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Organisms Diversity & Evolution

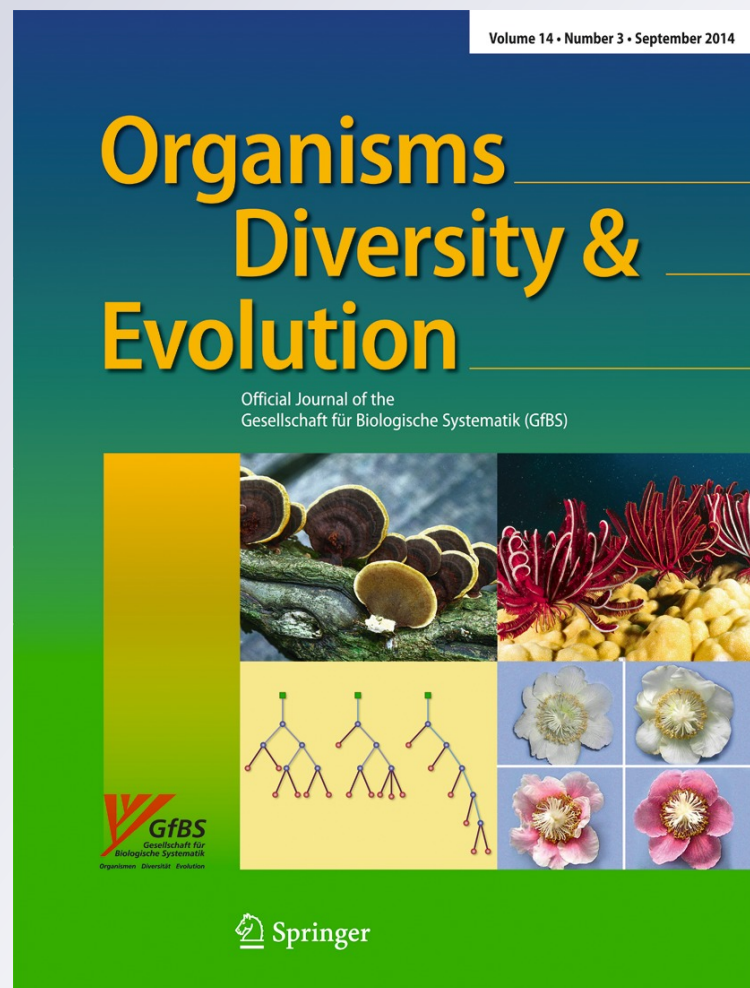
ISSN 1439-6092

Volume 14

Number 3

Org Divers Evol (2014) 14:295-306

DOI 10.1007/s13127-014-0168-4



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Where are you from, stranger? The enigmatic biogeography of North African pond turtles (*Emys orbicularis*)

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Received: 27 November 2013 / Accepted: 4 February 2014 / Published online: 20 February 2014
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Abstract The European pond turtle (*Emys orbicularis*) is a Nearctic element in the African fauna and thought to have invaded North Africa from the Iberian Peninsula. All North African populations are currently identified with the subspecies *E. o. occidentalis*. However, a nearly range-wide sampling in North Africa used for analyses of mitochondrial and microsatellite DNA provides evidence that only Moroccan populations belong to this taxon, while eastern Algerian and Tunisian pond turtles represent an undescribed distinct subspecies. These two taxa are most closely related to *E. o. galloitalica* with a native distribution along the Mediterranean coast of northern Spain

Electronic supplementary material The online version of this article (doi:10.1007/s13127-014-0168-4) contains supplementary material, which is available to authorized users.

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through southern France to western and southern Italy. This group is sister to a clade comprising several mitochondrial lineages and subspecies of *E. orbicularis* from Central and Eastern Europe plus Asia, and the successive sisters are *E. o. hellenica* and *E. trinacris*. Our results suggest that *E. orbicularis* has been present in North Africa longer than on the Iberian Peninsula and that after an initial invasion of North Africa by pond turtles from an unknown European source region, there was a phase of diversification in North Africa, followed by a later re-invasion of Europe by one of the African lineages. The differentiation of pond turtles in North Africa parallels a general phylogeographic paradigm in amphibians and reptiles, with deeply divergent lineages in the western and eastern Maghreb. Acknowledging their genetic similarity, we propose to synonymize the previously recognized Iberian subspecies *E. o. fritzjuergenobsti* with *E. o. occidentalis* sensu stricto. The seriously imperiled Moroccan populations of *E. o. occidentalis* represent two Management Units different in mitochondrial haplotypes and microsatellite markers. The conservation status of eastern Algerian pond turtles is unclear, while Tunisian populations are endangered. Considering that Algerian and Tunisian pond turtles represent an endemic taxon, their situation throughout the historical range should be surveyed to establish a basis for conservation measures.

