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Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758)

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Abstract

The phylogeny and phylogeography of *Emys orbicularis* was inferred from mitochondrial nucleotide sequences of the cytochrome *b* gene analysed by DNA sequencing and RNA heteroduplex analysis. Within the family Emydidae the monotypic genus *Emys* is affiliated with the nearctic taxa *Emydoidea blandingii* and *Clemmys marmorata*. The analysis of 423 individuals of *E. orbicularis*, originating throughout its distribution range, revealed a remarkable intraspecific differentiation in 20 different haplotypes with distinct geographical ranges. Maximum parsimony analysis produced a star-like phylogeny with seven main lineages which may reflect separations in the late Pliocene. The haplotype distribution examined by partial Mantel tests and analysis of molecular variance revealed a substantial effect of glacial periods. This historical perspective suggests the existence of multiple glacial refugia and considerable Holocene range expansion which was modulated by climatic traits. Further support is gained for the occurrence of long-term parapatry in glacial refugia.

Keywords: cytochrome *b*, *Emys orbicularis*, mitochondrial DNA, parapatry, phylogeography, Pleistocene

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Introduction

The west palaearctic terrapin fauna consists of only four species and is depauperate as compared with East Asia or North America, where the families Bataguridae and Emydidae are relatively diverse. *Mauremys caspica, M. leprosa,* and *M. rivulata* are the sole European representatives of the batagurid family, whereas *Emys orbicularis* is a member of the otherwise New World Emydidae. Among terrapins, *E. orbicularis* inhabits one of the largest ranges which comprises parts of northern Africa, the Mediterranean and moderate climatic regions of Europe and the Middle East up to the Aral Sea. Despite its wide distribution *E. orbicularis* has been regarded as a monotypic species for decades. Although variation in coloration had been known for some time (Arnold & Burton 1978), an intraspecific subdivision in 13 subspecies was proposed only recently by Fritz and coworkers (as reviewed in Fritz (1998)). While the northern part of the range is inhabited by the nominate subspecies exclusively, most intraspecific taxa occur in the south: North Africa (one subspecies), Iberia (two), Sardinia (one), Corsica (one), southern France–western Italy (one), southern Italy (one formally undescribed), Adriatic and Aegean coast (one), remaining Asia Minor and Georgia (three plus one formally undescribed), Caspian region (two). This diversity encouraged us to resolve the molecular phylogeography of this species.

The impacts of the cold Pleistocene climate on the holarctic fauna and flora were substantial and many species became extinct or lost large parts of their former ranges. Thermophilic reptiles of Europe were susceptible to low temperatures and could only survive in small restricted and climatically favoured areas in the southern extremities of Europe and adjacent areas, as described by Reinig (1937) and De Lattin (1949). According to their views, the actual distribution patterns of many European organisms largely depend on the location of glacial refugia and the course of postglacial recolonization.

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Although molecular markers substantially contribute to the understanding of biogeographical processes, comprehensive studies on the European fauna are still limited (for animals: Wallis & Arntzen (1989), Taberlet & Bouvet (1994), Cooper *et al*. (1995), Santucci *et al*. (1998), as summarized in Taberlet *et al*. (1998)). Studies on reptiles, temperature sensitive and less mobile than other terrestrial organisms, are lacking up to now.

However, work has already been carried out on the molecular phylogeography and phylogeny of turtles of other parts of the world (Lamb *et al*. 1989; Avise *et al*. 1992; Bowen *et al*. 1992, 1994; Allard *et al*. 1994; Osentoski & Lamb 1995; Walker *et al*. 1995, 1997; Encalada *et al*. 1996). These studies demonstrated the merits of mitochondrial DNA (mtDNA) to analyse phylogeographical patterns in turtles, although evolution rates and divergences tend to be low compared with other vertebrates (Avise *et al*. 1992; Martin & Palumbi 1993).

A preliminary study (Lenk *et al*. 1998) based on 187 specimens has already identified a remarkable intraspecific structure in *E. orbicularis* of geographically localized haplotypes. In the following study we present a sample of 423 specimens covering a major part of the species' range. The comparatively large and dense sampling scale allows the use of comprehensive statistical analyses to provide a framework for a detailed analysis of the history of this species. The methods used were DNA sequencing and RNA heteroduplex analysis; the latter has been previously adapted to this approach (Lenk & Wink 1997).

Materials and methods

Sampling and laboratory procedures

Samples were obtained from 423 specimens of *Emys orbicularis* and six related nearctic terrapin species (genera *Clemmys*, *Emydoidea*, and *Terrapene*) belonging to the emydine subfamily (Gaffney & Meylan 1988). Blood or muscle tissues (from ethanol-preserved animals) were taken and stored as described in Haskell & Pokras (1994) and Arctander (1988). Total genomic DNA was extracted following standard proteinase K and phenol–chloroform protocols (Sambrook *et al*. 1989).

Polymerase chain reaction methodology (PCR) was used to amplify a fragment containing the target sequence (1036 nt of the cytochrome *b* gene and 38 nt of the $tRNA_{Thr}$) following a procedure described previously (Lenk *et al*. 1998). The primers used were mt-A (Lenk & Wink 1997) in combination with CR12H (Lenk & Wink 1997) or H-15909 (5'-AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG-3').

Prior to DNA sequencing the PCR products were screened in order to sort out identical sequences that could be assigned to particular haplotypes. RNA heteroduplex analysis was used with the aid of the Mismatch Detect II Kit (Ambion 1418) as described in Lenk & Wink (1997).

