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Gene flow across secondary contact zones of the *Emys orbicularis* complex in the Western Mediterranean and evidence for extinction and re-introduction of pond turtles on Corsica and Sardinia (Testudines: Emydidae)

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Abstract

European pond turtles represent a phylogeographically deeply structured complex of distinct taxa. Here, we use mitochondrial DNA sequences (cytochrome *b* gene) and eight polymorphic microsatellite loci to investigate genetic differentiation and gene flow of Sicilian, Corsican and Sardinian pond turtles and of subspecies involved in two secondary contact zones in the Pyrenean region and Southern Italy. Mitochondrial and microsatellite differentiation is largely concordant in populations from the core regions of the distribution ranges of the studied taxa. Both marker systems provide no evidence for gene flow between Sicilian pond turtles (*Emys trinacris*) and Southern Italian subspecies of *E. orbicularis*. By contrast, in the contact zones limited gene flow occurs between distinct subspecies of *E. orbicularis*. Although the Southern Italian contact zone is significantly older than the Pyrenean contact zone of Holocene age, patterns of asymmetric introgression are similar. Introgressive hybridization leads to the exchange of mitochondria, but microsatellite data indicate only a few individuals with mixed ancestry. This suggests that incipient isolating mechanisms maintain largely discrete nuclear genomic gene pools. Furthermore, this implies that Southern Italy acted as a hotspot rather than as a melting pot of genetic diversity during the last glacial. Pond turtles from Corsica and Sardinia are not differentiated from continental populations of the subspecies *E. o. galloitalica*, neither in the mitochondrial nor in the quickly evolving microsatellite markers. As the fossil record argues for a continuous presence of pond turtles on both islands since the Middle Pleistocene, this suggests that the native island populations became extinct and the extant turtles were later introduced by prehistoric settlers. The lack of genetic differentiation of pond turtles from Corsica and Sardinia supports the view that the subspecies described from these islands are not valid.

Key words: Europe – phylogeography – secondary contact zone – hybridization – glacial refugium – speciation – genetic cluster analysis

Introduction

Secondary contact zones between closely related taxa allow insights into early stages of allopatric speciation. Studies on the genetic structure of contact zones are also particularly important for understanding the complex nature of hybridization processes (cf. Coyne and Orr 2004; Currat et al. 2008). In the absence of reproductive isolation, secondary contact may lead to complete admixture of parental taxa, while prezygotic (e.g., mating behaviour, ecological adaptations) and postzygotic (genetic incompatibilities) isolation mechanisms are expected to maintain the distinctiveness of parental gene pools, but may result in relatively narrow hybrid zones characterized by steep allele/haplotype frequency clines (Barton and Hewitt 1985; Orive and Barton 2002; Ballard and Whitlock 2004; Mallet 2005). However, introgression can reach far beyond such hybrid zones and has often been found to be asymmetric (see review in Currat et al. 2008).

Within the framework of the Biological Species Concept (e.g., Mayr 1942, 1963; Coyne and Orr 2004), subspecies may be understood as incipient species that are not yet reproductively isolated, so that in secondary contact zones extensive gene flow occurs and may ultimately lead to complete genetic amalgamation. European pond turtles of the *Emys orbicularis* complex are an attractive model for the study of gene flow in secondary contact zones. They inhabit one of the largest distribution ranges among all chelonians, extending from North Africa over the Iberian peninsula and parts of western, central, and eastern Europe eastwards to the Aral Sea (Fritz 2003). Previous morphological and genetic surveys demonstrated that pond turtles represent a highly differentiated complex of ten major evolutionary lineages with a clear phylogeographic structure (Lenk et al. 1999; Fritz 2003; Fritz et al. 2004, 2005a,b, 2007, 2009a; Fig. 1). Pond turtles from Sicily, representing the most basal mtDNA lineage and being distinct in nuclear genomic ISSR fingerprint profiles, have recently been removed from *E. orbicularis* as the distinct species *E. trinacris* (Fritz et al. 2005b). All other lineages are thought to constitute the polytypic species *E. orbicularis* with many recognized subspecies. Their subspecies status was inferred from narrow contact zones with morphologically intermediate turtles and sympatric occurrences of distinct mitochondrial lineages, suggestive of extensive gene flow (Fritz 1995, 1996, 2003; Lenk et al. 1999; Fritz et al. 2005a,b, 2007).

The phylogeographic structure of pond turtles largely corresponds to general phylogeographic patterns identified for Western Palaearctic biota (Hewitt 1996, 1999, 2000, 2001; Taberlet et al. 1998; Joger et al. 2007; Schmitt 2007). Two to three endemic turtle lineages occur on each of the southern European peninsulas and in Asia Minor. It is generally accepted that most of these lineages diverged during the Pliocene and that their current distribution was shaped during Pleistocene and Holocene range fluctuations (Fritz 1996, 2003; Lenk et al. 1999; Fritz et al. 2007, 2009a). The northern part of the distribution range has been recolonized in post-glacial times from two refuges in the south-eastern Balkans and the Black Sea region (Lenk et al. 1999; Sommer et al. 2009) according to the 'grasshopper' and 'bear patterns' of Hewitt

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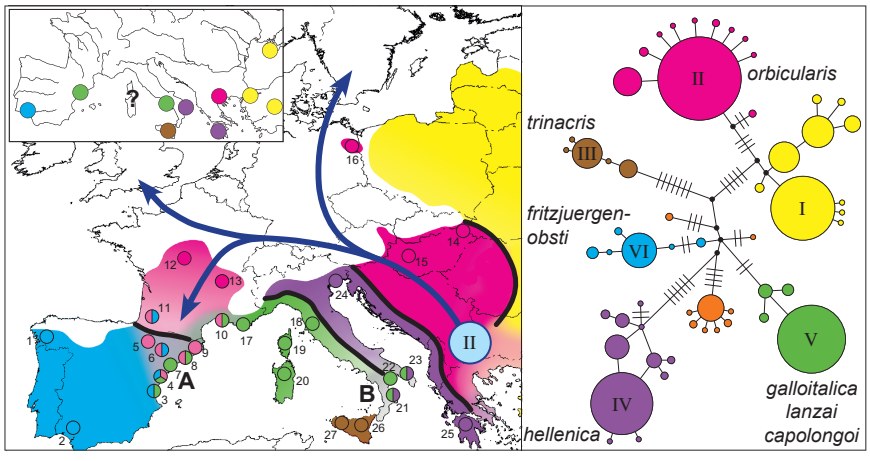

Fig. 1. Left: distribution of mitochondrial lineages of *Emys orbicularis* and *E. trinacris* (only Sicily) and location of studied populations. Numbers refer to Table 1. Colours indicate mitochondrial lineages and correspond to the haplotype network on the right; merging colours, secondary contact zones (A: Pyrenean contact zone; B: Southern Italian contact zone). Colours of pie charts symbolize haplotype lineages occurring in the respective population, but not their frequencies. Arrows indicate Holocene recolonization routes of *E. o. orbicularis* (mtDNA lineage II) from a refuge in the south-eastern Balkans (circle); the distribution gap in its extant range is the result of Holocene extinction (Fritz 1996; Sommer et al. 2007, 2009). Black bars represent mountain barriers. Inset shows approximate location of glacial refugia. Endemic mitochondrial haplotypes suggest that two refugia existed for *E. o. hellenica*, one in Southern Italy, and the other one in Greece. The extant circum-Adriatic distribution of *E. o. hellenica* resulted from a range expansion out of the Italian refuge (Fritz et al. 2007). Two distinct refugia have also been postulated for *E. o. galloitalica*, one on the Apennine peninsula and the other along the Spanish Mediterranean coast (Lenk et al. 1999; Mascort et al. 1999). Question mark shows the possible existence of refugia on Corsica and Sardinia. Right: median-joining network (Bandelt et al. 1999) for mtDNA haplotypes based on the data set (n = 1107; partial cytochrome *b* gene: 1031 bp) of Fritz et al. (2007). Circle size is a rough approximation of haplotype frequency. Small black circles denote missing node haplotypes. Each line joining haplotypes corresponds to one nucleotide substitution except when hatches across lines are present; then each hatch stands for one step. Species and subspecies names indicated; lineage V corresponds to three subspecies: *E. o. galloitalica* (continental Europe), *E. o. lanzai* (Corsica), and *E. o. capolongoi* (Sardinia). Lineage I (yellow) is not involved in the Western Mediterranean contact zones; orange lineages occur beyond the map sector. Figure modified from Sommer et al. (2009)

(2000), whereas most southern taxa expanded their ranges only moderately and remained more or less confined to their glacial refugia (Lenk et al. 1999; Fritz et al. 2007; Sommer et al. 2009; see also Fig. 1). Generally, southern subspecies of *E. orbicularis* (and *E. trinacris*) are distinctly smaller sized and lighter coloured than the northern subspecies *E. o. orbicularis*. Typical maximum shell lengths of turtles from the south lie between 12 and 17 cm, whereas adult individuals from the north reach 16–23 cm. Moreover, while pond turtles hibernate in the north and produce normally only one large clutch of up to 23 eggs per season, southern subspecies may be active all year and females lay up to three clutches of typically 4–8 eggs (Fritz 2003).

