fragments were separated by PAGE in a vertical apparatus (38 × 30 cm, Sequigene, Bio-Rad) for 1.5 h at 65 W. The denaturing gel [7 M urea, 6% acrylamide (Rotiphorese 40, 29 : 1, Roth) in 1× TBE buffer (10×: 1 M Tris, 0.83 M boric acid, 10 mM EDTA, pH 8.6)] had a thickness of 0.4 mm. After drying, the gel was scanned. Seventeen unambiguously scorable bands of 41 samples were transformed into a presence/absence matrix and analysed using the cluster algorithm NJ in PAUP* 4.0b10. Given that *M. caspica* and *M. rivulata* have diagnostic banding patterns, their ISSR profiles should be revealed as distinct clusters. Hybrids should share alleles of both species.

Results

Morphology

All stripe-necked terrapins studied during fieldwork, except the five putative hybrids from north-western Turkey (Gaziler village, Osmaneli district, Bilecik province), perfectly agreed with the species-diagnostic coloration and pattern characters outlined by Fritz & Wischuf (1997) and could be unambiguously assigned either to *M. caspica* or to *M. rivulata*, without any character overlap. Individuals of the three morphologically distinctive subspecies of *M. caspica* agreed with the descriptions of Fritz & Wischuf (1997). The putative *caspica* × *rivulata* hybrids resembled the eight hybrids described in Fritz & Wischuf (1997) in that species-diagnostic coloration and pattern characters of *M. caspica* and *M. rivulata* (see Fritz & Wischuf, 1997) occurred in combination in the same terrapin or characters were intermediate (Fig. 2).

Mitochondrial DNA

Ten haplotypes were found in *M. caspica* (Cmt1–Cmt10) and eight in *M. rivulata* (Rmt1–Rmt8); all five putative *caspica* × *rivulata* hybrids yielded the *caspica* haplotype Cmt9. Except in the putative hybrids, no shared mtDNA haplotypes between both species were found (Appendix S1). Most haplotypes of *M. caspica* occur allopatrically or parapatrically; only in a few localities was more than one haplotype found. In the southernmost part of the species' range (Bahrain) four haplotypes occurred together (Cmt1, Cmt2, Cmt5, Cmt6) and in the province of Kermanshah, Iran, the haplotypes Cmt3 and Cmt8 occurred in close proximity. In contrast, in many sites of *M. rivulata* more than one haplotype was present (Fig. 3;

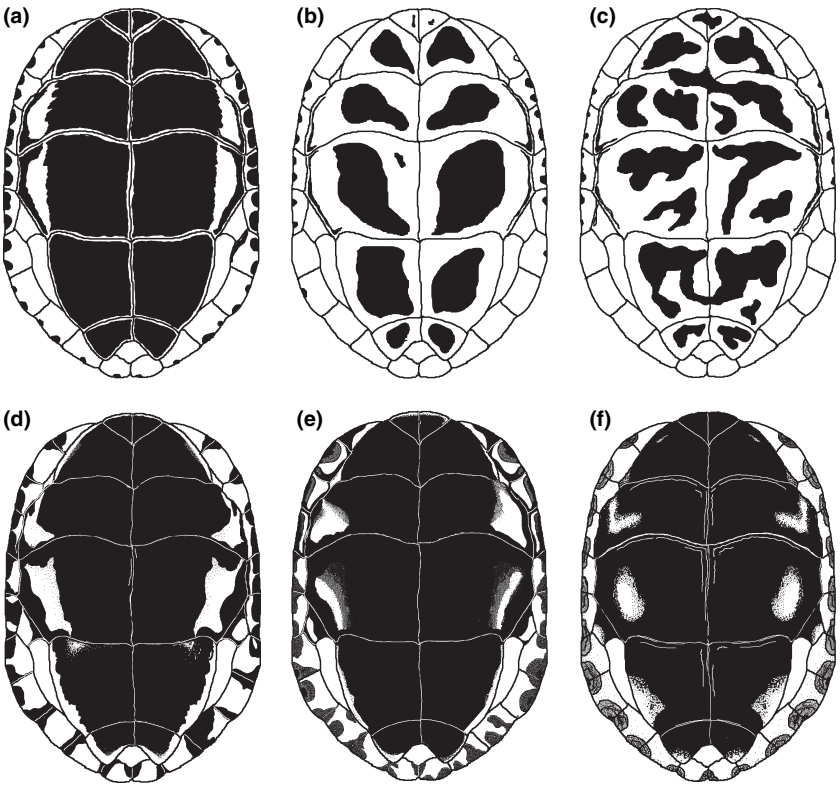

Fig. 2 Plastral pattern of (a) *Mauremys caspica caspica*, (b) *M. c. siebenrocki*, (c) *M. c. ventrimaculata*, (d) and (e) putative *caspica* × *rivulata* hybrids (Gaziler village, Osmaneli district, Bilecik province, Turkey), (f) *M. rivulata*. All specimens adult. Note that dark pigment on the bridge of *M. caspica* is confined to the scute seams whereas *M. rivulata* has a completely dark bridge. The adjacent submarginal scutes bear two massive specks in *M. caspica*; in *M. rivulata* a large ocellus lies on the seam between two submarginals so that two half ocelli are located on each scute.