Keywords Reptilia · Testudines · Emydidae · Phylogeography · Africa · Iberian Peninsula · Palaearctic

Introduction

The European pond turtle, *Emys orbicularis* (Linnaeus 1758), is distributed over a vast range embracing northwestern Africa and much of Europe and western Asia (Fig. 1). Sicilian pond turtles, formerly treated as conspecific with *E. orbicularis*, are today recognized as the distinct species *E. trinacris* Fritz et al. 2005



Fig. 1 *Top*: Distribution range of European and Sicilian pond turtles (*Emys orbicularis*, *E. trinacris*, shaded). *Bottom*: Sampling sites of pond turtles yielding haplotypes of mtDNA lineage VI in Europe (Fritz et al. 2007; Velo-Antón et al. 2011; present study) and of North African samples (present study). *Red sampling sites*: Morocco (Middle Atlas Mountains and Kenitra Province), *orange*: Morocco (Rif Mountains),

blue: eastern Algeria, Tunisia; sampling sites in the Doñana National Park (Spain) used for genotyping *encircled in yellow*. Possibly extinct populations (Gharb plain, Morocco, Fahd et al. 2009; Algiers Region, Algeria, Fritz 1994) are indicated by *question marks*. Moroccan regions mentioned in the text are: 1 Kenitra Province; 2 Rif Mountains (west); 3 Rif Mountains (east); 4 Middle Atlas Mountains

(Fritz and Havaš 2007; van Dijk et al. 2012), and these two species together constitute the only Palaearctic representatives of the otherwise strictly New World family Emydidae (McDowell 1964; Fritz and Havaš 2007; van Dijk et al. 2012). The ancestor of the extant *Emys* species is thought to have immigrated from North America via the Bering Bridge during the Miocene (Hutchison 1981; Fritz 1995, 1996, 2003). In North Africa, *E. orbicularis* is generally understood as a very recent invader

from Europe (Bons and Geniez 1996; Schleich et al. 1996; Lapparent de Broin 2000). The rich African freshwater turtle fauna is otherwise composed of softshell turtles (Trionychidae), pelomedusid side-neck turtles (Pelomedusidae) and the geoemydid *Mauremys leprosa* (Fritz and Havaš 2007; van Dijk et al. 2012), so that *E. orbicularis*, having Nearctic roots and immigrated from Europe, represents a doubly exotic element. The species occurs in North Africa in a patchy range with some

isolated populations in Morocco and, widely separated thereof, in eastern Algeria and northern Tunisia (Fig. 1). Whether *E. orbicularis* is extinct or still persists near Algiers, another isolated region for which a few historical records are known, remains unclear (Fritz 1994, 2003).

While the knowledge of the phylogeography and taxonomy of European and Sicilian pond turtles has increased steadily during the past 25 years (Fritz 1996, 1998, 2001, 2003; Lenk et al. 1999; Fritz et al. 2004, 2005, 2006a, 2007, 2009a; Sommer et al. 2007, 2009; Velo-Antón et al. 2007, 2008, 2011; Pedall et al. 2011), the North African populations remain poorly studied. For instance, several studies accumulated a large, nearly range-wide data set of more than 1,550 sequences of the mitochondrial cytochrome *b* (*cyt b*) gene for phylogeographic purposes (Lenk et al. 1999; Fritz et al. 2004, 2005, 2006a, 2007, 2009a; Sommer et al. 2009; Velo-Antón et al. 2011). However, not more than four of these sequences originated from North African turtles (three turtles from the Rif Mountains, Morocco, and one from the Middle Atlas Mountains, Morocco; Lenk et al. 1999; Fritz et al. 2007). The mitochondrial haplotypes of these Moroccan turtles closely resemble haplotypes endemic in Spain and Portugal. Pond turtles from other parts of the range represent nine distinct genetic lineages (Lenk et al. 1999; Fritz et al. 2007, 2009a), two of which penetrate the Iberian Peninsula in the Pyrenean region and hybridize there with the Iberian lineage (Lenk et al. 1999; Fritz et al. 2007; Pedall et al. 2011). In phylogenetic and parsimony network analyses, the Moroccan haplotypes came consistently out as basal to the Iberian haplotypes, suggesting that the latter are derived from the North African haplotypes. This has led to the speculation that *E. orbicularis* might have colonized the Iberian Peninsula from North Africa (Fritz et al. 2007). Such a scenario would conflict with the established view that the European pond turtle is a recent invader of North Africa (Bons and Geniez 1996; Schleich et al. 1996; Lapparent de Broin 2000).

In the present article, we examine this question by expanding the sampling considerably with 45 new samples from North Africa, including for the first time turtles from eastern Algeria and Tunisia. Based on this sampling, we analyze a robust genetic data set (approximately 3,500 bp of mitochondrial DNA and 15 microsatellite markers) to study the biogeography of European pond turtles in a region crucial for understanding the evolutionary history of both *E. orbicularis* and *E. trinacris* and, in particular, the colonization history of North Africa and the Iberian Peninsula.

Materials and methods

Sampling

Forty-five new samples (tissue and blood) of North African pond turtles were obtained. Among these were for the first

time samples from eastern Algeria ($n=14$) and Tunisia ($n=8$). With respect to Moroccan samples ($n=23$), 2 came from Kenitra Province, 7 from the Rif Mountains and 14 from the Middle Atlas Mountains. Considering the precarious conservation situation of *Emys orbicularis* in North Africa (Fritz 2003; Fahd et al. 2009), precise locality data are not published here. Approximate sampling sites are shown in Fig. 1. For comparison of population structuring, 24 Spanish samples from the Doñana National Park were used, representing a geographically close population from the European side of the Straits of Gibraltar.