This paper focuses on genetic variation of pond turtles in the Western Mediterranean. Two secondary contact zones are situated in this region. North and south of the Pyrenees and along the French Mediterranean coast lies a contact zone of three subspecies: *E. o. fritzjuergenobsti*, endemic in the Iberian peninsula (Velo-Antón et al. 2008); *E. o. galloitalica*, a subspecies distributed along the Mediterranean coast of northern Spain, southern France and the western Italian peninsula (Mascort et al. 1999; Fritz 2003); and *E. o. orbicularis*, a recent invader from central and south-eastern Europe (Fritz 1996; Lenk et al. 1999; Mascort et al. 1999; Fritz et al. 2007; Sommer et al. 2009). The other contact zone is situated in Southern Italy, where the Western Mediterranean subspecies *E. o. galloitalica* intergrades with *E. o. hellenica*, a subspecies with circum-Adriatic distribution (Fritz et al. 2005b, 2007, 2009a). In close geographical proximity occurs the Sicilian pond turtle, *E. trinacris*. Besides morphological features, each of these taxa is characterized by a distinct mitochondrial lineage (Lenk et al. 1999; Fritz et al. 2005a,b, 2007); however, the cryptic *E. trinacris* morphologically closely resembles *E. o. galloitalica* (Fritz et al. 2005b, 2006a). Two further subspecies have been described from Corsica (*E. o. lanzai*) and Sardinia (*E. o. capolongoi*) and are included in our analyses. Both resemble *E. o. galloitalica* morphologically and share with this subspecies the most frequent haplotype of the same mitochondrial lineage (Fritz 1995, 2003; Lenk et al. 1999). Consequently, their validity has been doubted (Zuffi 2000). However, the fossil record suggests that pond turtles have been present on Corsica and Sardinia at least since the Middle Pleistocene (Caloi et al. 1981; Hervet and Salotti 2000; Chesi et al. 2008), so that some genetic divergence should be expected.

We hypothesize that the Pyrenean hybrid zone is distinctly younger than the Italian hybrid zone because *E. o. orbicularis* is a Holocene invader of the Pyrenean region (Lenk et al. 1999; Sommer et al. 2009). By contrast, glacial refuges of *E. o. galloitalica* and *E. o. hellenica* were located on the Italian peninsula (Fritz et al. 2007), allowing gene flow much earlier, perhaps even prior to or during the last glaciation. Therefore, it should be expected that populations in the older Southern Italian contact zone possess a distinctly higher degree of introgressed genes.

Previous papers on the *E. orbicularis* complex mainly used morphological traits and mitochondrial DNA sequences to study geographical and interpopulational variation, while nuclear markers were rarely employed (Fritz 1995, 1996; Lenk et al. 1999; Fritz et al. 2004, 2005a,b, 2006a, 2007, 2009a). Microsatellite markers have been used before only for inferring differentiation between Iberian populations (Velo-Antón et al. 2008). However, in this study the northern Spanish contact zone of the Iberian subspecies with other taxa was not

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included. In the present paper we apply a comparative approach combining mtDNA sequence data and, as nuclear genomic marker, information from eight unlinked polymorphic microsatellite loci (Pedall et al. 2009). Mitochondrial and nuclear markers provide different, and complementary, information about the evolutionary history of the studied organisms. Variation of biparentally inherited microsatellite DNA indicates more recent events because of higher mutation rates and has the advantage of estimating population admixture at the individual level, while mtDNA is a powerful tool for tracing back deeper genealogical splits in maternal line (e.g., Zhang and Hewitt 2003; Brito and Edwards 2009). Using both marker systems, we address the following questions: (i) do patterns of introgression differ in the two contact zones of different age, (ii) does gene flow occur between Sicilian and Southern Italian pond turtles, and (iii) do rapidly evolving microsatellite markers reveal a genetic distinctness of Corsican and Sardinian populations from continental populations harbouring the same mitochondrial lineage? In doing so, populations from the core regions of the ranges of involved taxa serve as a yardstick for determining levels of introgression in the contact zones.

Materials and Methods

Sampling and *a priori* population assignment

Blood samples from 413 specimens of *Emys orbicularis* and *E. trinacris* representing 27 *a priori* defined populations mainly from the Western Mediterranean were studied. According to their collection sites, these populations were classified either as belonging to core regions of or to

Table 1. Genetic diversity of populations representing core regions and contact zones, based on eight unlinked microsatellite loci and 1031 bp of mitochondrial DNA (partial cytochrome *b* gene). Mitochondrial data originate from previous studies using the same samples (Lenk et al. 1999; Fritz et al. 2005b, 2006a, 2007). Numbers preceding localities refer to Figs 1 and 2; upper-case letters indicate countries. Groups used for hierarchical AMOVA are separated by lines. *n*, number of individuals; N_a, average number of alleles; *AR*, allelic richness; H_O, average observed heterozygosity; H_E, average expected heterozygosity; F_{IS}, inbreeding coefficient; hap. div., mitochondrial haplotype diversity; nuc. div., mitochondrial nucleotide diversity

		Microsatellites					mtDNA	
Group/Population	*n*	N_a	*AR*	H_O	H_E	F_{IS}	hap. div.	nuc. div.
Emys orbicularis fritzjuergenobsti								
1 – Pontevedra E	9	4.250	3.781	0.508	0.590	0.147	0.39	0.0004
2 – Doñana E	17	6.500	4.565	0.506	0.603	0.166	0.32	0.0005
Pyrenean contact zone								
3 – Valencia E	26	7.625	4.939	0.627	0.734	0.148*	0.44	0.0030
4 – Ebro E	19	7.625	4.992	0.652	0.725	0.103	0.45	0.0039
5 – Navarra E	15	4.250	3.545	0.595	0.615	0.033	0	0
6 – Estaña E	7	3.750	3.648	0.411	0.657	0.393*	0.29	0.0047
7 – Tarragona E	6	3.500	3.500	0.688	0.657	-0.051	0	0
8 – Sils E	14	4.375	3.782	0.696	0.639	-0.093	0.14	0.0019
9 – Girona E	8	2.875	2.695	0.328	0.369	0.117	0	0
10 – Camargue F	33	7.125	4.296	0.655	0.692	0.054	0.52	0.0069
11 – Aquitaine F	18	8.750	6.113	0.757	0.838	0.099	0.48	0.0034
Emys orbicularis orbicularis								
12 – Brenne F	8	5.625	5.029	0.734	0.756	0.031	0	0
13 – Rhone F	9	5.875	5.044	0.653	0.702	0.074	0.22	0.0002
14 – Tajba SK	10	5.750	4.914	0.750	0.759	0.013	0	0
15 – Danube H	25	9.625	6.188	0.845	0.842	-0.004	0.22	0.0002
16 – Brandenburg D	20	9.125	5.915	0.699	0.816	0.147*	0	0
Emys orbicularis galloitalica								
17 – Var F	19	7.500	5.245	0.671	0.725	0.077	0	0
18 – Pisa I	16	8.250	5.919	0.758	0.815	0.072	0	0
Emys orbicularis lanzai								
19 – Corsica F	11	7.375	5.791	0.648	0.751	0.143	0	0
Emys orbicularis capolongoi								
20 – Sardinia I	10	5.875	4.870	0.563	0.686	0.188	0	0
Southern Italian contact zone								
21 – Neto I	18	8.250	5.777	0.715	0.776	0.081	0.79	0.0055
22 – Basilicata I	8	5.250	4.753	0.563	0.752	0.265*	0.25	0.0002
23 – Southern Apulia I	26	10.000	6.352	0.740	0.834	0.115*	0.65	0.0022
Emys orbicularis hellenica								
24 – Adria North	18	7.875	5.359	0.546	0.766	0.294*	0	0
25 – Greece	12	8.250	5.754	0.625	0.712	0.128	0.76	0.0019
Emys trinacris								
26 – Sicily Nebrodi I	10	4.625	3.990	0.500	0.609	0.186	0	0
27 – Sicily West I	21	8.750	5.741	0.600	0.759	0.214*	0.09	0.0001

*Significantly different from zero after Bonferroni correction.