The PCR products of at least one specimen per locality were sequenced as described in Lenk *et al*. (1998) or using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science, RPN 2438/RPN 2538) in combination with an automated sequencer (Pharmacia, ALF-Express). The sequencing primers were mt-A, mt-B, mt-C, mt-D (Wink 1995), L-14943 (Lenk *et al*. 1998), L-15601 (5'-CCATTCTACGCTCAATCCC-3'), and H-15909. All sequences were read by eye and aligned manually. The nucleotide sequence data reported here will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the Accession nos AJ131407–AJ131432.

Phylogenetic and statistical analysis

The program package MEGA (Kumar *et al*. 1993) was used to estimate genetic distances and to calculate sequence statistics. Maximum parsimony and maximum likelihood searches were conducted with the heuristic search approach of PAUP* 4.0 (Swofford 1998) using the 'tree-bisection–reconnection' swapping algorithm. For maximum parsimony the default settings were applied. The assumptions of the maximum likelihood procedure were specified to allow for six substitution types (Lanave *et al*. 1994) and a gamma distributed among-site rate variation (Yang 1994) with four categories (shape parameters to be estimated for the data set). All calculations were run with changing outgroup compositions: all six nearctic species together, separately, or without outgroup. Bootstrap analyses (500 replicates) were conducted to examine the robustness of tree bifurcations with the maximum parsimony algorithm.

To get an idea about phylogeographical trends which might have driven the postglacial range expansion, the actual distribution pattern was analysed by employing partial Mantel tests (Mantel 1967; Thorpe 1991). Partial Mantel tests evaluate the association between the observed phylogeographical structure with patterns predicted by different hypotheses, while simultaneously excluding the confounding effects of intercorrelation between hypotheses (Thorpe *et al*. 1994). First, genetic identities of localities were scored for input into distance matrix format (based on the patristic distances of an unrooted parsimony tree). Localities of less than 50 km geographical distance were pooled. Various putative range patterns served as test hypotheses representing final states of a colonization process. These reconstructions were made by allocating each lineage to a specific refugium, defining most probable pathways of colonization to avoid physical barriers (Fig. 1) and employing alternative scenarios to control

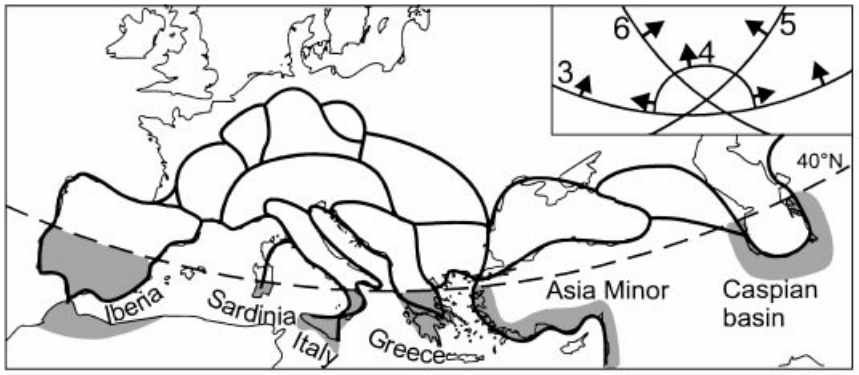

Fig. 1 A scheme to reconstruct the post-glacial colonization process as used for partial Mantel tests. It shows the approximate locations of glacial refugia (grey) and the putative colonization pathways (bold lines) which were defined by considering the physiography of the species' range. The upper-right box displays temporary limits to control the expansion fronts along pathways according to scenarios 3–6 (see text). The mode of shifting is indicated by arrows.

colonization reconstructions. The scenarios were: (1) constant expansion rate across all lineages and pathways; (2) like (1), but contemporary northern lineages assumed to be categorically superior in the northern part of the range; temporary northern border controlled by a factor shifting parallel to the geographical latitude (3); radially (4); with 45° inclination (5); or −45° inclination (6) to the geographical latitude (Fig. 1).

The position on a pathway where two fronts meet marks the distribution boundary between adjacent test groups. The theoretical identities of all localities were resampled according to these hypotheses and alternative distance matrices were calculated as described above. Associations between these and the dependent matrix were examined with partial Mantel tests with 10 000 permutations using a program developed by R. Thorpe. A sequential Bonferroni (Rice 1989) procedure was applied to all *P*-values, to adjust for the number of simultaneous tests.

Population genetic structure was inferred by analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) provided within the program package ARLEQUIN 1.0 (Schneider *et al*. 1997) using both haplotype statistics (based on haplotype frequencies only) and sequence statistics (which incorporate sequence divergence between haplotypes). The program calculates *F*-statistic equivalents and variance components on both modes. Spatial patterns found to be significant in the partial Mantel tests were used to define test groups. Variance components and Φ-values among geographical test groups, among populations within geographical test groups, and within populations were tested with 10 000 replicates.

Results

Nucleotide variation and genetic distances

Twenty haplotypes were observed, based on cytochrome *b* and $tRNA_{Thr}$ sequences among the 423 specimens of *Emys orbicularis*. Of 1074 aligned sites, 50 were variable with 47 transitions and three transversions; 46 sites were parsimony informative. Sequence divergence (Tamura & Nei 1993) among haplotypes ranged from 0.09% to 1.71% (Table 1).