DNA extraction, PCR and sequencing of mtDNA, alignment

Total DNA from the North African samples and two representatives of each other mtDNA lineage of *Emys orbicularis* and *E. trinacris* (Table S1) was isolated using standard proteinase K and phenol chloroform protocols (Sambrook and Russell 2001), the DTAB method (Gustincich et al. 1991) or the InnuPrep DNA Mini Kit (Analytik Jena, Jena, Germany).

PCR was used to amplify five different mtDNA fragments (12S, 16S, *cyt b*, D-loop, ND4+adjacent DNA coding for tRNAs) and performed in a final volume of 20 μ l containing 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany) with the buffer recommended by the supplier, 2.5 mM MgCl₂ (Bioron), 0.5 mM of each dNTP (Thermo-Scientific, St. Leon-Rot, Germany), 0.5 μ M of each primer (Table S2) and 10–50 ng of total DNA.

The *cyt b* fragment was amplified using the primer pair CytbG and mt-f-na, resulting in a DNA fragment of approximately 1,250 bp. When PCR failed, two alternative primer pairs were applied to generate two shorter overlapping fragments (CytbG and mt-E-Rev2: approximately 870 bp; mt-C-For2 and mt-f-na: approximately 800 bp). Thermocycling conditions for all three primer combinations were the same. The other mtDNA fragments were amplified using a single primer pair (Table S2). Thermocycling protocols are summarized in Table S3.

PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Stauf, Germany) and sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers indicated in Table S2. Resulting sequence data were aligned in BIOEDIT 7.0.5.2 (Hall 1999) and individually checked. GenBank accession numbers are listed in Table S1.

Haplotype analyses and phylogenetic inference

Previous phylogeographic studies on European pond turtles relied largely on sequence variation of the *cyt b* gene (Lenk et al. 1999; Fritz et al. 2004, 2005, 2007, 2009a; Velo-Antón

et al. 2011). To compare our 45 new North African turtles to the comprehensive data set of 1,551 *cyt b* sequences published by Fritz et al. (2009a) and Velo-Antón et al. (2011), a parsimony network (TCS 1.21; Clement et al. 2000) was calculated using a 1,031-bp-long alignment of 1,596 sequences and the default 95 % probability threshold. In addition, uncorrected *p* distances among and within mitochondrial lineages were obtained using MEGA 6.06 (Tamura et al. 2013).

For further examination of phylogenetic relationships of the North African turtles, the *cyt b* data were concatenated with the other mtDNA fragments, resulting in an alignment of 3,475 bp length of the following partitions: 12S (395 bp), 16S (537 bp), D-loop (645 bp), *cyt b* (1,031 bp), ND4 (682 bp), tRNA-His (70 bp), tRNA-Ser (66 bp) and tRNA-Leu (49 bp). In addition to the data of all North African samples, each Iberian haplotype and two representatives of all other mitochondrial lineages of *Emys orbicularis* and *E. trinacris* (see Fritz et al. 2007, 2009a) were included. Previously published sequences of all other emyde genera served as outgroups (Table S1).

To determine the best partitioning for phylogenetic inference, three partition schemes (no partitioning, partitioning by DNA fragment and maximum partitioning; Table S4) were compared using the Bayesian information criterion (BIC) according to Li et al. (2008). In the second and third partition schemes, the short tRNA fragments were pooled in a single partition of 185 bp. In the maximum partitioning scheme, each codon of protein-coding genes and each non-protein-coding DNA area corresponded to a distinct partition, except for the lumped DNA coding for tRNAs.

Bayesian trees were computed for each scheme using MRBAYES 3.2.1 (Ronquist et al. 2012), with the best evolutionary model for each partition established in jMODELTEST 0.1.1 (Posada 2008) by the BIC (Table S4). Models were defined by the number of substitution types (nst) and the substitution rates for each partition, but without setting priors for the alpha parameter, points of invariance and rate matrix, allowing these parameters to be estimated by MRBAYES. Phylogenetic analyses were performed using two parallel runs (each with four chains). The chains ran for 10 million generations with every 500th generation sampled and reached a split frequency below 0.05. The calculation parameters were analyzed using a burn-in of 2.5 million generations to assure that both runs converged. Subsequently, only the plateau of the most likely trees was sampled using the same burn-in, and a 50 % majority rule consensus tree was generated. The posterior probability of any individual clade in this consensus tree corresponds to the percentage of all trees containing that clade and is a measure of clade frequency and credibility.

In addition to Bayesian inference (BI), maximum likelihood (ML) calculations were run using RAXML 7.2.6 (Stamatakis 2006) and the default GTR+G model. For each partition scheme, five independent ML searches were

performed using different starting conditions and the fast bootstrap algorithm to explore the robustness of the phylogenetic trees by comparing the best trees. Subsequently, 1,000 non-parametric thorough bootstrap replicates were computed and the values plotted against the best tree.

In all cases, the trees were rooted with *Glyptemys*, a genus constituting the sister group of the remaining emyde according to Wiens et al. (2010). The maximum partitioning scheme delivered the most accurate phylogenetic inference for both BI and ML (Table S5).