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contact zones between the taxa occurring in the study region (Fig. 1; Table 1; Supporting Information: Appendix S1). The core populations consist only of morphologically homogenous turtles with mitochondrial haplotypes of the same lineage. In the contact zones, turtles harbouring haplotypes of distinct lineages occur syntopically or in close proximity; these individuals are often morphologically intermediate between the parental taxa (Lenk et al. 1999; Fritz 2003; Fritz et al. 2005a, 2007).

Genotyping and mitochondrial haplotyping

Total DNA was isolated using standard proteinase K and phenol chloroform protocols (Sambrook and Russell 2001). Eight microsatellite loci (msEo29, msEo41, GmuD107, msEo21, GmuD16, msEo2, GmuD88, GmuD55 of Pedall et al. 2009) were analysed for each individual; there was no evidence for linkage disequilibrium between any of these loci (Pedall et al. 2009). Multiplex PCRs and genotyping on a MegaBACE 500 DNA analysis system were performed as described in Pedall et al. (2009). Data of mitochondrial haplotypes (1031 bp cytochrome *b* gene) of the same samples were available from previous papers; haplotype nomenclature follows these studies (Lenk et al. 1999; Fritz et al. 2005b, 2006a, 2007). Accordingly, haplotypes clustering in phylogenetic analyses in distinct clades represent mitochondrial lineages that are numbered with Roman numerals. Individual haplotypes of each lineage bear consecutive letters. For haplotypes of used samples and their GenBank accession numbers, see Supporting Information (Appendix S1).

Genetic cluster analysis

For inferring population structuring based on unlinked genetic markers, several Bayesian algorithms exist. However, different methods may obtain conflicting results with respect to the number of genetic clusters (K) and the degree of admixture between clusters, and none of the methods is clearly superior to the others (Pearse and Crandall 2004; Chen et al. 2007; Frantz et al. 2009). In particular, genetic structure may be overestimated in data sets with an underlying pattern of isolation-by-distance (Frantz et al. 2009). Consequently, it is crucial to examine whether genetic divergence increases with geographical distance. For doing so, geographical coordinates of all 27 *a priori* defined populations were used to calculate a geographical distance matrix with the software GEOGRAPHIC DISTANCE MATRIX GENERATOR 1.2.3 (http://biodiversityinformatics.amnh.org/open_source/gdmg/download.php). Isolation-by-distance patterns were then assessed using log-transformed geographical distances and pairwise F_{ST} values obtained with ARLEQUIN 3.1 (Excoffier et al. 2005) following the approach by Rousset (1997) to account for two-dimensional habitat distribution. To analyse the correlation between both matrices, a Mantel test was run using the internet platform ISOLATION BY DISTANCE WEB SERVICE 3.16 (Jensen et al. 2005; http://ibdws.sdsu.edu/~ibdws/) and the option 'genetic distances/similarities' with 30 000 randomizations. The results suggested a positive correlation of genetic divergence and geographical distance ($r = 0.1892$; $p = 0.03$). Therefore, the parallel application of a spatially explicit Bayesian clustering method, allowing the usage of geographical information as additional prior, and a non-explicit method is advisable to explore the robustness of the partitions obtained by each method (Frantz et al. 2009).

As spatially non-explicit Bayesian method, the Gibbs sampler algorithm as implemented in STRUCTURE 2.1 (Pritchard et al. 2000; Falush et al. 2003) was chosen. Under this method, the main criterion for delimiting genetic clusters is the search for groups in Hardy–Weinberg equilibrium and linkage equilibrium. This search is conducted for each locus separately, which allows detecting genetic admixture. For estimation of K, posterior probabilities [highest lnP(D)] and the ΔK method of Evanno et al. (2005) were used. The ΔK method is known to be more reliable, but to detect only the uppermost level of population structure, corresponding to more inclusive clusters with finer substructure (i.e., when population relationships can be described as hierarchical island model; Evanno et al. 2005). Posterior probabilities were estimated for $K = 1$ to $K = n + 3$, where $n = 27$ was the number of *a priori* defined populations. For assessing the number of population clusters, an admixture scenario with allele frequencies correlated was chosen, because there is morphological evidence for intergradation in contact zones of distinct *Emys orbicularis* subspecies (Fritz 2003). The burn-in was set to 10^5 and the number of further MCMC runs to 5×10^4. Calculations were repeated 10 times for each K; convergence of likelihood values was reached after the burn-in. To visualize obtained results for clusters and individual admixture, barplots were produced.

As spatially explicit clustering method, the Metropolis-Hastings algorithm as implemented in BAPS 5.3 (Corander et al. 2008a,b) was applied. Its main criterion is the search for clusters that are similar in their allelic profile. In a first step (so-called mixture analysis), individuals are clustered according to their overall allelic profile and geographical origin (spatial clustering). Then, in a second step (admixture analysis), the level of gene flow between clusters is inferred by estimating the probability that a certain allele of a specimen might originate from another cluster. Accordingly, using microsatellite data and geographical coordinates for each sample the number of genetic clusters was estimated with five independent runs for different putative maximum numbers of K, ranging from $K = n$ to $K = n + 10$, where $n = 27$ was again the total number of *a priori* defined populations (mixture analysis). Subsequently, the probability of genetic admixture between each cluster was estimated (settings: minimum size of population: 5; 500 simulations from posterior allele frequencies; reference individuals: 50 with 100 iterations) and used to produce individual barplots to depict individual admixture, similar to the STRUCTURE output. In addition, BAPS was used to estimate the pairwise Nei's genetic distances (Nei 1972) between the genetic clusters and to create a Neighbour Joining (NJ) tree based on these.

Diversity and divergence of populations

For microsatellite and mitochondrial markers, diversity indices were estimated. Using FSTAT 2.93 (http://www2.unil.ch/popgen/softwares/fstat.htm), the inbreeding coefficient F_{IS} was computed for each microsatellite locus and population; furthermore, the average over all loci was calculated for each population to infer reduction of population heterozygosity. Statistical tests with Bonferroni corrections (Rice 1989) were conducted to analyse whether F_{IS} values differ significantly from zero using randomized data sets (5000 randomizations). In populations represented by < 10 individuals, F_{IS} values were treated with caution because of small sample size and resulting weak statistical power, however. FSTAT 2.93 was also employed to estimate allelic richness (AR) per locus and population. Mean number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosity (mean values derived from individual microsatellite loci) and population-specific mitochondrial haplotype and nucleotide diversities were calculated in ARLEQUIN 3.1.

To assess the degree of similarity between all 27 *a priori* defined populations and only between the core populations, allele frequencies at all eight microsatellite loci were used to perform a principle component analysis (PCA) with the software PCAGEN 1.2 (http://www2.unil.ch/popgen/softwares/pcagen.htm); the significance of PCA axes was tested using 1000 permutations.

Seven groups representing core populations of distinct taxa and two additional groups corresponding to populations from each of the two contact zones were studied (Table 1). For two data sets, one including all of these nine groups and the other only the seven groups of core populations, levels of global genetic divergence for microsatellites (settings: distance matrix, number of different alleles) and mtDNA (settings: distance matrix, pairwise differences) were assessed by a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) in ARLEQUIN 3.1. To determine divergences between the groups representing core populations or contact zone populations, as well as within these groups, the same software was used to calculate pairwise F_{ST} values for both marker systems. In addition, pairwise Rho_{ST} values were produced for microsatellite data using R_{ST} CALC 2.2 (Goodman 1997). F_{ST} values are more accurate for populations with high levels of gene flow, while Rho_{ST} values better reflect differentiation of populations with longer divergence times (Balloux and Lugon-Moulin 2002). Furthermore, on the basis of microsatellite data, chord distances (D_C; Cavalli-Sforza and Edwards 1967), D_A distances (Nei et al. 1983), and standard

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distances (D_S; Nei 1972) were calculated for all 27 *a priori* defined populations using POPULATIONS 1.2.30 (http://www.cnrs-gif.fr/pge). With these values, NJ trees were built and their robustness tested using 1000 bootstrap replicates and allele frequencies over individuals.