When the six nearctic terrapin species were included, 242 sites were variable including 183 transitions, 37 transversions, and 22 positions with transitions plus transversions; 132 sites were parsimony informative. Genetic distance estimates (Table 1) ranged between 5.79% (*Clemmys muhlenbergi* and *C. insculpta*) and 11.45% (*C. guttata* and *E. orbicularis*).

Light strands had the following nucleotide compositions: A, 30.4–31.6%; C, 29.9–31.7%; G, 11.7–12.5%; and T, 25.3–26.9%. The strong bias against guanine is characteristic for mitochondrial but not for the nuclear genes (e.g. Desjardins & Morais 1990). Deletions, insertions or inversions were not encountered.

Phylogenetic relationships

Maximum parsimony and maximum likelihood analyses produced largely concordant trees. Within the *Emys* clade seven major lineages, denoted with Roman numerals, were unequivocally detected by both methods. Each lineage included one to several closely related haplotypes, whose monophyly was supported by high bootstrap values. However, only weak cladogenetic resolution was obtained among these lineages as indicated by short internal branches or low bootstrap support, respectively (Fig. 2). The only exception was the clade comprising lineages I and II which was defined as a monophyletic group. Although less supported by bootstrap analysis lineage III tended to take up a basal position within *E. orbicularis* in all reconstructions. If exclusively *Emys* haplotypes were subjected to maximum parsimony analysis, a single most parsimonious unrooted tree of 55 steps in length (Fig. 3) was produced. It showed a star-like phylogeny with six long branches. Significant secondary bifurcations appeared

Table 1 Genetic distance table of *Emys orbicularis* lineages and six related species. For *E. orbicularis* the haplotypes which appeared ancestral to the respective lineage according to the parsimony tree were selected. Genetic divergence estimates (Tamura & Nei 1993) are presented in the lower-left part, the absolute number of substitutions and number of transitions (in parentheses) in the upper-right part. The diagonal presents maximum within-lineage divergence estimates (Tamura & Nei 1993)

Haplotype/taxon	1	2	3	4	5	6	7	8	9	10	11	12	13
1 Ia	**0.0037**	6 (5)	16 (14)	15 (14)	12 (11)	13 (12)	13 (12)	104 (79)	91 (75)	107 (85)	64 (51)	87 (67)	76 (64)
2 IIa	0.0056	**0.0019**	18 (17)	17 (17)	14 (14)	15 (15)	15 (15)	108 (84)	93 (76)	110 (89)	65 (53)	91 (70)	76 (65)
3 IIIb	0.0151	0.0171	**0.0009**	15 (14)	12 (11)	13 (12)	15 (14)	105 (80)	90 (72)	102 (80)	61 (50)	82 (60)	72 (60)
4 IVc	0.0142	0.0161	0.0142	**0.0037**	11 (11)	12 (12)	12 (12)	100 (76)	88 (71)	100 (79)	68 (56)	84 (63)	73 (62)
5 V	0.0113	0.0133	0.0113	0.0104	—	7 (7)	9 (9)	101 (77)	93 (76)	101 (80)	69 (57)	87 (66)	74 (63)
6 VIc	0.0123	0.0142	0.0123	0.0113	0.0066	**0.0028**	10 (10)	101 (77)	91 (74)	100 (79)	66 (54)	82 (61)	75 (64)
7 VIIa	0.0123	0.0142	0.0142	0.0113	0.0085	0.0094	**0.0009**	103 (79)	96 (79)	106 (85)	68 (56)	90 (69)	77 (66)
8 *Terrapene ornata*	0.1072	0.1119	0.1084	0.1028	0.1039	0.1039	0.1065	—	104 (81)	99 (70)	108 (80)	103 (78)	109 (82)
9 *Clemmys insculpta*	0.0927	0.0949	0.0914	0.0892	0.0946	0.0925	0.0983	0.1069	—	109 (87)	90 (71)	59 (49)	92 (76)
10 *Clemmys guttata*	0.1110	0.1145	0.1056	0.1030	0.1044	0.1032	0.1103	0.1006	0.1127	—	101 (78)	105 (79)	99 (77)
11 *Clemmys marmorata*	0.0635	0.0647	0.0604	0.0677	0.0687	0.0656	0.0679	0.1114	0.0910	0.1033	—	91 (66)	66 (53)
12 *Clemmys muhlenbergi*	0.0887	0.0930	0.0828	0.0849	0.0883	0.0828	0.0919	0.1058	0.0579	0.1076	0.0921	—	87 (65)
13 *Emydoidea blandingii*	0.0766	0.0767	0.0723	0.0734	0.0744	0.0755	0.0777	0.1121	0.0935	0.1009	0.0652	0.0879	—