Microsatellites

Our North African samples (Table S1) plus 24 samples from the southern Spanish Doñana National Park were genotyped at 16 previously characterized microsatellite loci, which are unlinked and highly polymorphic in *Emys orbicularis* (Ciofi et al. 2009; Pedall et al. 2009). Microsatellite DNA was amplified using four multiplex PCRs (Table S6) in a final volume of 10 μ l containing 0.5 units *Taq* polymerase (Bioron) together with the buffer recommended by the supplier, 1.5 mM MgCl₂ (Bioron), 0.2 mM of each dNTP (Thermo-Scientific), 2 μ g of bovine serum albumin (Thermo-Scientific) and 20–40 ng of total DNA. Each multiplex reaction contained a specific set of primers at a specific concentration: Multiplex reaction I contained primers for locus msEo21 (0.132 μ M each), msEo29 (0.4 μ M each), msEo41 (0.4 μ M each) and GmuD107 (0.1 μ M each); multiplex reaction II contained primers for msEo2 (0.2 μ M each), GmuD16 (0.4 μ M each), GmuD55 (0.2 μ M each) and GmuD88 (0.1 μ M each); multiplex reaction III contained primers for Emys2 (0.4 μ M each), Emys4 (0.6 μ M each), Emys5 (0.4 μ M each) and Emys11 (0.2 μ M each); multiplex reaction IV contained primers for Emys1 (0.4 μ M each), Emys6 (0.5 μ M each), Emys7 (0.2 μ M each) and Emys8 (0.5 μ M each). Thermocycling conditions are summarized in Table S7.

Fragment lengths were determined on an ABI 3130xl Genetic Analyzer (Applied Biosystems) using the GeneScan-600 LIZ Size Standard (Applied Biosystems) and the software PEAK SCANNER 1.0 (Life Technologies, Carlsbad, CA).

Genetic cluster analysis and diversity indices for microsatellite data

Our microsatellite data were analyzed with an unsupervised Bayesian clustering approach as implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000; Hubisz et al. 2009) using the admixture model and correlated allele frequencies. However, the Emys2 locus was excluded because allele sizes could not be determined unambiguously. STRUCTURE searches in the data set for partitions that are, as far as possible, in Hardy-

Weinberg equilibrium and linkage equilibrium. The upper bound for calculations was arbitrarily set to $K=10$, a value clearly beyond the number of North African sampling regions plus the Doñana National Park (Fig. 1). The most likely number of clusters (K) was determined using the maximum posterior probability method (Pritchard et al. 2000) and the ΔK method (Evanno et al. 2005). Calculations were repeated ten times for each K using a MCMC chain of 750,000 generations for each run, including a burn-in of 250,000 generations. Population structuring and individual admixture were visualized using the software DISTRUCT 1.1 (Rosenberg 2004).

Clusters inferred by STRUCTURE were further examined by calculating pairwise F_{ST} values and analyses of molecular variance (AMOVAs) using ARLEQUIN 3.5.1.2 (Excoffier et al. 2005). In addition, clusters were characterized by genetic diversity indices. For this purpose, allele frequency tables were produced using CONVERT 1.31 (Glaubitz 2004) and subsequently taken to calculate the total number of alleles per cluster, the mean number of alleles per locus and to count the total number of private alleles per cluster. FSTAT 2.9.3.2 (Goudet 1995) was employed to estimate F_{IS} values and mean values for allelic richness per locus and population. Observed and expected heterozygosities were estimated for each locus in ARLEQUIN and used to calculate the arithmetic means.

Results

mtDNA analyses

In parsimony network analysis, North African *cyt b* sequences were assigned to two distinct clusters (Fig. 2), indicating a previously unknown major phylogeographic break within North Africa. Moroccan sequences clustered together with Iberian haplotypes in mtDNA lineage VI sensu Lenk et al. (1999), and the Moroccan haplotypes had a basal position compared to the Iberian ones. By contrast, Algerian and Tunisian sequences were assigned to mtDNA lineage IX sensu Fritz et al. (2005, 2007), which was known before only from a zoo turtle without locality data. Among the Moroccan sequences were two previously unknown haplotypes (VIh, VIi), and also among the Algerian and Tunisian sequences were two new haplotypes (IXb, IXc).

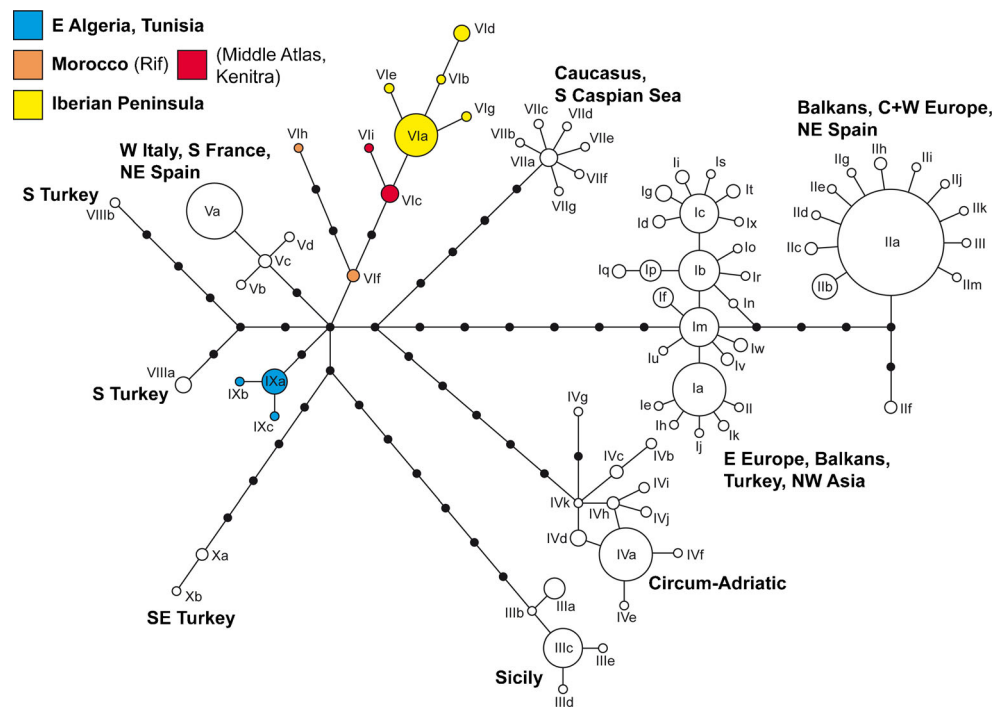
The geographical distribution of haplotypes suggests a weak phylogeographic structuring within each lineage occurring in North Africa. The most common Moroccan haplotype is VIc ($n=16$), which was found in 14 turtles from the Middle Atlas and two from Kenitra Province. Haplotype VIc differs from the most frequent Iberian haplotype VIa in just one mutational step. Also the newly discovered Moroccan haplotype VIi differs in one step from VIc. Haplotype VIi was found in one turtle from the Middle Atlas. Most turtles from the Rif Mountains ($n=9$) harbored haplotype VIi, regardless