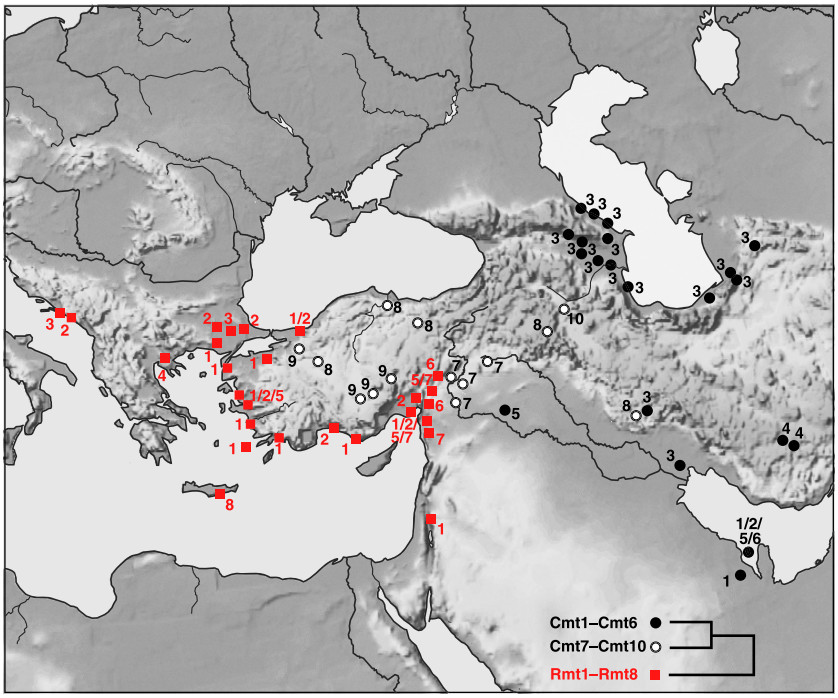

Fig. 3 Distribution of mtDNA haplotypes of *Mauremys caspica* (western clade, open circles; eastern clade, solid black circles) and *M. rivulata* (solid red squares). Numbers indicate individual haplotypes; numbers separated by slashes, syntopic occurrence of respective haplotypes. Note high diversity around the Persian Gulf.

Appendix S1). Uncorrected *p* distances and ML distances of haplotypes are summarized in Table 1.

All tree-building methods revealed an identical branching pattern; haplotypes of each species correspond to a distinct clade (Fig. 4). However, under BA and ML the monophyly of *M. caspica* haplotypes is unexpectedly weakly supported (posterior probability of 0.82, bootstrap support of 65%), whereas under parsimony and NJ high bootstrap support of 100% occurs. Monophyly of *M. rivulata* haplotypes is well-supported under all methods. Within *M. caspica*, two clades occur. One weakly supported clade (Cmt1–Cmt6) contains haplotypes from the eastern and southern parts of the range; the other clade (Cmt7–Cmt10), moderately to well-supported, comprises sequences of terrapins from Anatolia and adjacent westernmost Iran; both clades occur in the province of Kermanshah, western Iran (Cmt3 and Cmt8). Structure within the *M. rivulata* clade is weak; Rmt5 and Rmt6 are suggested with weak support as sister to the other haplotypes.

Networks of the two species are highly distinct. Using TCS, their parsimony networks are not connected if 90–95% thresholds are applied (Fig. 5). If a connection is enforced, haplotypes of the two species are separated by a minimum of 50 mutational steps (52 in the MJ network created by NETWORK 4.2.0.1). In *M. caspica* two distinct clusters occur, corresponding to the two clades in phylogenetic analyses. In parsimony (Fig. 5) and MJ network analyses (not shown), haplotypes of the two clusters are separated by at least 10 mutational steps.

Table 1 Uncorrected *p* distances and ML distances (percentages; means and ranges) for cyt *b* of *Mauremys leprosa* (outgroup sequences), *M. caspica* (haplotypes Cmt1–Cmt10) and *M. rivulata* (haplotypes Rmt1–Rmt8).

	M. leprosa	Cmt1–Cmt10	Cmt1–Cmt6	Cmt7–Cmt10	Rmt1–Rmt8
M. leprosa	**0.91 (–)/0.95 (–)**	14.55 (13.46–16.20)	14.49 (13.46–16.20)	14.65 (13.84–15.42)	21.61 (20.50–22.62)
Cmt1–Cmt10	7.34 (7.03–7.64)	**0.86 (0.10–1.49)/0.96 (0.10–1.88)**	n/a	n/a	8.42 (7.61–10.06)
Cmt1–Cmt6	7.28 (7.03–7.53)	n/a	**0.45 (0.10–0.79)/0.47 (0.10–0.80)**	1.43 (1.02–1.88)	8.54 (7.61–10.06)
Cmt7–Cmt10	7.44 (7.23–7.64)	n/a	1.29 (0.96–1.49)	**0.19 (0.10–0.29)/0.19 (0.10–0.29)**	8.25 (7.67–8.78)
Rmt1–Rmt8	7.53 (7.33–7.74)	5.08 (4.81–5.30)	5.05 (4.81–5.30)	5.12 (5.00–5.29)	**0.30 (0.10–0.58)/0.30 (0.10–0.60)**

Uncorrected *p* distances are given below, ML distances above the diagonal. The within-group divergence is given in bold on the diagonal (uncorrected *p* distances/ML distances). n/a, not applicable.