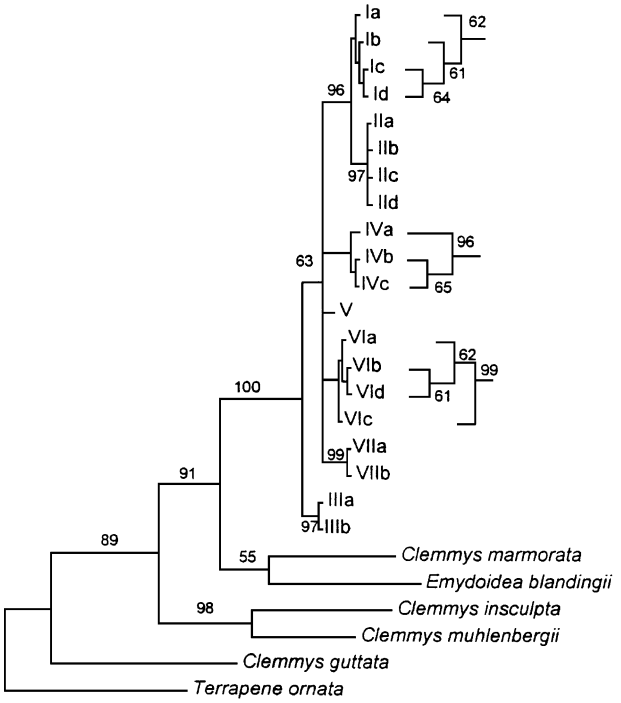

Fig. 2 A 50% majority rule consensus phylogram showing the results of the maximum parsimony analysis, if all nearctic species were set to outgroup. Originally 21 shortest trees with 378 steps in length were found (CI: 0.72, HI: 0.28, RI = 0.74, RC: 0.53). Bootstrap values derived from 500 replicates are indicated at each node of the tree or the bifurcation schemes at the right.

in the branches leading to lineages I and II. Among all basal lineages, V exhibited the shortest branch length.

C. marmorata and *Emydoidea blandingii* were shown as closest relatives to *E. orbicularis*. The three species represented a well-supported monophyletic clade. However, the position of *C. marmorata* conflicted in both methods. Maximum likelihood allocated it as sister taxon to *Emys* (not shown), whereas maximum parsimony indicated a closer relationship to *E. blandingii*. Other members of the genera *Clemmys* and *Terrapene* fell into basal clades, indicating that *Clemmys* represents a paraphyletic group, and that this genus may require a taxonomic revision.

If varying outgroups were employed, the same intraspecific order, but with differing root positions (Fig. 3) along branches III or IV was gained. *C. marmorata* or *E. blandingii*, the closest relatives, systematically produced trees with lineage III as basal offbranch. However, all root positions were largely acentric, if compared with the basal splitting of the main lineages of *E. orbicularis*. Outgroup rooting led to a remarkable reduction of the basal branch, which appeared to be unnatural (probably due to multiple reversals, as indicated by strikingly different consistency indices in Figs 2 and 3). For this reason the basal position of lineage III (Fig. 2), although supported in 63% bootstrap replicates, remains problematic.

The geographical distribution of haplotypes

As indicated in Fig. 4 the extensive sequence polymorphism

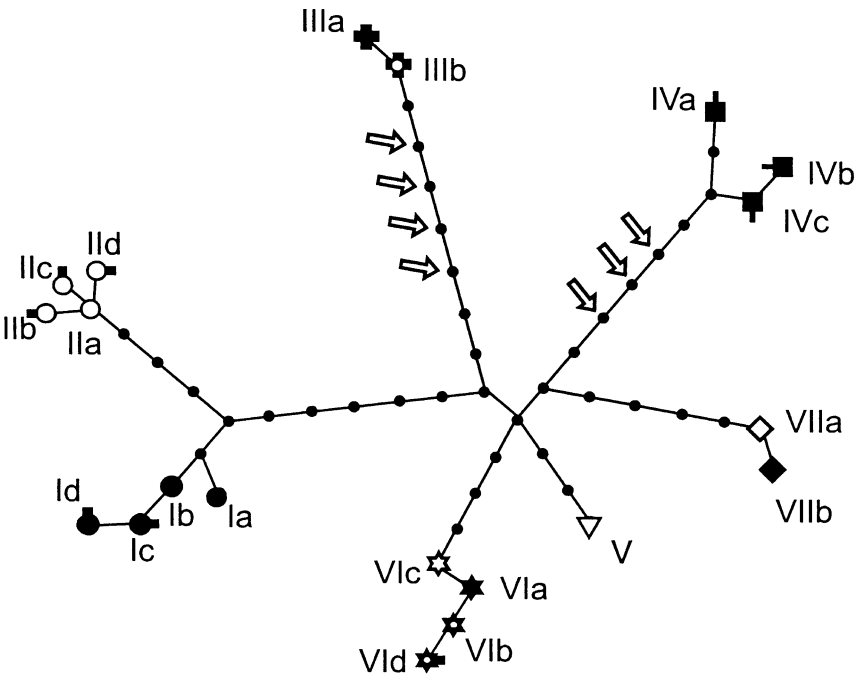

Fig. 3 An unrooted parsimony tree of all *Emys orbicularis* haplotypes exclusively. A single most parsimonious tree of 55 steps in length was obtained (CI: 0.91, HI: 0.09, RI: 0.96, RC: 0.87). Each step between two circles represents one nucleotide substitution. Black circles and symbols denote missing and existing haplotypes, respectively. The arrows indicate all potential positions of roots, when different outgroup species were employed.