of whether the turtles originate from the western or eastern Rif Mountains. One turtle from the Rif Mountains yielded haplotype VIh, which differs in three mutational steps from VIi (Fig. 2; Table S1). The most common haplotype among Algerian and Tunisian sequences is IXa ($n=18$), which was found at all three collecting sites in easternmost Algeria and Tunisia. Haplotype IXb was found twice and occurred only in easternmost Algeria; haplotype IXc was also found twice, but in northeastern Tunisia (Table S1). Haplotypes XIb and XIc differ from XIa in one mutational step each (Fig. 2).

The uncorrected p distance between distinct haplotypes of lineages VI ($n=9$) and IX ($n=3$) amounts on average to 0.66 %. This falls into the lower range observed among the ten mitochondrial lineages of *E. orbicularis* and *E. trinacris* (Table S8). Within lineage VI, an average divergence of 0.31 % is observed, and within lineage IX, 0.13 %. The five Iberian haplotypes of lineage VI differ on average by 0.37 % from the four Moroccan haplotypes of lineage VI. Among the Iberian haplotypes an average divergence of 0.18 % occurs, while the Moroccan haplotypes differ by 0.32 %, reflecting the greater number of mutational steps among Moroccan sequences (Fig. 2).

Phylogenetic analyses using ML and BI and the concatenated mtDNA sequences of 3,475 bp length yielded completely identical trees. They mirrored the differentiation between Moroccan plus Iberian pond turtles on the one hand and Algerian plus Tunisian turtles on the other (Fig. 3). Haplotypes of each group clustered in two well-supported clades (corresponding to haplotypes of mtDNA lineages VI and IX, respectively), which were placed together with haplotypes of mtDNA lineage V (*E. o. galloitalica*, distributed in western Italy, southern France and northeastern Spain) in a well-supported, more inclusive Western Mediterranean clade. This Western Mediterranean clade was with high support sister to a clade comprising the mtDNA lineages I, II, VII, VIII and X from Central and Eastern Europe and Asia. These lineages agree with *E. o. orbicularis*, *E. o. eiselti*, *E. o. persica* and an undescribed subspecies from southern Turkey (Fritz et al. 2009a). The successive sister groups were the circum-Adriatic lineage IV (*E. o. hellenica*) and the Sicilian lineage III (*E. trinacris*). Within the Western Mediterranean clade, the placement of each of the three included clades was badly resolved, and a sister group relationship of the Moroccan-Iberian clade and the Algerian-Tunisian clade was only weakly supported. Within the Moroccan-Iberian clade, haplotypes from the Iberian Peninsula constituted a weakly supported clade, which was firmly embedded within Moroccan sequences. If the mtDNA sequences are related to the currently recognized subspecies of *E. orbicularis* (Fig. 3), the sequences of the North African subspecies *E. o. occidentalis* correspond to two distinct mitochondrial clades (VI and IX). Moreover, *E. o. occidentalis* is paraphyletic with respect to the Iberian subspecies *E. o. fritziuergenobsti*.

Fig. 2 Parsimony network for mitochondrial haplotypes of *Emys orbicularis* (mtDNA lineages I and II, IV–X sensu Lenk et al. 1999; Fritz et al. 2007, 2009a) and *E. trinacris* (mtDNA lineage III) based on 1,031 bp of the *cyt b* gene of 1,596 samples. Symbol size very roughly reflects haplotype frequency. Black circles indicate missing haplotypes; each line connecting haplotypes corresponds to one mutational step. Introduced populations harboring lineage V also occur on Corsica and Sardinia (Pedall et al. 2011) and on the Balearic Islands, where lineage II is found as well (Lenk et al. 1999)



Microsatellite analyses

According to the maximum posterior probability method (Pritchard et al. 2000), the most likely number of clusters (K) for the pond turtles from North Africa and the Doñana National Park (Spain) is four ($K=4$; Fig. S1), whereas the ΔK method (Evanno et al. 2005) suggested $K=2$ (Fig. S2). However, the ΔK values show for $K=4$ a pronounced second peak, giving the maximum posterior probability approach at least some support. Based on this evidence, the inferred population structuring for $K=2$ and $K=4$ is presented here (Fig. 4).