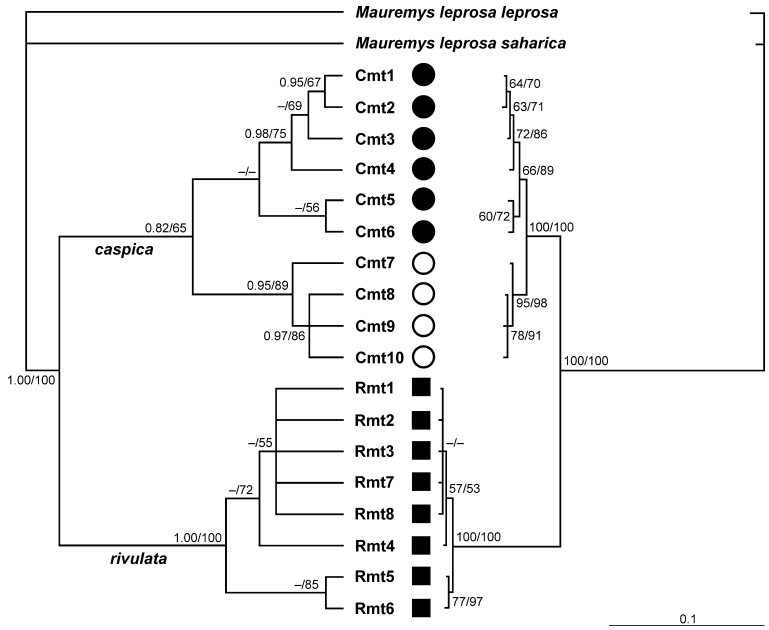

Fig. 4 Phylogenetic hypothesis for mtDNA haplotypes of *Mauremys caspica* and *M. rivulata*, rooted with *M. l. leprosa* and *M. l. saharica* as revealed under BA, ML, MP (single most parsimonious tree, CI = 0.9044, RI = 0.9714; tree length = 136) and NJ. Left, cladogram with BA posterior probabilities equal to or > 0.95 (except in one crucial node with lower value) and ML bootstrap values > 50. Right, BA phylogram with MP and NJ bootstrap values > 50. Dashes, support values < 0.95/50. Symbols for haplotypes correspond to Fig. 3 except that *M. rivulata* is represented by black squares. For haplotype codes, see Appendix S1.

Within haplotypes of the Cmt1–Cmt6 cluster, a maximum of nine steps occurs; within the Cmt7–Cmt10 cluster three steps, indicating a higher diversity in haplotypes from the southern and eastern parts of the range. Under coalescent theory, Cmt1 is the ancestral *caspica* haplotype (outgroup probability: 0.2876). Cmt1 was found in three terrapins from the south-easternmost part of the range (Bahrain: MTD T 571; Saudi Arabia: MTD T 3504–3505; Appendix S1).

In contrast, phylogeographic differentiation within *M. rivulata* is decidedly weak. The haplotypes show no obvious geographically correlated distribution. While haplotypes of *M. caspica* differ in 1–22 mutational steps, haplotypes of *M. rivulata* differ only in one to six steps. Six of the eight haplotypes of *M. rivulata* differ only in one nucleotide; the most differentiated haplotypes Rmt5 and Rmt6, distinct by three to six steps from other haplotypes, occur in south-eastern Turkey and the Turkish Aegean region (Appendix S1). The most frequent haplotype Rmt1 ($n = 17$), under coalescence ancestral to all other *rivulata* haplotypes (outgroup probability: 0.5079), has a wide geographic distribution from Greece to Jordan (Fig. 3).

Nuclear DNA

The reconstructions of the gametic C-mos haplotypes using the EM algorithm (ARLEQUIN 3.1) resulted in 24 haplotypes (13 in *M. caspica* and 11 in *M. rivulata*). None of the haplotypes is shared between both species with the exception of the putative *caspica* × *rivulata* hybrids. Most of the putative hybrids possessed *M. caspica* haplotypes, only in one case was a *M. rivulata* allele found in a hybrid individual (specimen MTD T 3334; Appendix S1).

In network analyses sequences of *M. caspica* were assigned to only 11 haplotypes (Cn1–Cn11) and sequences of *M. rivulata* to only nine haplotypes (Rn1–Rn9; Fig. 6; Appendix S1). This is due to the fact that some of the haplotypes inferred by ARLEQUIN differed only by missing data ('Ns'). These sequences were included in complete haplotypes. Network haplotype Cn1 represents three sequence types that differ only in the presence of Ns at otherwise invariable positions, assuming that sites with missing data do not represent unique mutations. Haplotypes Rn1 and Rn2 differ only in position 35. Arbitrarily included in haplotype Rn1 were five alleles with an N at this position (extracted from specimens MTD T 247, T 279 and T 362) that could also belong to haplotype Rn2 or yet another undiscovered haplotype, different in position 35. Similarly, three alleles from specimens MTD T 275 and T 276 were assigned to haplotype Rn6, although these sequences might in fact be haplotype Rn7.