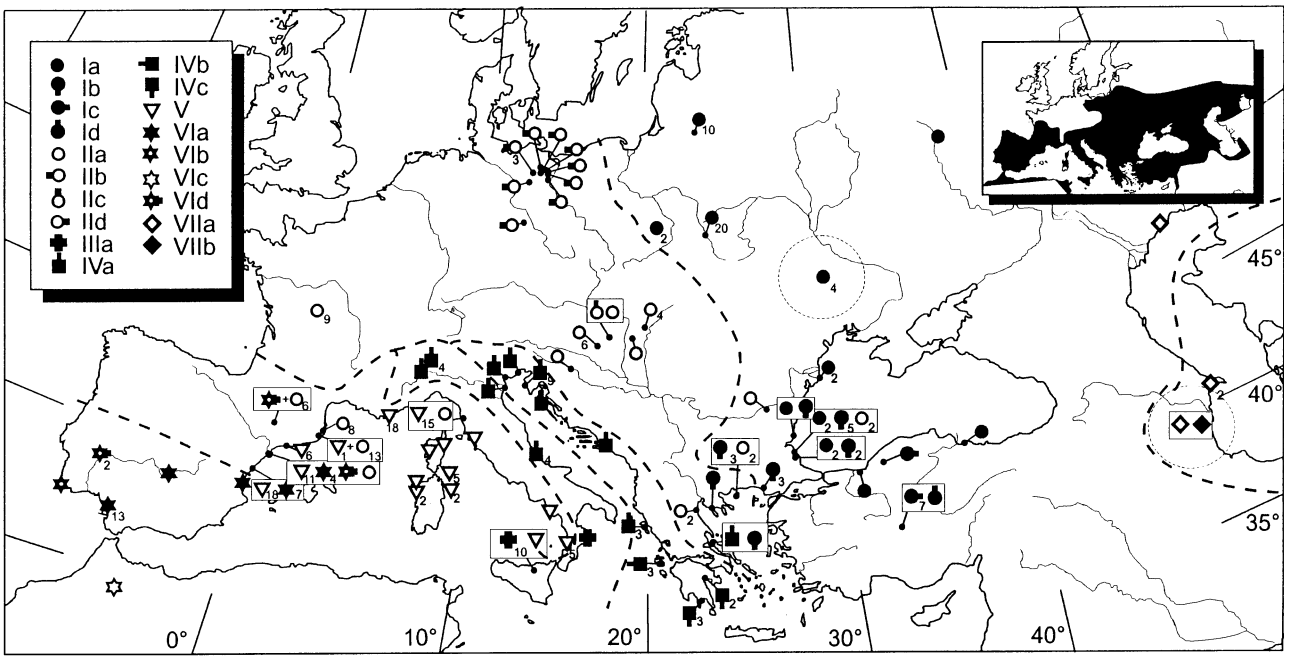

Fig. 4 Geographical distribution of 313 *Emys orbicularis* haplotypes. The numbers by the symbols indicate the frequencies of haplotypes per locality (symbols without numbers represent single specimens), symbols in boxes mark polymorphic populations, dashed circles denote inexact localities. Dashed lines mark the range partition according to hypothesis 4a used for AMOVA. The upper-left box indicates the haplotype symbols, the upper-right box shows the recent range of *E. orbicularis*. Apart from these 313 specimens data from another 110 specimens from localities of Spain: Menorca (V_{19}), Mallorca (V_{12}, IIa_8); Italy: Castel Porziano (IVa_{15}, V_5); France: Camargue (IIa), Lyon (IIa); Denmark, different localities (Ia_6, Ib, IId); and Germany, different localities (Ia_{14}, Ib, IIa_{10}, IIIa, IIIb, IVa_{11}, V_2, VIa) were obtained, but not usable for phylogeographical analyses (see Lenk *et al*. (1998)) and excluded.

detected among *E. orbicularis* was characterized by many localized haplotypes. They were situated in eastern Europe and Asia Minor (lineage I), central Europe and central Balkan (II), southern Italy (III), around the Adriatic Sea (IV), the northwest coast of the Mediterranean (V), Iberia and Northern Africa (VI), and the Caspian region (VII). Some lineages showed subdivisions on a finer scale: in the parsimony tree (Fig. 3) the North African haplotype VIc appeared ancestral to haplotypes VIa, b, d which occurred on the Iberian peninsula; the southwest Greece haplotypes IVc and IVb were spatially separated from IVa which occupied the remaining range of lineage IV; in east Germany the widely distributed haplotype IIa was replaced by its putative descendant IIb; in Asia Minor Ic and Id seemed to occupy the more central part, while Ib and Ia were restricted to the coast. V was the sole lineage showing no geographical subdivision.

Partial Mantel tests

As time calibrations on the basis of genetic distances (see below) suggested a preholocene origin for each of the seven lineages, seven distinct haplotypes should have existed after the last cold stage in southern refugia at least. On the basis of their restricted distributions five of seven lineages could be assigned unequivocally to specific refugia (Iberia, Italy, Greece, Asia Minor, Caspian region). The assignment for two lineages (II, V) remained ambiguous. Thus, before testing alternative scenarios the glacial arrangements of mitochondrial lineages had to be examined. Seven potential arrangements were therefore raised (types a–g; Table 2) and tested simultaneously under each scenario. Congruence was found across all scenarios to reject hypotheses based on arrangement types b–g (Table 3). The allocation of lineage V to an Italian refugium and II to a Greek refugium, as suggested under arrangement type a, yielded the sole model with a significant association to the extant phylogeographical pattern.

When the associations of the six expansion hypotheses were tested simultaneously under arrangement type a, all but one hypothesis were rejected, as denoted in Table 3, lower part. Hence, the sole scenario which retained significance in combination with arrangement type a was 4.