Results

Isolation-by-distance and population structure

Microsatellite data for all 413 turtles representing the 27 *a priori* defined populations were analysed for clusters using STRUCTURE and the spatially explicit approach of BAPS. In STRUCTURE, the highest mean posterior probability was obtained for $K = 29$ (Supporting information: Table S1), but the corresponding genetic structure was biologically difficult to explain. We conclude that K was overestimated, as known to occur in STRUCTURE using the likelihood approach (Falush et al. 2003; Evanno et al. 2005), in particular when genetic divergence is influenced by isolation-by-distance (Frantz et al. 2009), as in our data set. In contrast to the likelihood approach, the ΔK method of Evanno et al. (2005) resulted only in $K = 5$ (Supporting information: Table S1). Then, turtles of the core populations representing *Emys orbicularis fritzjuergenobsti*, *E. o. orbicularis*, *E. o. hellenica*, and *E. trinacris* were assigned to distinct clusters (Fig. 2: blue, red, violet, and brown clusters, respectively). A further cluster (green) corresponded to the populations Tarragona, Sils, and Girona from the eastern part of the Pyrenean contact zone. Turtles from populations in the southern Pyrenean contact zone (Valencia, Ebro, Navarra, Estaña) were either entirely or mostly assigned to the blue 'Iberian' cluster otherwise corresponding to *E. o. fritzjuergenobsti*, whereas nearly all individuals from contact zone populations north of the Pyrenees (Camargue, Aquitaine) were placed in the red cluster corresponding to *E. o. orbicularis*. Notably, populations of the subspecies *E. o. galloitalica* (Var, Pisa), *E. o. lanzai* (Corsica), and *E. o. capolongoi* (Sardinia) did not appear as distinct clusters. Rather, for these samples mixed ancestries of the violet and green clusters were inferred. By contrast, populations of the Southern Italian contact zone were mainly assigned to the violet cluster, otherwise matching *E. o. hellenica*. To explore the possibility that the unexpected mixed ancestries of the populations of *E. o. galloitalica*, *E. o. capolongoi* and *E. o. lanzai* could be an artefact from the chosen $K = 5$, additional analyses with $K = 6$ as upper bound were run. Then, however, similar results were obtained

Fig. 2. Diagram combining individual mtDNA data (horizontal bands, 1031 bp of cytochrome *b* gene; top) with individual barplots from STRUCTURE (centre, $K = 5$) and BAPS (bottom, $K = 15$) based on variation of eight unlinked microsatellite loci. Numbers preceding population names refer to Fig. 1 and Table 1. Bold vertical lines denote populations, taxa, and contact zones. Colours of mitochondrial lineages and STRUCTURE clusters correspond to Fig. 1; graded primary colours (red, green, violet, blue) of BAPS clusters indicate more inclusive groups corresponding to STRUCTURE clusters (see Fig. 3). Note the mixed ancestry of core populations of *Emys orbicularis galloitalica*, *E. o. lanzai*, and *E. o. capolongoi* in the STRUCTURE barplots; for further differences of both Bayesian methods, see Fig. 3. Membership proportions shown from runs (STRUCTURE: $n = 10$, BAPS: $n = 5$) with best likelihood value

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(Supporting information: Fig. S1), and the sixth cluster corresponded not to *E. o. galloitalica* or the island populations, but to two populations from the Pyrenean contact zone (Sils, Girona). Populations of *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi* were still revealed as of admixed origin.

In contrast to STRUCTURE, the spatially explicit BAPS analyses resulted in a higher estimate of $K = 15$ (p = 99%). Nevertheless, the general pattern revealed by BAPS agreed quite well with the STRUCTURE results. A NJ tree based on Nei's genetic distances between the clusters showed that the 15 BAPS clusters are placed in four more inclusive groups (Fig. 3). With a few exceptions, these four groups corresponded to the five STRUCTURE clusters, suggesting that the higher K of BAPS mirrored mainly genetic substructuring within the entities revealed by STRUCTURE. This is in line with the insights derived from simulation studies by Evanno et al. (2005). These authors found that STRUCTURE typically infers genetic entities of higher order when a hierarchical substructure is present. Major differences between the STRUCTURE and BAPS approaches include, however, that BAPS associated the Camargue population from the northern Pyrenean contact zone not with core populations of *E. o. orbicularis* (Brenne, Rhone, Danube, Brandenburg, Tajba) and the population Aquitaine, but with southern contact zone populations (Tarragona, Sils, Girona) and core populations of *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi*. In the Camargue population, mitochondrial haplotypes of two genetic lineages occur, one corresponding to *E. o. orbicularis* and the other to *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi*, so that their allocation to either group makes biological sense. Moreover, for core populations of *E. o. galloitalica* (Var, Pisa), *E. o. lanzai* (Corsica), and *E. o. capolongoi* (Sardinia) BAPS did not suggest a mixed ancestry as STRUCTURE had done. Instead, these populations were placed together into the same, distinct cluster within a more inclusive group also containing the three populations from the southern Pyrenean contact zone (Girona, Tarragona, Sils) and the Camargue population. Finally, the BAPS cluster representing *E. trinacris* was found to be highly divergent from any other cluster based on Nei's genetic distances. This is reflected by its very long branch that occurs within a more inclusive group containing also populations from the Southern Italian contact zone and of *E. o. hellenica*. The allocation of the *E. trinacris* cluster within these populations indicates that their considerable genetic distance from *E. trinacris* is smaller than the distances between the Sicilian species and other populations of *E. orbicularis*.

Genetic diversity

Diversity indices derived from microsatellite data for single contact zone populations were not extraordinarily high when compared to core populations (Table 1). This is in line with the rareness of individuals with mixed ancestry in contact zone populations as revealed by STRUCTURE and BAPS (Fig. 2). By contrast, the values for mitochondrial haplotypes were distinctly higher in the contact zone populations, mirroring the occurrence of distinct mitochondrial lineages there (Table 1). An overall deficiency of heterozygous specimens was found in seven populations. However, since there is no obvious correlation with core or contact zone populations, this could be the result of a Wahlund effect, i.e., a reduced overall heterozygosity in the respective populations because of a division into subpopulations, even though the latter may be in Hardy–Weinberg equilibrium.

Divergence between single populations, taxa, and contact zones

When only core populations were used for PCA, all taxa were returned as distinct clusters except the Corsican and Sardinian subspecies *Emys orbicularis lanzai* and *E. o. capolongoi* (Fig. 4, top). The clusters of *E. trinacris* (Sicily) and *E. o. fritzjuergenobsti* (Iberian peninsula) were highly distinct from all others. When the populations from the secondary contact zones were processed additionally, five major clusters were revealed (Fig. 4, bottom) corresponding to (i) populations from the core range of *E. o. fritzjuergenobsti* (Doñana, Pontevedra), (ii) populations from the Ebro Valley and Valencia (Navarra, Estaña, Ebro, Valencia), i.e., from the southern part of the Pyrenean contact zone, (iii) the two core populations of *E. trinacris* (Sicily West, Sicily Nebrodi), (iv) populations from the Southern Italian contact zone (Neto, Basilicata, Southern Apulia) plus core populations of *E. o. hellenica* from Greece and the northern Adriatic Sea, and (v) a diverse assemblage comprising core populations of