Under both network algorithms, the 11 *M. caspica* haplotypes differ in one to six steps using the most likely and direct pathways (for haplotype frequencies, homozygous and heterozygous individuals, see Appendix S1). The rare haplotypes Cn4–Cn11 are confined to the Caucasus region, Iran, the Euphrates–Tigris region (Syria, Turkey) and Bahrain. In Central Anatolia only the most common and widely distributed haplotype Cn1 was found (Fig. 7).

The nine *M. rivulata* haplotypes differ by one to nine mutation steps. These haplotypes group into two distinct clusters, being separated by the ancestral and interiorly

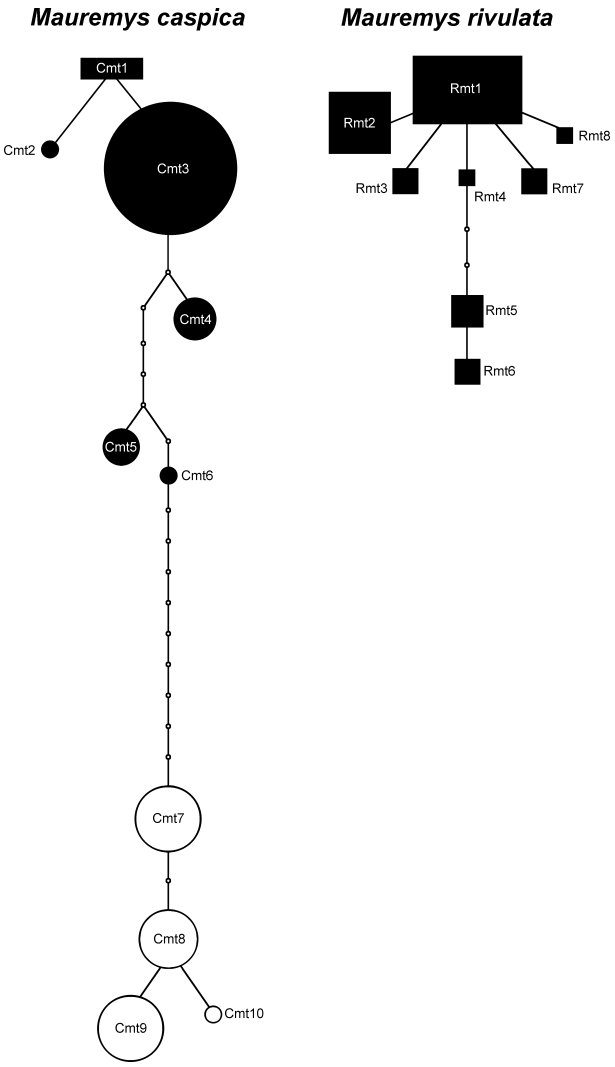

Fig. 5 Parsimony networks (TCS 1.21) of mtDNA haplotypes (cyt *b*) of *Mauremys caspica* and *M. rivulata*. Topology obtained with the MJ algorithm (NETWORK 4.2.0.1) is identical; subnets are connected then via 52 mutation steps. Haplotypes with biggest outgroup probability shown as rectangles on the top; other haplotype symbols correspond to Figs 3 and 4. Symbol size, approximate haplotype frequency; missing haplotypes, small circles. Each line between symbols and haplotypes indicates one mutation step. For haplotype frequencies and occurrence of haplotypes, see Appendix S1. Network of *M. caspica* includes haplotypes of putative *caspica* × *rivulata* hybrids (Cmt9; $n = 5$).

located *caspica* haplotype Cn1 (TCS outgroup probability: 0.1236). Both *rivulata* clusters correspond nearly perfectly to distinct geographic groupings. The five Jordanian *M. rivulata* (MTD T 275–279), from the southern range border, yielded with one exception the unique haplotypes Rn6–Rn9. In one heterozygous terrapin (sample MTD T 279) one frequent allele (Rn1 or Rn2), otherwise occurring in samples from the Balkans and Turkey (Appendix S1), was found together with Rn9. Except for the haplotypes of the Jordanian terrapins, there is no obvious geographical accumulation of rare haplotypes in *M. rivulata* (Fig. 7).

Four of the five putative *caspica* × *rivulata* hybrids (MTD T 3331–3333, 3335) were homozygous for the most frequent *caspica* allele Cn1 and one heterozygous terrapin (MTD T 3334) had, besides Cn1, the common *rivulata* allele Rn4.