Population genetic structure according to hypothesis 4a (see Fig. 4) inferred with AMOVA revealed a significant geographical partition as evidenced by both haplotype

Table 2 Seven hypothetical spatial arrangement types (a–g) of mitochondrial lineages during the last glaciation (as shown in Fig. 1) as used for partial Mantel tests

Refugia	Iberia			Italy		Greece			
Subrefugia	West	East	Sardinia	West	East	West	East	Asia Minor	Caspian
Type a	VI		—	V	III	IV	II	I	VII
Type b	VI		V	III		IV	II	I	VII
Type c	VI	V	—	III		IV	II	I	VII
Type d	VI	II	V	III		IV	I		VII
Type e	VI	II	—	V	III	IV	I		VII
Type f	VI	II	V	III		IV		I	VII
Type g	VI	II	—	V	III	IV		I	VII

Table 3 Partial Mantel association tests to examine the postglacial expansion process: null hypothesis probabilities for the partial regression between the spatial genetic composition and causal hypotheses. The hypotheses are represented by different types of mitochondrial lineage arrangements in glacial refugia (a–g; Table 1) in combination with putative scenarios modulating the postglacial expansion (1–6, Fig. 1). Each Mantel test entails 10 000 randomizations. * indicates significance beyond $P < 0.05$ after sequential Bonferroni correction. The six columns of the upper table present the results of six partial Mantel tests to examine the most probable lineage arrangement during the last glacial. Significant associations to the actual haplotype distribution could be obtained in those reconstructions which were based on arrangement type a only. The lower part displays the probabilities of scenarios 1–6 based on arrangement type a. The reconstruction based on scenario 4 and arrangement type a is the sole hypothesis which retains a significant association to the actual distribution pattern of haplotypes

	Scenario					
Arrangement type	1	2	3	4	5	6
a	0.0006*	0.0003*	0.0037*	0.0001*	0.0001*	0.0142
b	0.0212	0.0177	0.0119	0.6790	0.0247	0.0226
c	0.8774	0.2414	0.8106	0.1319	0.2834	0.3871
d	0.4062	0.2316	0.1391	0.1613	0.0296	0.0258
e	0.7090	0.3423	0.2449	0.9418	0.7473	0.1335
f	0.8246	0.6189	0.5496	0.1807	0.8172	0.4924
g	0.7097	0.6633	0.4725	0.7723	0.3847	0.4632

Scenario	Arrangement type a
1	0.1260
2	0.0351
3	0.0141
4	0.0001*
5	0.8541
6	0.2405

frequency and sequence statistics. However, on the basis of sequence statistics a more conspicuous population differentiation was evident as indicated on Φ_{ST}s and variance components (Table 4). While 38.8% of the total variation was explained by haplotype frequency differences among groups, the comparable value was 62.1% when sequence divergence was considered. In contrast, variation among populations was higher when haplotype frequencies were taken into account. This remarkable shift was due to numerous closely related haplotypes within many clades which provided less sequence differentiation but substantially contributed to haplotype diversity. *E. orbicularis* is thus a species showing discontinuous divergence patterns in geographical distribution. This is probably due to long-term extrinsic barriers to gene flow corresponding with category Ia of Avise *et al*. (1987).

Table 4 Mitochondrial variation in *Emys orbicularis* by haplotype frequencies and sequence divergence. The level of genetic variation of the three sources, among groups, among populations within groups, and within populations, were examined by AMOVA. Variance components and percentages of variation of each hierarchical level are indicated. The lower part of the table contains fixation indices and the significance of fixation indices as well as variance components after permutation tests

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among geographical test groups				
Frequencies	6	52	0.18 Va	38.84
Sequence divergence	6	940	3.74 Va	62.11
Among populations within geographical test groups				
Frequencies	64	58	0.20 Vb	43.14
Sequence divergence	64	455	1.59 Vb	26.51
Within populations				
Frequencies	241	21	0.09 Vc	18.00
Sequence divergence	241	164	0.69 Vc	11.37
Total				
Frequencies	311	130	0.47	
Sequence divergence	311	1560	6.02	
Fixation indices				
Frequencies	Φ_{SC}:	0.71	Vb and Φ_{SC}: $P < 0.00001$	
Sequence divergence		0.70	Vb and Φ_{SC}: $P < 0.00001$	
Frequencies	Φ_{ST}:	0.82	Vc and Φ_{ST}: $P < 0.00001$	
Sequence divergence		0.89	Vc and Φ_{ST}: $P < 0.00001$	
Frequencies	Φ_{CT}:	0.39	Va and Φ_{CT}: $P < 0.00001$	
Sequence divergence		0.62	Va and Φ_{CT}: $P < 0.00001$	

Discussion

The origin of Emys orbicularis

A phylogenetic analysis of some emydid turtles (Bickham *et al*. 1996) based on 16S rRNA sequences had already revealed that the monotypic genus *Emys* is a sister group to *Clemmys marmorata* and *Emydoidea blandingii*. Our findings based on the cytochrome *b* and $tRNA_{Thr}$ genes corroborate this study in identifying *C. marmorata* and *E. blandingii* as closest extant taxa to *Emys*, although morphologically based phylogenies are not concordant (Gaffney & Meylan 1988; Burke *et al*. 1996).