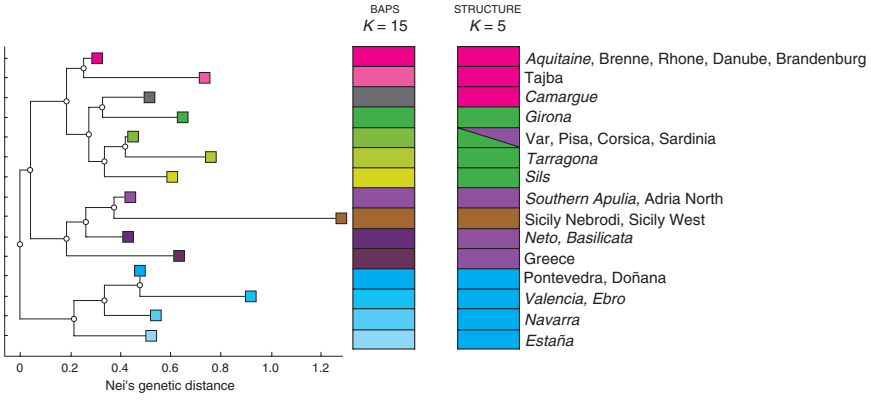

Fig. 3. Neighbour Joining tree for the 15 clusters obtained from BAPS using Nei's genetic distances. Colour coding on the right compares the cluster assignment of the 27 *a priori* defined populations by BAPS with the five clusters obtained from STRUCTURE (ΔK method). Italics indicate populations from contact zones. Graded primary colours (red, green, violet, blue) of BAPS colour patches indicate more inclusive groups corresponding to STRUCTURE clusters. Divided colour patch for the populations Var, Pisa, Corsica, and Sardinia symbolizes their mixed ancestry in STRUCTURE analyses. Note their assignment to a non-admixed, distinct cluster by BAPS. Furthermore, the allocation of the brown and grey BAPS clusters are in conflict with the STRUCTURE results (see text)

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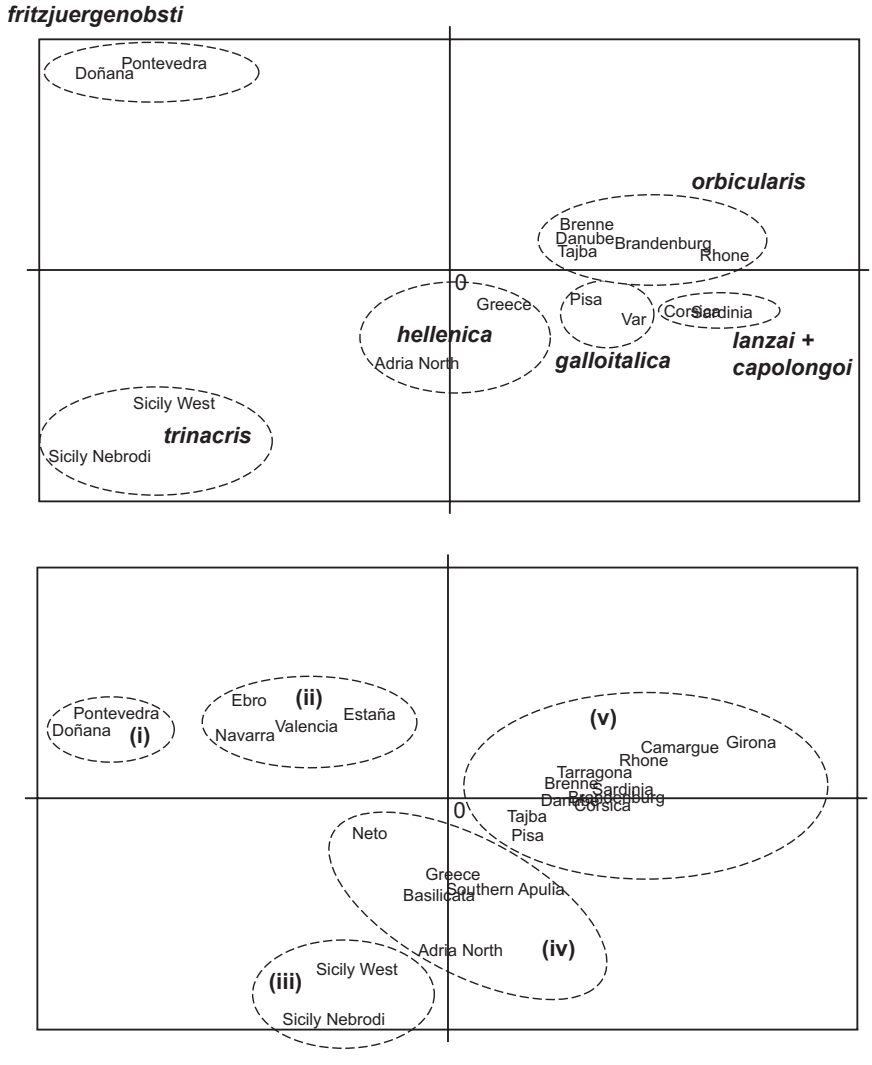

Fig. 4. Top: PCA cluster diagram for core populations. The *x*-axis explains 22% and the *y*-axis 15% of variation. Bottom: PCA cluster diagram for all 27 populations (core populations and populations from secondary contact zones). Populations Aquitaine, Sils and Var are invisibly hidden in centre of cluster (v). The *x*-axis explains 26% and the *y*-axis 18.5% of variation. In both analyses, the first two axes are significant ($p = 0.001$ each, based on 1000 permutations)

E. o. orbicularis (Brenne, Rhone, Tajba, Danube, Brandenburg), of *E. o. galloitalica*, *E. o. lanzai*, and of *E. o. capolongoi* along with populations from the northern and north-eastern part of the Pyrenean contact zone (Tarragona, Sils, Girona, Camargue, Aquitaine). Notably, the majority of contact zone populations do not appear as distinct clusters (except Ebro, Navarra, Valencia, Estaña), but group together with core populations of either *E. o. hellenica* or of *E. o. orbicularis*, *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi*.

Hierarchical AMOVA revealed significantly different patterns for microsatellite and mitochondrial markers, with much more pronounced intergroup and interpopulation differentiation in mtDNA (Table 2). When only the seven groups comprising core populations were considered, distinctly higher F_{CT} values occurred than when also the two groups of contact zone populations were included, suggestive of an impact of gene flow in the contact zones.

With respect to microsatellites, average F_{ST} and Rho_{ST} values between groups representing core and contact zone populations range between 0.02–0.31 and 0.01–0.57, respectively (Table 3). The lowest between-group values occur when populations representing *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi* are compared. Their values (F_{ST}: 0.02–0.05; Rho_{ST}: 0.01–0.09) resemble the within-group variation of

Table 2. Results of hierarchical AMOVA analyses of microsatellite and mitochondrial data to test for geographical differentiation in European pond turtles. Two different data sets were analyzed: (1) The seven groups corresponding to the core populations of distinct taxa plus the two groups corresponding to populations from the two contact zones and (2) only the seven groups representing core populations. For delineation of groups, see Table 1

		Between groups			Between populations within groups			Between populations (no groups considered)		
Groups	Marker	F_{CT}	Variance	Variation (%)	F_{SC}	Variance	Variation (%)	F_{ST}	Variance	Variation (%)
(1) Core and contact zone populations	Microsatellites	0.07*	0.3	7	0.12*	0.4	11	0.18*	2.9	82
	mtDNA	0.61*	3.6	61	0.61*	1.4	24	0.85*	0.9	15
(2) Core populations	Microsatellites	0.13*	0.5	13	0.07*	0.2	6	0.19*	2.9	81
	mtDNA	0.94*	6.6	94	0.73*	0.3	4	0.93*	0.1	2

*Significant at the $p < 0.001$ level.

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Table 3. Differentiation between and within populations from core regions and contact zones based on microsatellite variation. Below diagonal, mean pairwise F_{ST} values; above diagonal, mean pairwise Rho_{ST} values. On the diagonal in bold, within-group differentiation (mean pairwise F_{ST} value/mean pairwise Rho_{ST} value). For pairwise F_{ST} and Rho_{ST} values of individual populations, see Supporting Information (Tables S2 and S3)

	Emys orbicularis fritzjuergenobsti	Pyrenean contact zone	*Emys orbicularis orbicularis*	*Emys orbicularis galloitalica*	*Emys orbicularis lanzai*	*Emys orbicularis capolongoi*	Southern Italian contact zone	*Emys orbicularis hellenica*	*Emys trinacris*
E. o. fritzjuergenobsti	**0.07/0.07**	0.37	0.50	0.47	0.48	0.41	0.41	0.51	0.45
Pyrenean contact zone	0.25	**0.20/0.27**	0.27	0.20	0.19	0.29	0.21	0.37	0.36
E. o. orbicularis	0.26	0.17	**0.09/0.13**	0.28	0.26	0.44	0.32	0.57	0.50
E. o. galloitalica	0.25	0.14	0.12	**0.04/0.05**	0.01	0.06	0.08	0.32	0.28
E. o. lanzai	0.28	0.15	0.12	0.04	*	0.09	0.10	0.38	0.31
E. o. capolongoi	0.31	0.16	0.15	0.05	0.02	*	0.19	0.41	0.51
Southern Italian contact zone	0.23	0.18	0.14	0.11	0.11	0.12	**0.07/0.10**	0.23	0.29
E. o. hellenica	0.28	0.23	0.18	0.13	0.17	0.18	0.13	**0.15/0.32**	0.49
E. trinacris	0.30	0.28	0.23	0.21	0.23	0.27	0.19	0.23	**0.07/0.14**

*No average value because only a single population was analyzed.