ISSR fingerprinting

Thirteen of the 17 unambiguously scorable bands were polymorphic, two of which were unique to *M. caspica* (B, K) and seven to *M. rivulata* (A, D, F, G, H, N, Q; Appendix S2); the fragment G occurred only in one *M. rivulata* from Jordan (MTD T 276). Among the five putative *caspica* × *rivulata* hybrids were the only two individuals (MTD T 3333, 3334) that shared diagnostic amplicons of both species. NJ cluster analysis (Fig. 8) shows *M. caspica* and *M. rivulata* to be clearly distinct; the putative *caspica* × *rivulata* hybrids cluster either with *M. caspica* (MTD T 3333–3335) or with *M. rivulata* (MTD T 3331, 3332).

Discussion

Discordance of datasets

A striking finding of our investigation was the discordance of morphology, mitochondrial and nuclear genomic markers. Phylogenetic analyses for the mitochondrial cyt *b* gene resulted in clearly distinct monophyletic trees for *M. caspica* and *M. rivulata*, but in a paraphyletic topology for the nuclear C-mos gene of both species (Figs 4–6). Omland *et al.* (2006) offered a model of successive temporal lineage sorting, leading from originally completely intermixed haplotypes of recently separated populations over stages of intermediate polyphyly to reciprocally monophyletic units. If this model is used for the C-mos tree of *M. caspica* and *M. rivulata*, the species have reached the allotypic stage, sharing no haplotypes. The reciprocal monophyly of their mitochondrial gene trees is likely to reflect the smaller effective population size, strictly maternal inheritance and absence of interindividual recombination of the mitochondrial genome (e.g. Ballard & Whitlock, 2004; Omland *et al.*, 2006), with the result that complete sorting is more rapidly achieved.

Whereas species-diagnostic coloration and pattern characters perfectly match species-specific genetic markers of *M. caspica* and *M. rivulata*, the disagreement of genetic markers and morphology is obvious for the subspecies of *M. caspica*. None of the subspecies corresponds to a distinct genetic lineage. Fritz & Wischuf (1997) recognized three subspecies, the northern *M. caspica caspica* and two southern subspecies,

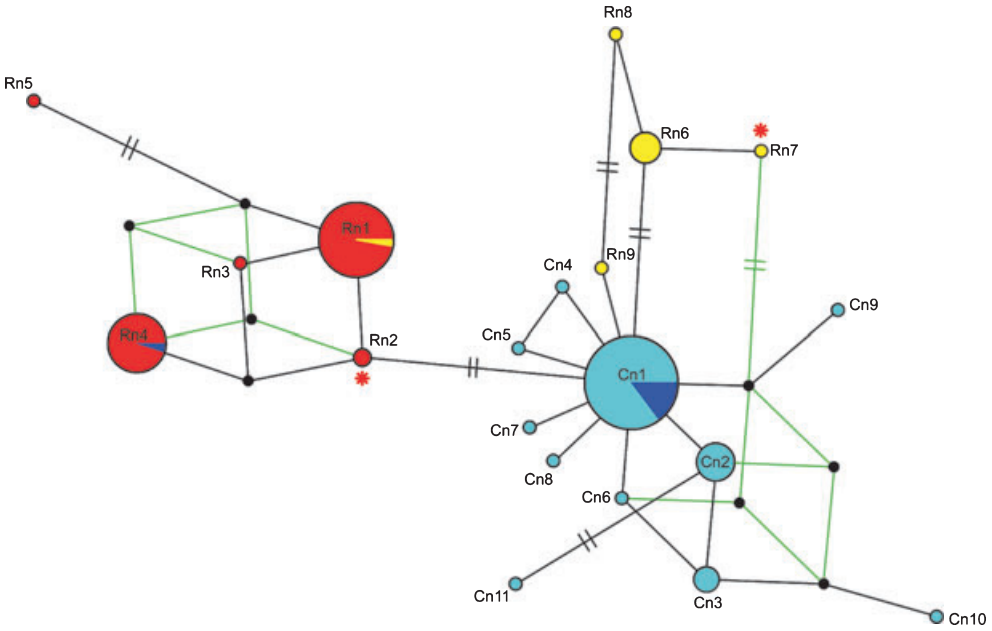

Fig. 6 Median-joining network (NETWORK 4.2.0.1) of C-mos haplotypes of *Mauremys caspica* and *M. rivulata*, representing all equally parsimonious solutions. Pathways of sparse network black; pathways occurring only in full network green. The parsimony network obtained with TCS 1.21 matches the full MJ network. Symbol size, approximate haplotype frequency. Each line joining haplotypes indicates one nucleotide substitution except when hatches across lines are present; then each hatch indicates one step. *Mauremys caspica*, light blue; *M. rivulata* from Jordan, yellow; *M. rivulata* from other parts of the range, red; putative *caspica* × *rivulata* hybrids, dark blue; missing node haplotypes, black. Stars indicate possible allocation of five alleles (MTD T 247, 279, 362) to Rn2 instead of Rn1 and of three alleles (MTD T 275, 276) to Rn7 instead of Rn6. See also text and Appendix S1.