Because *E. orbicularis* is the single Old World representative of the otherwise strictly New World terrapin family Emydidae, a nearctic radiation centre for this group is plausible (Fritz 1998). Earliest fossil remains of *Emys* in Kazakhstan date back to the middle Miocene (12 million years; Chkhikvadze 1989). The putative ancestor of this genus, however, had to pass the Bering Bridge much earlier, because it became climatically impassable about 20 million years ago. This hypothesis largely agrees with assumptions based on fossil snakes (Szyndlar 1991) and amphibians (Maxson *et al*. 1975). Hutchison (1981) proposed that ancestors of *Emys* entered Asia between 15 and 29 million years ago. Assuming that *Emys, C. marmorata, and Emydoidea*, differentiated 20 million years ago, a substitution rate of 0.3–0.4% sequence divergence per 1 million years can be obtained. This value is in agreement with other findings on turtle mtDNA evolution (0.4% sequence divergence/million years; Avise *et al*. 1992; Bowen *et al*. 1993; Lamb & Lydeard 1994).

According to this calibration the divergence leading to the extant lineages of *Emys* occurred approximately 3.0–4.1 million years ago, as the average genetic distance between the seven lineages amounts to $\bar{x}_p = 1.23\%$. Considering that *E. orbicularis* lives in zones of Mediterranean and moderate climates, the tropical conditions of the Pliocene period were unfavourable and prevented an earlier expansion across Europe. However, a more suitable climate with marked seasonal shifts was established in Europe 3.2 million years ago (Suc 1984). It is likely that this important change gave rise to a sudden radiation, as reflected by the star phylogeny in Fig. 3. Indeed the average sequence divergence among the main lineages (1.23%), when calibrated against 3.2 million years, would suggest an evolution rate of 0.38% per million years which agrees with our previous calibration.

The quaternary history of E. orbicularis

During the climatic oscillations of the Pleistocene the range of *E. orbicularis* probably became recurrently fragmented with isolates along a slender belt throughout southern Europe. This belt has been shaped by cold climates to the north (Frenzel 1967) and by barriers of inappropriate

habitats (Mediterranean Sea and the deserts of North Africa) to the south. Southern Europe, however, is highly structured by mountain blocks and maritime embayments which may contribute to range fragmentation and genetic isolation. *E. orbicularis* reflects this situation by a maximum haplotype diversity on a West–East transect extending throughout southern Europe and the Middle East (Fig. 4).

Partial Mantel tests (Table 3) indicate that southern Italy (V and III) and Greece (IV and II) served as refugia for two distinct lineages simultaneously. In principle, two reasons are possible: (i) the mentioned refugia harboured polymorphic assemblages and lineage sorting during expansion would have produced the present monomorphic populations; (ii) the refugia were subdivided into allo/parapatric subrefugia, which already consisted of monomorphic populations. The latter case is perhaps more likely than the maintenance of single but polymorphic populations in glacial refugia. Because of the uniparental inheritance of mtDNA, Avise *et al*. (1987) argued that the evolution of haplotypes is 'self-pruning', due to the continuous elimination of the paternal mitochondrial genomes in every new generation. It is to be expected that this tendency is enforced during periods of range fragmentation and decreasing population sizes (Hewitt 1996).

The vicariance of comparatively old lineages in Italy and Greece not only confirms the refugia hypothesis (e.g. Hewitt 1996) of European species to survive the glacials, but also proposes a remarkable extent of such populations and stability over long periods of time. Population fluctuations as caused by climatic changes increase the risk of extinctions and replacements by adjacent assemblages and hence would have contributed to the loss of genetic diversity. Despite this vicarious lineages in Italy and Greece support the perspective of permanently favourable conditions in the southern extremities since the Pliocene.

Effective barriers are supposed to evoke such genetic breaks. Italy and Greece have been interspersed with mountains and marine trenches since the Pliocene at least (e.g. Schröder 1986; Doutsos *et al*. 1987; Santucci *et al*. 1996) possibly acting as barriers to gene flow. Yet, coastal corridors could have promoted genetic exchange and also marine straits represent no absolute barriers for *E. orbicularis* as indicated by closely related haplotypes (Fig. 4) on both sides of the Strait of Gibraltar (3.5–3.0 million years old; Rögl & Steininger 1983).

Wright (1978) and Endler (1977) argued that evolution could act in species with semi-isolated populations linked by low gene flow. We therefore suggest that the observed vicariance has been maintained under allo/parapatric conditions. The synergism of low mobility and poor genetic exchange appears strong enough to evoke genetic distinctness and to prevent gene pools from amalgamating.

Zones of mtDNA overlap

Our data demonstrate that extensive mitochondrial sympatry is possible among adjacent mitochondrial lineages, including one zone in northeast Iberia and one in the southern Balkans.

In general, mtDNA of terrapins and tortoises reveals no extensive overlapping zones, and strong geographical structure is the rule (Lamb *et al*. 1989; Lamb & Avise 1992; Osentoski & Lamb 1995; Walker *et al*. 1995). However, some of these studies showed that closely related haplotypes can share a common range. In *E. orbicularis* a similar pattern was observed in the Aegean region where haplotypes Ia, Ib, IIa, and IV meet. Maximum overlap was found between related haplotypes like Ia/Ib and IIa/Ib (Fig. 4).

More distinct lineages (II, V, and VI) meet in a secondary contact zone in northeast Spain (Fig. 4). IIa originated from the Aegean region and V from southern Italy. Both refugia are fairly distant from northeast Iberia. It indicates that the indigenous Iberian lineage VI expanded only to a small extent, whereas the allochthonous haplotypes II and V would have entered Iberia after bypassing the Pyrenees. It is beyond the scope of this study to compare the different contact zones of *E. orbicularis*, but we would like to point out that this remarkable overlap is situated apart from the putative core refugia in the very south. Perhaps they arose via extensive range movements between forms with different origins and different histories (see below).