Under $K=2$, all Moroccan turtles are lumped together with the ones from the Doñana National Park (Spain). The second cluster is constituted by Algerian and Tunisian turtles, without any indication of admixture. An AMOVA revealed that 67.6 % of the molecular variance occurred within and 32.4 % between the two clusters, corresponding to an F_{ST} value of 0.324 between the two entities.

Under $K=4$, the Algerian-Tunisian cluster remains unchanged. However, the Moroccan turtles are assigned to two distinct clusters (Rif Mountains vs. Middle Atlas Mountains plus Kenitra) and the Spanish turtles to a fourth cluster, with weak individual admixture among the Moroccan and Spanish clusters. Compared to the turtles from the Doñana National Park, the North African clusters did not show any sign of a reduced genetic diversity (Table S9). According to an AMOVA, 67.5 % of the molecular variance occurred within and 32.5 % among the four clusters. Pairwise F_{ST} values ranged from 0.220 to 0.408 (Table S10). The Algerian-

Tunisian cluster was the most differentiated one, with fixation indices of 0.327–0.408, while between the Moroccan and Spanish clusters lower values were observed (0.220–0.291). It should be noted that the two Moroccan clusters differed by a higher value (0.291) than when each of the two Moroccan clusters was compared to the Spanish one (0.220 and 0.230).

Discussion

Biogeography

Our most prominent finding is that North African pond turtles represent two completely distinct genetic entities (mtDNA lineages VI and IX), which are supported by mitochondrial and microsatellite data (Figs. 2, 3, 4). One entity corresponds to Moroccan pond turtles (lineage VI) and the other to eastern Algerian plus Tunisian turtles (lineage IX).

Moreover, our additional mitochondrial sequence data yield a much better resolved phylogeny compared to previous studies relying on the mitochondrial *cyt b* gene alone (Lenk et al. 1999; Fritz et al. 2005, 2007, 2009a; Spinks and Shaffer 2009) or using three slowly evolving nuclear genes (Spinks and Shaffer 2009). Our data confirm the sister group relationship of *Emys orbicularis* and the Sicilian species *E. trinacris* (Fig. 3; Fritz et al. 2005, 2007, 2009a). The mitochondrial lineages representing the polytypic *E. orbicularis* cluster in three moderately to well-supported clades (Fig. 3) with a branching pattern indicating a hitherto unknown biogeographic scenario. A Western Mediterranean clade includes the two

Fig. 3 Maximum likelihood tree based on 3,475-bp mtDNA of *Emys orbicularis* and *E. trinacris* using the maximum partitioning scheme (Table S4). The topology inferred by Bayesian analyses was completely identical. For *E. orbicularis*, currently recognized subspecies indicated. Note that haplotypes of *E. o. occidentalis* are paraphyletic with respect to *E. o. fritzjuergenobsti*; see Discussion. Numbers above nodes are thorough bootstrap values greater than 50; numbers below nodes, Bayesian posterior probabilities greater than 0.95. Asterisks indicate maximum support under both methods. For each sample, the mitochondrial haplotype is given, preceded by the lab code or in one case by the GenBank accession number (GU646002). Haplotype nomenclature follows Lenk et al. (1999) and Fritz et al. (2005, 2007, 2009a). Identical haplotypes marked with asterisks are identical in their cyt b sequences, but differ elsewhere. Outgroups removed for clarity (branching patterns correspond to expectations; see Wiens et al. 2010)