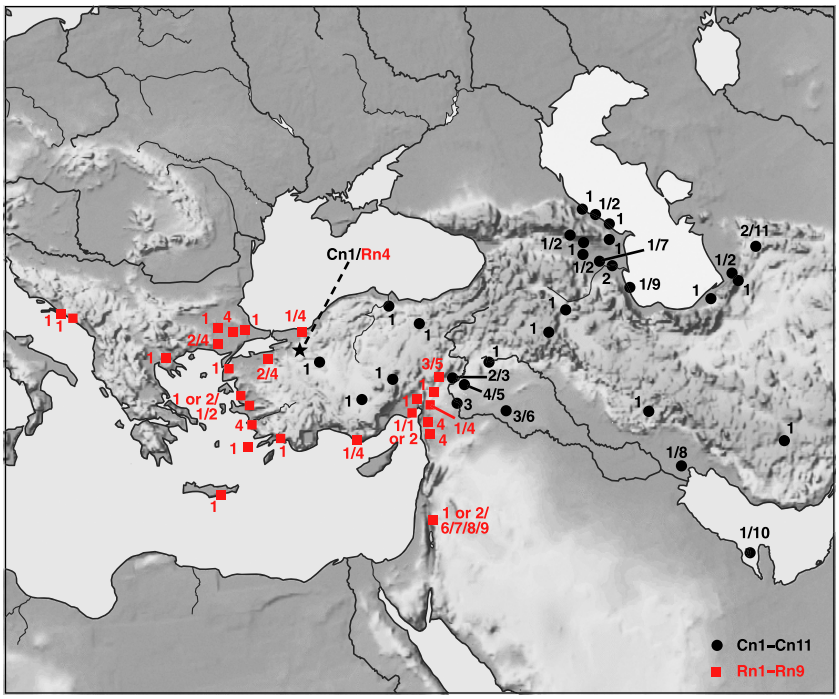

Fig. 7 Distribution of C-mos haplotypes of *Mauremys caspica* (black circles) and *M. rivulata* (red squares). Numbers indicate individual haplotypes; numbers separated by slashes, syntopic occurrence of respective haplotypes. Hybrid population sharing alleles of both species (star). Note that rare haplotypes of *M. caspica* do not occur in the westernmost part of the range (Central Anatolia); haplotypes Rn6–Rn9 of *M. rivulata* are restricted to the southernmost part of the range (Jordan).

M. c. siebenrocki and *M. c. ventrimaculata*, differing mainly in coloration and pattern characters (Fig. 2). The two southern subspecies are distinctly lighter coloured than the northern *M. c. caspica*; their plastra having smaller dark blotches. Our study revealed two mtDNA clades within *M. caspica*, one distributed in the west and the

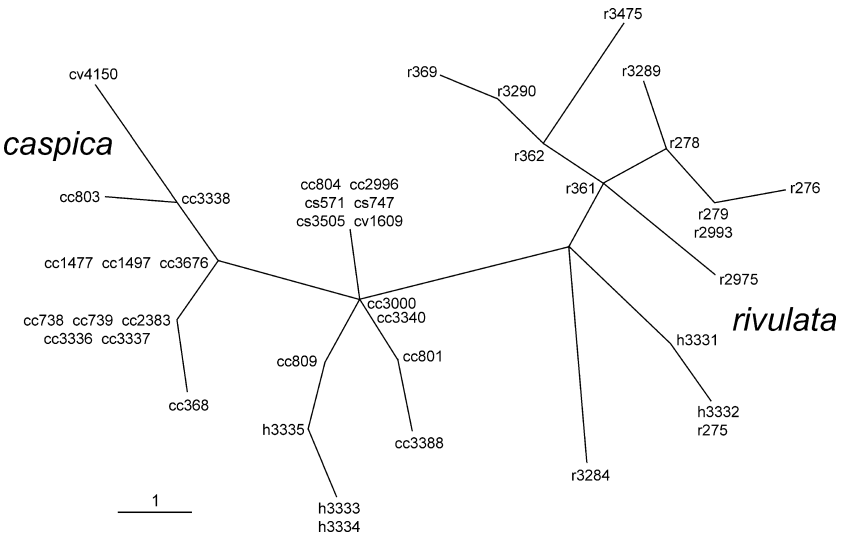

Fig. 8 Neighbour-joining tree based on presence/absence of 17 ISSR amplicons in *Mauremys caspica* and *M. rivulata*. Branch length, pairwise distances; for explanation of sample numbers see Appendix S2. Letters preceding sample numbers: cc, *Mauremys caspica caspica*; cs, *M. c. siebenrocki*; cv, *M. c. ventrimaculata*; h, putative *caspica* × *rivulata* hybrids; r, *M. rivulata*.

other in the east and south of the range, but their distribution does not agree with that of any subspecies (Figs 1 and 3). The eastern and southern clade comprises the light-coloured populations of *M. c. siebenrocki* and *M. c. ventrimaculata* as well as dark-coloured populations of *M. c. caspica* from the Caucasus region and the Middle East; the western clade corresponds to dark-coloured populations of *M. c. caspica* from Anatolia and adjacent eastern regions. Our four samples of *M. c. ventrimaculata*, endemic in the endorheic Maharloo basin of Iran, harboured the unique mtDNA haplotype Cmt4, embedded in the 'southern and eastern clade' (Fig. 4).