The course of re-immigration

In general, phylogeographical patterns are considered to be the result of a multifactorial process, being somewhat arbitrary and variable among different species (Taberlet *et al*. 1998). Our study presents an attempt towards the recognition of general trends in the phylogeography of the European pond turtle, while applying simple phylogeographical models with a limited number of variables.

Partial Mantel tests suggest that populations in the centre of the ancient range, the southern Balkans, were favoured either in their expansion potential, their northern distribution limits or both. This scenario implies an ecological factor which is optimal in the centre and decreases in the periphery of the range. But which parameters are causal?

We suppose that the thermo-sensitive reptiles were preferably affected by climate. Apart from the latitude-dependent temperature gradient, a second gradient of continental climate conditions was established across Europe during the postglacial period (Kutzbach & Guetter 1986). While the annual temperature regime of eastern Europe exhibits marked seasonal changes, the Atlantic influence in western Europe has a compensating effect resulting in cooler summers. Hence, summer temperatures in the

continental climate regions offer more suitable thermal conditions for reptiles (Hecht 1928; Spellerberg 1976) and, as a consequence, may have promoted postglacial expansion. In the most eastern regions, however, the suitable conditions are countered by extreme continental climates like the increasing aridity. Balkan populations as the main source for the recolonization of Europe is not a unique feature of *E. orbicularis*. Other organisms like *Zootoca* (Guillaume *et al*. 1997) or *Corthippus* (Cooper *et al*. 1995) obviously share some distribution patterns and pathways of re-immigration with *E. orbicularis*.

It is noteworthy that only turtles bearing lineages I and II which differentiated later than the other lineages (i.e. in Pleistocene times) developed strategies to use short but hot continental summers. In the northern parts of the range they normally deposit a single big clutch per year (Jabłoński & Jabńłońska 1998), whereas two or more small clutches are the rule in southern regions (Bannikow 1951; Kotenko & Fedorchenko 1993; Fritz *et al*. 1995). The feature is paralleled by significant morphological traits. Although dark coloration or large body size can be found in some southern subspecies of *E. orbicularis*, the northern parts of the distribution range are exclusively inhabited by large and dark-coloured forms attributed to the subspecies *orbicularis* (Fritz 1992).

Conclusions

Terrestrial and freshwater reptiles are, because of their limited dispersal capacities and temperature dependence, sensitive indicators for the study of biogeographical processes. Inferences from the geographical organization of genomic markers substantially contribute to both the historical geography and the biogeography of organisms.

As mentioned above, only a few phylogeographical studies deal with entire ranges of European-wide distributed species. In this context *Emys orbicularis* appears to be one of the most fragmented vertebrate species in the western palaearctic region. We deduce this fact from some characteristic life-history traits, like the low competition by other terrapin species, relative low dispersal capacities, and high longevity that could shelter local populations from extinctions during short-term habitat disruption.

The glacial periods had important impacts on the phylogeography of *E. orbicularis*, including a recurrent withdrawal from central Europe. Refugia are supposed to have been located in southern Europe and the Middle East. Our study confirmed that during the last pleniglacial *Emys* was actually present in all southern European peninsulas, but that hypothetical refugia north of 40°N latitude are unfounded. Thus, no evidence can be found that regions others than tiny areas in the extreme south of Europe and adjacent Asia were climatically suitable for *Emys* during the pleniglacial.

The current distribution of haplotypes suggests a postglacial expansion that substantially diverges from symmetric immigration models which imply a phylogeographical structure dependent simply on migration distance or a south–north temperature gradient. In contrast, *E. orbicularis* shows the trend of a centrifugal expansion mode probably due to declining ecological factors in the periphery of the range.

The deep genetic divergence between the main mitochondrial lineages indicates that striking speciation processes had already taken place. It could be argued that at least lineages III, V, and VI represent distinct species, as morphological (Fritz 1998) and molecular data correspond across the subspecies ranges. In order to decide this taxonomic issue, the *Emys* complex needs to be studied using nuclear loci, with particular emphasis on the contact zones of the lineages identified in this study.

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This work is part of the PhD of Peter Lenk devoted to the microevolution, phylogeny, and conservation genetics of the European pond turtle. He conducted the molecular work in the laboratories of Michael Wink. Peter Lenk is also working on the microevolution of some European colubrid snakes and the molecular phylogeny of Viperine snakes. Uwe Fritz is Deputy Director and Curator of the Herpetological Department of the Staatliches Museum für Tierkunde Dresden and provided most of the samples. His special interest is taxonomy and variability of chelonians from the Palaearctis and South East Asia. Ulrich Joger, Curator of Vertebrates at the Hessian State Museum, Darmstadt, Germany also teaches zoology and desert ecology at the University of Darmstadt. Running research projects include molecular phylogenies of mammals, reptiles, and amphibians, herpetofaunistic exploration of several African and Asian countries, ecology of amphibians and of desert reptiles. He is concerned with the application of molecular methods to the phylogeny and microevolution of several reptile groups. Michael Wink is Director of the Institut für Pharmazeutische Biologie at the University of Heidelberg. In addition to research projects in phytochemistry and chemical ecology he runs a laboratory to study the molecular evolution and ecology of animals and plants.