E. o. galloitalica (F_{ST}: 0.04; Rho_{ST}: 0.05) and all are lower, mostly distinctly lower, than the other between-group values (F_{ST}: 0.11–0.31; Rho_{ST}: 0.08–0.57). By contrast, the within-group variation of the populations of the Pyrenean contact zone is considerable (F_{ST}: 0.20; Rho_{ST}: 0.27) and falls in the range observed between populations of the majority of distinct taxa (F_{ST}: 0.12-0.31, mean: 0.22; Rho_{ST}: 0.26–0.57, mean: 0.42; differences between *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi* not considered). However, the within-group variation in the Southern Italian contact zone is clearly lower (F_{ST}: 0.07; Rho_{ST}: 0.10). This is in line with the observation that populations in the Pyrenean contact zone comprise individuals belonging to distinct microsatellite clusters, whereas most Southern Italian individuals are at the microsatellite level pure *E. o. hellenica* (Fig. 2).

In comparison with microsatellite data, F_{ST} values for mitochondrial haplotypes are distinctly higher except for *E. o. galloitalica*, *E. o. lanzai*, and *E. o. capolongoi*, which are not differentiated (Table 4). Between-group values for populations of other taxa or contact zones range from 0.36 to 0.97 (mean: 0.72). Within-group values in populations representing single taxa range from 0 (*E. o. galloitalica*) to 0.94 (*E. trinacris*); the values for the two contact zones are within this range.

The NJ trees using different distance measures (D_A, D_C, D_S) on the basis of microsatellite data resulted in very similar topologies and bootstrap support values; the D_A tree serves as example (Fig. 5). As a rule, all core populations cluster together and basal branches are mostly short, with their topologies only weakly supported by the bootstrap. Only the *E. trinacris* populations occur on a long basal branch with high support; similarly high support is, however, obtained for the core populations of *E. o. fritzjuergenobsti* (Doñana, Pontevedra). The latter two populations and the populations from the Pyrenean contact zone south of the Pyrenees (Valencia, Ebro, Navarra, Estaña) branch off from the only other long basal branch, albeit with weak support. Other populations from the eastern and northern Pyrenean contact zone are associated, with weak support, with core populations of *E. o. orbicularis*.

Discussion

Our combined analyses of variation of eight microsatellite loci and mtDNA sequences corroborate that European pond turtles represent a phylogeographically deeply structured complex of distinct taxa. Our data show that *Emys trinacris* is a highly distinct evolutionary lineage, without gene flow with Southern Italian subspecies of *E. orbicularis*, despite the close proximity of Sicily and Calabria and the observation that sea straits constitute otherwise no genetic barrier to Western Palaearctic freshwater turtles (Lenk et al. 1999; Mantziou

Table 4. Mean pairwise F_{ST} values between populations from core regions and contact zones based on mitochondrial haplotypes (1031 bp cytochrome *b*; below diagonal). On the diagonal in bold, within-group values. For pairwise F_{ST} values of individual populations, see Supporting Information (Table S4)

	Emys orbicularis fritzjuergenobsti	Pyrenean contact zone	*Emys orbicularis orbicularis*	*Emys orbicularis galloitalica*	*Emys orbicularis lanzai*	*Emys orbicularis capolongoi*	Southern Italian contact zone	*Emys orbicularis hellenica*	*Emys trinacris*
E. o. fritzjuergenobsti	**0.01**								
Pyrenean contact zone	0.66	**0.38**							
E. o. orbicularis	0.78	0.41	**0.38**						
E. o. galloitalica,	0.85	0.60	0.96	**0**					
E. o. lanzai	0.82	0.58	0.95	0	*				
E. o. capolongoi	0.81	0.57	0.95	0	0	*			
Southern Italian contact zone	0.52	0.46	0.65	0.43	0.37	0.36	**0.39**		
E. o. hellenica	0.65	0.68	0.77	0.84	0.80	0.80	0.49	**0.56**	
E. trinacris	0.81	0.83	0.93	0.97	0.97	0.97	0.67	0.80	**0.94**

*No average value because only a single population was analyzed.

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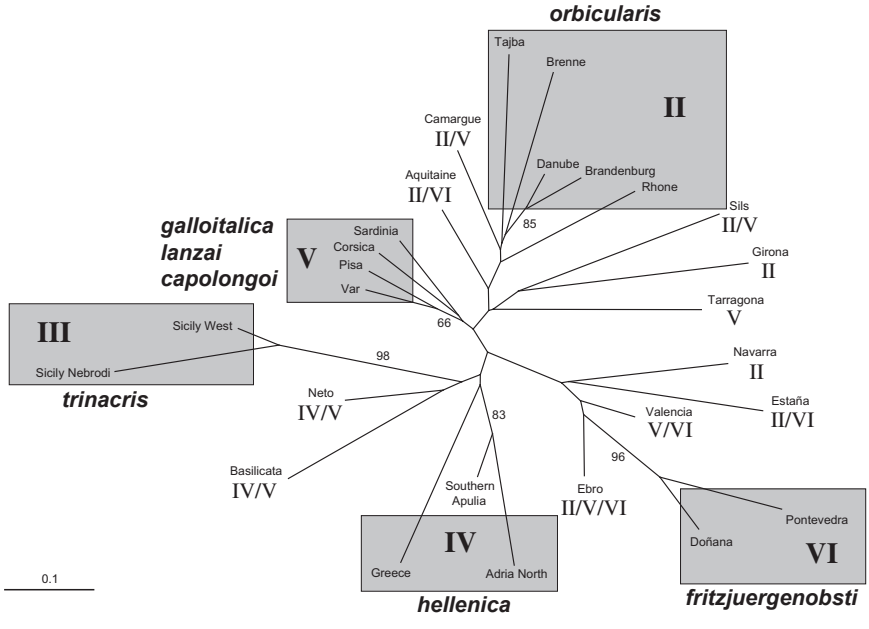

Fig. 5. Neighbour Joining tree based on D_A distances of individual populations (microsatellite data). Core populations are shown in grey boxes. Roman numerals indicate mitochondrial lineages occurring in respective population. Numbers along branches are bootstrap values greater than 50

et al. 2004; Fritz et al. 2006b). Negligible gene flow in contact zones of subspecies of *E. orbicularis* occurs, but is asymmetrical and mostly restricted to mitochondrial introgression. Pond turtles from Corsica and Sardinia (*E. o. lanzai*, *E. o. capolongoi*) are undifferentiated from the continental subspecies *E. o. galloitalica* in mitochondrial and microsatellite markers (see below). For core populations of *E. o. fritzjuergenobsti*, *E. o. orbicularis*, *E. o. hellenica*, and for *E. trinacris*, all applied methods found similar differentiation patterns of the two marker systems. These morphologically distinctive taxa (Fritz 2003; Fritz et al. 2005b), each also characterized by another mitochondrial lineage (VI, II, IV and III, respectively), were independently identified as distinct entities by STRUCTURE and BAPS analyses, PCA and different distance measures of microsatellite data, underlining their evolutionary distinctness. The different degree of differentiation of mtDNA and microsatellite data revealed by hierarchical AMOVA (Table 2), as expected, reflects the smaller effective population size of the mitochondrial genome (e.g., Ballard and Whitlock 2004), resulting in faster lineage sorting and a higher level of fixation for the mitochondrial marker. The two Bayesian methods for analysing microsatellite data yielded, with exceptions that concern mainly *E. o. galloitalica* and the Corsican and Sardinian populations (see below), largely congruent results. Generally, the spatially explicit analyses using the software BAPS unravelled a finer pattern than STRUCTURE (Figs 2 and 3).