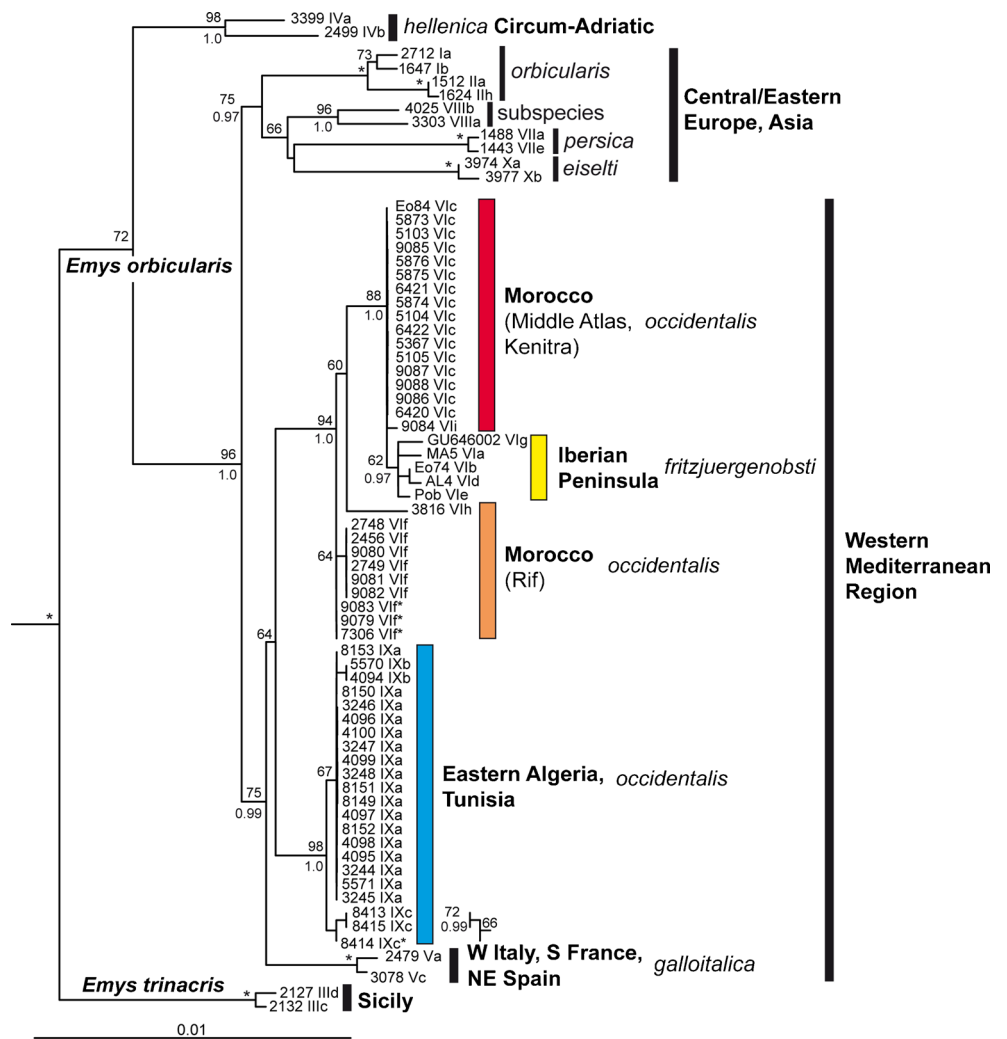
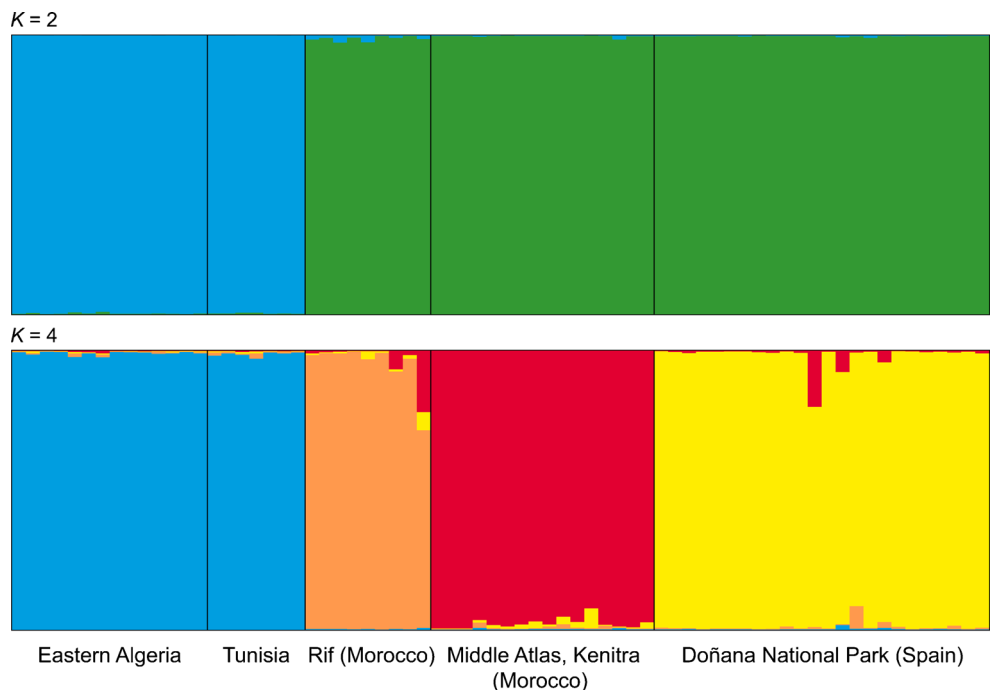


Fig. 4 Inferred population structure of European pond turtles from North Africa and the Doñana National Park (Spain) for $K=2$ (top) and $K=4$ (bottom) using 15 microsatellite loci. Shown are the STRUCTURE runs with the best probability values. Distinct clusters are color-coded. Within each cluster, an individual turtle corresponds to a vertical segment that reflects its ancestry. Mixed ancestries are indicated by differently colored sectors, corresponding to inferred genetic percentages of the corresponding clusters. Sampling regions are separated by black lines



lineages VI (Morocco, Iberian Peninsula) and IX (Algeria, Tunisia) plus lineage V. The latter lineage is distributed along the Mediterranean coast of northern Spain through southern France to western and southern Italy and corresponds to the subspecies *galloitalica* (introduced populations also occur on Corsica and Sardinia; Lenk et al. 1999; Fritz et al. 2005, 2007; Pedall et al. 2011). This well-supported Western Mediterranean clade is sister to another clade containing several mtDNA lineages (I, II, VII, VIII, X) occurring in the eastern part of the distribution range of *E. orbicularis*, and the successive sister group is constituted by lineage IV having a circum-Adriatic distribution. This suggests that there was a sequence of vicariance events, leading first to the separation of the Sicilian *E. trinacris* (lineage III), then to the evolution of lineage IV (*E. o. hellenica*), and then to a split between a western and an eastern stock, which diverged later into the remaining eight mtDNA lineages of *E. orbicularis* identified with all other currently recognized subspecies of *E. orbicularis* (Fig. 3), including the North African populations.