Phylogeography

The restriction of rare C-mos haplotypes of *M. caspica* to the Near and Middle East as well as the endemic C-mos alleles Rn6–Rn9 of *M. rivulata* in Jordan (Fig. 7) suggest that the two species diverged in western Asia and that Europe and Central Anatolia, regions with low nDNA diversity, were colonized from a south-eastern or eastern radiation centre. This is in accord with the fossil record and mtDNA differentiation (see below). *Mauremys* are known from the Medial to Upper Miocene of the Transcaucasus (Chkhikvadze, 1983, 1989; Danilov, 2005) and the United Arab Emirates (de Lapparent de Broin & van Dijk, 1999), while the oldest records for the Balkan Peninsula and Crete date only to the Pleistocene (Simonelli Cave, Crete: Kotsakis, 1977; Megalopolis, Peloponnese: Melentis, 1966; Bachmayer & Symeonidis, 1970), implying recent arrival of *M. rivulata* there. This could explain its weak mtDNA differentiation revealed in this study as well as in a previous investigation focusing on *M. rivulata* from Greece (Mantziou *et al.*, 2004). Compared with the complex phylogeographic differentiation of another co-distributed terrapin, *Emys orbicularis*, in the Balkans and western Asia (Fritz *et al.*, 2007b), comprising several highly distinct lineages, the uniformity of *M. rivulata* mtDNA haplotypes is striking and supportive of rapid dispersal from a west Asian centre. Three of the eight haplotypes of *M. rivulata* are confined to western Asia (Rmt5–Rmt7), including the most distinct ones; two haplotypes are shared between Asia and Europe (Rmt1–Rmt2), and the three haplotypes known only from Europe (Rmt3–Rmt4, Rmt8) differ only in one mutation step from the most frequent and widely distributed haplotype Rmt1 (Figs 3 and 5).

With two distinct clades, phylogeographic differentiation of the mitochondrial genome of *M. caspica* is distinctly more pronounced than that of *M. rivulata*. In the southernmost part of the range of *M. caspica*, around the Persian Gulf, occur all six mtDNA haplotypes of the 'eastern and southern clade' (Cmt1–Cmt6). According to coalescent theory, Cmt1 is ancestral to all other haplotypes of *M. caspica* (Fig. 5). In contrast, generally only one haplotype per locality is found in more northerly regions. Likewise, in *M. rivulata* only in the southernmost part of the range do the distinct nuclear alleles Rn6–Rn9 occur together with the widespread Rn1 or Rn2. Both cases resemble the well-known pattern of southern genetic richness and northern purity of many western Palaearctic taxa. In Europe, this pattern is thought to reflect rapid Holocene dispersal from glacial refuges (e.g. Hewitt, 1996, 2000; Taberlet *et al.*, 1998). We suppose that in west Asia this is the result of older dispersal and vicariance events, because the Caucasus region as well as the south coast of the Caspian Sea are well-known glacial refuges and biodiversity hotspots (e.g. de Lattin, 1967; Tuniyev, 1990, 1995; Myers *et al.*, 2000), and it seems highly unlikely that stripe-necked terrapins invaded these regions only in the Holocene. Evidence for the long-lasting existence of terrapins is also provided by the fossil record (Chkhikvadze, 1983, 1989).

Table 2 Morphological and molecular characters in *Mauremys caspica* × *rivulata* hybrids from Bilecik province, Turkey.

Specimen	Morphology	mtDNA (cyt *b*)	nDNA (C-mos)	Diagnostic ISSR amplicons	ISSR cluster analysis
MTD T 3331	Intermediate	*Caspica* Cmt9	*Caspica* Cn1	*Rivulata*	*Rivulata*
MTD T 3332	Intermediate	*Caspica* Cmt9	*Caspica* Cn1	*Rivulata*	*Rivulata*
MTD T 3333	Intermediate	*Caspica* Cmt9	*Caspica* Cn1	*Caspica* + *rivulata*	*Caspica*
MTD T 3334	Intermediate	*Caspica* Cmt9	*Caspica* + *rivulata* Cn1 + Rn4	*Caspica* + *rivulata*	*Caspica*
MTD T 3335	Intermediate	*Caspica* Cmt9	*Caspica* Cn1	*Caspica*	*Caspica*

The phylogenetically differentiated clade of haplotypes Cmt7–Cmt10, nearly entirely confined to Anatolia, suggests that a distinct refuge existed there. This is unexpected considering the high altitude of this region (~1000 m above the sea level in the Anatolian Plateau), surely with severe climatic conditions during Pleistocene glacials. The uniformity of nDNA alleles in Central Anatolia (Fig. 7) could reflect that these populations underwent a glacial bottleneck.

The co-occurrence of both mtDNA clades of *M. caspica* in the upper and lower Euphrates and Tigris catchment basins (Fig. 3) calls for further research. This pattern could be the result of distinct Holocene range expansions, downstream from the western refuge in Anatolia and upstream from the south-eastern refuge around the Persian Gulf, leading to admixture of both dispersal waves along the courses of the Euphrates and Tigris rivers.