In agreement with the extant subspecies distribution and mitochondrial data (Mascort et al. 1999; Fritz et al. 2005b, 2007), microsatellites suggest that distinct glacial microrefugia existed on the Iberian and Italian peninsulas (for *E. o. fritzjuergenobsti* and *E. o. galloitalica*, and for *E. o. galloitalica* and *E. o. hellenica*, respectively). For one Pyrenean contact zone population (Tarragona) no admixture and no introgressed mtDNA were found (Fig. 2). This implies that the Tarragona population represents a relict of pure Iberian *E. o. galloitalica*. BAPS revealed distinct clusters for Iberian and Italian populations of *E. o. galloitalica* and for Italian and Greek populations of *E. o. hellenica* (Figs 2 and 3), supporting the view that for each subspecies two distinct refugia were located in these regions (Fritz et al. 2007; Fig. 1, see also below).

Introgression patterns in the two contact zones of different age

The Pyrenean contact zone of the *Emys orbicularis* complex agrees well with one of the major European suture zones resulting from Holocene range expansions (Taberlet et al. 1998; Hewitt 1999; Schmitt 2007). The Southern Italian contact zone must be significantly older, by more than the factor 10, when it is considered that refugia of *E. o. galloitalica* and *E. o. hellenica* were located there (Fritz et al. 2007) and that the last glacial began about 110 000 years ago and ended about 10 000 years ago (Ehlers and Gibbard 2004).

In recent years, there is growing evidence that the classic southern European refugial centres are composed of several microrefugia (also often referred to as 'refugia within refugia'; e.g., Gómez and Lunt 2007; Joger et al. 2007; Schmitt 2007; Rull 2009), and this is also true for the Italian peninsula and Sicily. However, in contrast to the well-studied Iberian peninsula (review in Gómez and Lunt 2007), the subdivision of the Apennine peninsula and Sicily into microrefugia is still less recognized. For instance, Schmitt (2007) believes in a recent review that the phylogeographic patterns of the Italian peninsula and the whole circum-Adriatic region are considerably simpler than that of the Iberian peninsula. This is most probably an underestimation because of the later awoken interest for the phylogeography of Italian and Balkan biota. In the past ten years, for a considerable number of taxa evidence accumulated for distinct microrefugia in the Apennine peninsula and Sicily, and for many distinct phylogroups a contact zone exists in Southern Italy, suggestive of an important, but often overlooked, suture zone there. Distinct Italian microrefugia have been reported for at least three plant species (*Arabis alpina*: Ansell et al. 2008; *Fagus sylvatica*: Vettori et al. 2004; *Quercus petraea*: Bruschi et al. 2003), the Italian crayfish (*Austropotamobius italicus*: Fratini et al. 2005), scorpions (*Euscorpius carpathicus* complex: Salomone et al. 2007),

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two salamanders (*Salamandrina perspicillata* and *S. terdigitata*: Canestrelli et al. 2006), four frogs (*Bufo viridis* subgroup: Stöck et al. 2008; *Hyla intermedia*: Canestrelli et al. 2007; *Pelophylax lessonae*: Canestrelli and Nascetti 2008; *Rana italica*: Canestrelli et al. 2008), several reptiles (*Emys orbicularis* complex: Fritz et al. 2005b, 2007; two lizards, *Lacerta viridis* complex: Böhme et al. 2007; *Podarcis sicula*: Podnar et al. 2005; three snake species, *Hierophis viridiflavus*: Rato et al. 2009; *Vipera aspis*: Ursenbacher et al. 2006; Barbanera et al. 2009; *Zamenis longissimus* complex: Lenk and Wüster 1999; Lenk et al. 2001), the hedgehog (*Erinaceus europaeus*: Seddon et al. 2001), and the greater mouse-eared bat (*Myotis myotis*: Ruedi et al. 2008). However, heretofore *Vipera aspis* was the only reptile species from this region in which gene flow has been studied using both mitochondrial and microsatellite data (Barbanera et al. 2009). The results of this paper indicated mitochondrial introgression from one viper subspecies into the other.

Also in both of the contact zones of the *E. orbicularis* complex, mitochondrial and microsatellite introgression are discordant and seem to follow the same general pattern, despite the different age of the contact zones and contrary to the expectation that populations from the older Southern Italian contact zone are more introgressed. This suggests that Southern Italy acted as a hotspot rather than as a melting pot of genetic diversity during the last glacial, as the much larger Iberian peninsula. The same has been reported for trees and shrubs (Petit et al. 2003), whereas for certain amphibians the Italian peninsula acted simultaneously as hotspot and melting pot (Canestrelli et al. 2008).

In both contact zones, haplotypes of distinct mitochondrial lineages may occur together, or mitochondrial haplotypes may be combined with pure nuclear gene pools of another genetic lineage. By contrast, microsatellites reveal that individuals of mixed ancestry or recent immigrants are rare (Fig. 2). However, the STRUCTURE analyses suggest for the Southern Italian contact zone that mainly in the Neto population individuals with mixed ancestry occur, but these are inferred to have been genetically impacted by Iberian pond turtles (*E. o. fritzjuergenobsti*). Since the BAPS analyses did not confirm any influence from Iberian turtles, we cannot exclude that this is a methodological problem. Also the PCAs of microsatellite data indicate largely homogenous nuclear gene pools and associate the populations from the Southern Italian contact zone tightly with *E. o. hellenica* (Fig. 4, bottom: cluster iv). In any case, it is of interest that BAPS placed the *E. o. hellenica* population from the northern Adriatic coast together with pond turtles from southern Apulia in the same cluster (Figs 2 and 3), despite considerable geographical distance. By contrast, the Greek population of *E. o. hellenica* was distinct, which is also mirrored by distance methods (Fig. 5). This is in line with the earlier reported exclusive occurrence of endemic mitochondrial haplotypes in Greece. Based on this finding, it has been suggested that two glacial refuges existed for *E. o. hellenica*, one in Greece and the other in Southern Italy, and that the more northern Adriatic coasts were colonized in the Holocene from Southern Italy (Fritz et al. 2007). This is supported by the new microsatellite data from this study.

Asymmetric introgression of nuclear and mitochondrial markers, as observed across both secondary contact zones of *E. orbicularis*, has been found in many species. As a rule, mtDNA introgresses more easily and more frequently than nuclear loci after range expansions (e.g., Currat et al. 2008). A variety of factors has been discussed that might cause such asymmetric patterns (Barton and Hewitt 1985; Orive and Barton 2002; Mallet 2005; Currat et al. 2008), including neutral mechanisms (i.e., genetic drift), assortative mating, sex-biased gene flow, and selective forces including sex-biased hybrid survival (Haldane's rule). Since *E. orbicularis* lacks sex chromosomes (Matthey and van Brink 1957; Ivanov 1973; Belcheva et al. 1992), sex-biased mitochondrial introgression owing to Haldane's rule seems not to be a likely explanation of the observed asymmetric pattern.

We presume that the absence of complete nuclear genomic admixture in our studied contact zone populations is due to incipient isolating mechanisms rather than other factors, such as sex-specific differences in dispersal. When the rapid Holocene range expansion of *E. o. orbicularis* is considered (Fritz 1996; Sommer et al. 2007, 2009), it is obvious that long-distance dispersal may occur. Observations on home ranges in extant Italian and northern populations (review in Fritz 2003) suggest, however, that dispersal of both sexes is normally low. We assume, moreover, that occupied habitats impede the establishment of immigrating turtles, minimizing their genetic impact on local populations. Both could explain the relative stability of the old contact zone in Southern Italy, with a steep genetic gradient resembling that of the much younger Pyrenean contact zone. In the latter, a further factor could play a role for shaping the observed pattern of mitochondrial introgression. The invading subspecies *E. o. orbicularis* attains a greater body size than the local Iberian subspecies *E. o. fritzjuergenobsti* or *E. o. galloitalica* (> 20 cm versus 15 cm maximum shell length, Fritz 2003). Choice experiments have shown that pond turtle males prefer the odour of larger females, which promise a higher reproductive success (Poschadel et al. 2006). Such mate choice could be responsible for the predominance of *orbicularis* mitochondria in many contact zone populations (Fig. 2).

Corsican and Sardinian pond turtles: native or not?