Hybridization

Nuclear genomic markers confirmed that the terrapins from Bilecik province, Turkey exhibiting morphological characters of *M. caspica* and *M. rivulata* represent a hybrid population (Table 2). In most of southern Turkey the contact zone of both terrapin species coincides with the Taurus Mts, forming an insurmountable barrier. In many parts of western Turkey the ranges of the two species are separated by small distribution gaps. The two extinct hybrid populations in south-eastern Turkey and adjacent Syria (Fritz & Wischuf, 1997) inhabited an area where river courses and valleys allowed direct contact (Fig. 1). Likewise, the newly discovered hybrid population in north-western Turkey occurs in a region where the west-east direction of mountain chains allows contact of inland (*M. caspica*) and coastal populations (*M. rivulata*). The lack of any morphological or genetic evidence for wide-ranging gene flow suggests that the observed hybridization is an exceptional and localized phenomenon, resulting from secondary contact.

Conclusions

Speciation of *M. caspica* and *M. rivulata* fits the well-known allopatric speciation model (e.g. Mayr, 1942, 1963; Coyne & Orr, 2004), representing a textbook example of the parapatric phase of speciation (e.g. Butlin, 2005). Genetic markers and the fossil record suggest for both species a western Asian origin. The complex geodynamic and climatic changes in the Neogene (Rögl, 1998; Popov *et al.*, 2004; Harzhauser *et al.*, 2007) are likely to have caused geographic segregation of ancestral populations of both species in this region. Today, the adaptation of *M. rivulata* to a warm Mediterranean climate and of *M. caspica* to a winter-cold continental climate is probably playing the key role in spatial segregation of the two species (Fritz & Wischuf, 1997).

Clear phylogeographic differentiation within *M. caspica* reflects earlier stages of speciation and provides evidence for three distinct refuges in the Near and Middle East (Central Anatolia, south coast of Caspian Sea, Gulf of Persia). The distribution of the southern and eastern clade of *M. caspica* (haplotypes Cmt1–Cmt6) along the Persian Gulf and the south coast of the Caspian Sea reveals that the Zagros Mts do not constitute an important biographic barrier, in sharp contrast to the Taurus Mts in southern Turkey that separate *M. caspica* and *M. rivulata* (Fig. 1). Also in two other western Palaearctic terrapin species (*E. orbicularis*, Fritz *et al.*, 2007b; *M. leprosa*, Fritz *et al.*, 2006a) mountain chains are the most important phylogeographic divides, underlining that further research is needed for understanding the striking distribution of the southern and eastern clade within *M. caspica*.

The discordance of morphologically defined subspecies of *M. caspica* and mitochondrial differentiation implies that the dark coloration of northern populations does not correspond to subspecific differentiation. Also in another western Palaearctic terrapin species, *E. orbicularis*, northern populations comprise only dark-coloured individuals, whereas individuals from southern populations are in part distinctly lighter coloured (Fritz, 2001; Fritz *et al.*, 2006c), suggestive of dark terrapins being favoured by selection in the north. Geographically correlated genetic differentiation does exist, as reflected by the two distinct mtDNA clades within *M. caspica*. From a taxonomic point of view, an option could therefore be to redefine the currently recognized subspecies. The type localities of all available names of *M. caspica* (Fritz & Havaš, 2007) lie within the range of the eastern and southern mtDNA clade however, so that a comprehensive taxonomic revision is needed because no name is available for the mainly Anatolian clade.

Hybridization between *M. caspica* and *M. rivulata* is extremely rare. Apart from three hybrid populations (Fritz & Wischuf, 1997; this study), there is no evidence for gene flow. This was unexpected considering the frequent cases of hybridization and introgression of mitochondrial genomes in other, in part even distantly related, chelonian species (Guicking *et al.*, 2002; Lara-Ruiz *et al.*, 2006; Farias *et al.*, 2007; Fong *et al.*, 2007; Praschag *et al.*, 2007; Spinks & Shaffer, 2007; see also reviews in Schilde *et al.*, 2004; Buskirk *et al.*, 2005 and Bowen & Karl, 2007; Stuart & Parham, 2007) and suggests that in chelonians effective intrinsic isolation mechanisms exist, developed during speciation. The increasing frequency of hybridization in other, mainly tropical chelonians in recent years could therefore reflect the breakdown of ecological isolation due to large-scale human disturbance.

Acknowledgments

Daniel Frynta, Mario Herz, Pavel Široký and Paul Vercammen provided samples or assisted during collection of samples. Thanks go to Massimo Delfino for his help with the fossil record of *Mauremys* and to Anke Müller for laboratory work. Dinçer Ayaz's work was supported by the TÜBITAK grant TBAG-2402 (103T189).

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1 *Mauremys* samples and their mitochondrial and nuclear genomic haplotypes.

Appendix S2 ISSR marker bands of *Mauremys caspica* and *M. rivulata* using the primer $(GACA)_4$.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1420-9101.2007.01485.x

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Received 7 September 2007; revised 15 November 2007; accepted 26 November 2007