With respect to the core populations of *Emys orbicularis galloitalica* and the two island subspecies *E. o. lanzai* and *E. o. capolongoi*, conflicting results were obtained from the two Bayesian methods. These taxa are morphologically very similar (Fritz 1995, 2003; Schulze and Fritz 2003). Moreover, all three share – unlike other subspecies – haplotypes of the same mitochondrial lineage V (Lenk et al. 1999; Fritz et al. 2007; Table 4), and their validity has therefore been doubted (Zuffi 2000). Supportive of this view, the spatially explicit BAPS analyses ($K = 15$) placed populations of all three subspecies together in the same distinct cluster (Fig. 2), and F_{ST} and Rho_{ST} values close to zero (Table 3) indicate no differentiation between them as well. Also the STRUCTURE analyses ($K = 5$ and $K = 6$) found the three taxa not differentiated, but suggested for all a mixed ancestry of predominantly two gene pools, one corresponding to *E. o. hellenica* and the other being confined to populations occurring in the Pyrenean contact zone in the north-east of the Iberian peninsula ($K = 5$: Tarragona, Sils, Girona, Figs 1–2; $K = 6$: Tarragona, Supporting Information: Fig. S1), i.e., to populations from one of the putative glacial refugia of *E. o. galloitalica*. This clash of the two Bayesian methods is difficult to interpret, but might reflect the different criteria used by these algorithms to find genetic clusters and admixture between them (see Material and Methods). Further research using a denser sampling and additional genetic

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markers is needed to clarify this situation, in particular since STRUCTURE suggested a gradual decrease of the genetic influence of *hellenica* with increasing distance from Southern Italy. At least, the two Bayesian approaches have in common that the two island populations and the French and Italian core populations of *E. o. galloitalica* are not differentiated. When it is considered that microsatellites evolve rapidly and that the Corsican and Sardinian populations are exposed to genetic drift, such a completely absent differentiation is surprising.

The Mediterranean fauna has been heavily impacted and altered by humans for thousands of years (e.g., Masseti 2009), and also the prehistoric or historic introduction of several chelonian species to Mediterranean islands and southern Spain is well known (*Testudo marginata* in Sardinia: Bringsøe et al. 2001; *E. orbicularis*, *T. graeca* and *T. hermanni* in the Balearic Islands: Buskirk et al. 2001; Cheylan 2001; Fritz 2003; Fritz et al. 2009b) or likely (*T. graeca* and *T. hermanni* in Sardinia; *T. hermanni* in Corsica; *T. graeca* in Spain and Sicily: Fritz et al. 2009b; Giacalone et al. 2009). However, in contrast to these cases, several fossil turtle remains of different ages suggest a continuous presence of *E. orbicularis* on Sardinia from the Late Pleistocene (Tyrrhenian stage) to prehistoric times (Caloi et al. 1981; Chesi et al. 2008); from Corsica exists a single record from the Middle Pleistocene (Hervet and Salotti 2000) and then much younger findings in an archaeological context (Copper Age; Cheylan 1988). Consequently, there is some evidence that *E. orbicularis* is native on Corsica and Sardinia. However, if the extant populations should be native there, this would necessitate unexpected slow evolutionary rates of microsatellite DNA and be in conflict with the finding that BAPS revealed even for several populations of Holocene age distinct gene pools (Figs 2 and 3: Pyrenean contact zone, Camargue, Valencia + Ebro, Navarra, Estaña). It is obvious that this situation warrants further research, but that a non-parsimonious explanation of extinction on the islands and later re-introduction by prehistoric settlers has to be considered.

Conclusions

Mitochondrial and microsatellite markers indicate largely congruent differentiation patterns in European pond turtles. They revealed no gene flow between Sicilian pond turtles (*Emys trinacris*) and the Southern Italian subspecies of *E. orbicularis*, supporting full species status for Sicilian pond turtles under the Biological Species Concept (e.g., Mayr 1942, 1963; Coyne and Orr 2004). By contrast, limited gene flow occurs in the Western Mediterranean secondary contact zones of *E. orbicularis* subspecies that are congruent with two major phylogeographic suture zones, one being located in the Pyrenean region, and the other in Southern Italy. Although the Southern Italian contact zone is at least of Late Pleistocene age and, therefore, significantly older than the Holocene Pyrenean contact zone, introgression patterns are similar and largely restricted to the exchange of mitochondria, whereas microsatellite DNA indicates that individuals of mixed ancestry or recent immigrants are rare. This suggests that incipient isolation mechanisms prevent also in the old Southern Italian contact zone extensive gene flow or genetic amalgamation and that Southern Italy acted more as hotspot than as melting pot of genetic diversity during the last glacial. Pond turtles from Corsica and Sardinia are not differentiated from continental populations of the subspecies *E. o. galloitalica*, neither in mitochondrial nor in microsatellite markers. Although the fossil record suggests a continuous presence of pond turtles on Corsica and Sardinia since the Middle Pleistocene, this could indicate that the native island populations became extinct and that the extant turtles were introduced later. Moreover, the lack of genetic differentiation of Corsican and Sardinian pond turtles supports the view that the island populations do not represent valid subspecies.

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Zusammenfassung

Genfluss in sekundären Kontaktzonen des Emys orbicularis-Komplexes im Westmediterran und Hinweise auf Aussterben und Wiederansiedlung von Sumpfschildkröten in Korsika und Sardinien (Testudines: Emydidae)

Europäische Sumpfschildkröten stellen einen phylogeographisch stark strukturierten Komplex distinkter Taxa dar. In der vorliegenden Arbeit verwenden wir mitochondriale DNA-Sequenzen (Cytochrom *b*-Gen) und acht polymorphe Mikrosatelliten-Loci um die genetische Differenzierung und möglichen Genfluss bei sizilianischen, korsischen und sardischen Sumpfschildkröten sowie bei Unterarten zu untersuchen, die in zwei sekundären Kontaktzonen in der Pyrenäen-Region und in Süditalien aufeinandertreffen. Bei Populationen aus dem Kernareal der untersuchten Taxa zeigen beide Markersysteme weitgehend übereinstimmende Differenzierungsmuster. Es gibt keine Hinweise auf Genfluss zwischen sizilianischen Sumpfschildkröten (*Emys trinacris*) und den in Süditalien verbreiteten Unterarten von *E. orbicularis*. In den Kontaktzonen kommt es dagegen zu einem beschränkten Genfluss zwischen verschiedenen Unterarten von *E. orbicularis*. Obwohl die süditalienische Kontaktzone deutlich älter als diejenige in den Pyrenäen ist, sind in beiden ähnliche asymmetrische Introgressionsmuster zu verzeichnen, die weitgehend auf die mitochondriale Ebene beschränkt sind. Die Mikrosatelliten-Daten deuten dagegen nur auf wenige Schildkröten mit gemischter Abstammung hin. Dies lässt vermuten, dass in der Entfaltung begriffene Isolationsmechanismen helfen, weitgehend voneinander getrennte kerngenomische Genpools aufrechtzuerhalten. Dies deutet ferner darauf hin, dass Süditalien im letzten Glazial ein Mannigfaltigkeitszentrum genetischer Diversität war und dass es dort keinesfalls zu einer kompletten genetischen Durchmischung der Refugialpopulationen kam. Korsische und sardische Sumpfschildkröten sind von kontinentaleuropäischen Populationen der Unterart *E. o. galloitalica* weder mitochondrial noch bei den schnell evolvierenden Mikrosatelliten-Markern differenziert. Da es auf beiden Inseln seit dem Mittelpleistozän Fossilnachweise gibt, spricht dies dafür, dass die ursprünglichen Inselpopulationen ausstarben und die heutigen Sumpfschildkröten später durch prähistorische Siedler eingeführt wurden. Die fehlende genetische Differenzierung der Sumpfschildkröten von Korsika und Sardinien spricht außerdem gegen die Validität der von dort beschriebenen Unterarten.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Individual STRUCTURE barplots based on variation of eight unlinked microsatellite loci ($K = 6$).

Table S1. Mean lnP(D) values for different *K*s and their ΔK values. Maximum values indicated in bold and asterisked.

Table S2. Pairwise F_{ST} values for microsatellite data of the 27 *a priori* defined populations of *Emys orbicularis* and *E. trinacris*. Bold numbers stand for the individual populations as outlined in Fig. 1 and Table 1.

Table S3. Pairwise Rho_{ST} values for microsatellite data of the 27 *a priori* defined populations of *Emys orbicularis* and *E. trinacris*. Bold numbers stand for the individual populations as outlined in Fig. 1 and Table 1.

Table S4. Pairwise F_{ST} values for mtDNA data of the 27 *a priori* defined populations of *Emys orbicularis* and *E. trinacris*. Bold numbers stand for the individual populations as outlined in Fig. 1 and Table 1.

Appendix S1. List of samples, their mitochondrial haplotypes and coordinates of *a priori* defined populations.

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