In this context it must be emphasized that it remains entirely unclear when and from where *E. orbicularis* invaded North Africa. The close phylogenetic relationship of the two North African lineages VI and IX with lineage V from the northwestern Mediterranean coast (Fig. 3) suggests that the ancestor of the two North African lineages originated somewhere in western Europe and that lineages VI and IX diverged only in North Africa, because lineage IX is endemic there. Furthermore, we found a very close relationship between Moroccan and Iberian pond turtles (Fig. 4: top), and with respect to mitochondrial data, sequences of Iberian turtles are phylogenetically nested among Moroccan sequences. This relationship is also confirmed by a parsimony network analysis, in which the Moroccan sequences are basal to the Iberian sequences (Figs. 2 and 3), indicating that Iberian pond turtles have evolved from North African founders and not vice versa, as traditionally assumed (Bons and Geniez 1996; Schleich et al. 1996; Lapparent de Broin 2000). The occurrence of two genetically deeply divergent lineages in North Africa, together with the fact that Iberian pond turtles seem to be derived from North African ancestors, implies that European pond turtles have been present in North Africa longer than on the Iberian Peninsula and that after an initial invasion of North Africa by pond turtles from an unknown European source region, there was a phase of diversification in North Africa, followed by a later re-invasion of Europe from Morocco. This fits with the finding that Iberian haplotypes of lineage VI are less diverse than Moroccan ones (Fig. 2), and with a decrease of genetic diversity from south to north in Iberia (Velo-Antón et al. 2008).

Theoretically, there are two source regions for the initial invasion of North Africa, but both are not very likely: (1) The Iberian Peninsula, which would necessitate a later massive

extinction event there, followed by a re-colonization from North Africa, and (2) the Apennine Peninsula and Sicily (as hypothesized in the opposite direction for green toads; Stöck et al. 2008). However, Sicily is occupied by a genetically totally different pond turtle, *E. trinacris* (lineage III), which is not closely related to any African or Western Mediterranean lineage (Fig. 3).

Moreover, fossil evidence currently does not support the long presence of *Emys* in North Africa. Although the fossil record of European pond turtles in Europe is extensive (e.g., Fritz 1995; Hervet 2000; Delfino 2002; Sommer et al. 2007; Bailón 2011; Georgialis and Kear 2013), there are only a few Holocene finds known from North Africa (Algeria and Tunisia; Lapparent de Broin 2000), which was interpreted as evidence for a very recent colonization of North Africa. The oldest records from the Iberian Peninsula are considerably older, dating back to the Lower Pleistocene (approximately 1.5–1.2 million years ago; Fig. S3), and at first glance this seems to support the traditional hypothesis that European pond turtles were present on the Iberian Peninsula for a long time and invaded North Africa from there. However, due to the fragmentary character of the oldest Iberian fossils (Bailón 2011; Blasco et al. 2011), it is impossible to determine whether they represent the ancestor of the extant Spanish and Portuguese pond turtles or possibly another lineage that became extinct and was later replaced by invaders from North Africa. The first Iberian fossils that can be unambiguously morphologically identified with the extant turtles are much younger and only 370,000–320,000 years old (Jiménez Fuentes 1980; Fritz 1995).

Normally, fossils are considered to indicate the long-term occurrence of the relevant species in the fossiliferous region. However, it has been shown twice that putatively long-established local populations of turtles and tortoises became extinct and were replaced much later – in these cases – by introduced conspecifics (*Emys orbicularis* and *Testudo hermanni* in Corsica and Sardinia: Giacalone et al. 2009; Pedall et al. 2011; Perez et al. 2014). Taking this complex situation into account, we believe it is prudent not to consider the current fossil record as evidence for any biogeographic scenario. In addition, this situation prevents us from using the fossil record of *Emys* to date the mitochondrial phylogeny. Our exploratory calculations using fossils of North American emydines lead to such excessive confidence intervals with respect to the *Emys* lineages that we refrain from publishing these results here.

In general, the differentiation pattern found for Iberian and North African pond turtles mirrors a common paradigm for North African amphibians and reptiles, with Moroccan populations often being well-differentiated from their eastern Algerian and Tunisian counterparts (e.g., *Pleurodeles* spp., Carranza and Arnold 2004; Veith et al. 2004; *Hyla meridionalis*, Recuero et al. 2007; *Mauremys leprosa*, Fritz

et al. 2006b; *Testudo graeca*, Fritz et al. 2009b; *Chamaeleo chamaeleon*, Dimaki et al. 2008; *Timon* spp., Paulo et al. 2008; *Coronella girondica*, Santos et al. 2012; *Macroprotodon* spp., Carranza et al. 2004; *Malpolon* spp., Carranza et al. 2006; *Natrix maura*, Guicking et al. 2008; *Vipera latastei* complex, Velo-Antón et al. 2012), while Moroccan and Iberian populations can be often very similar (e.g., *Pleurodeles waltl*, Carranza and Arnold 2004; Veith et al. 2004; *Mauremys leprosa*, Fritz et al. 2006b; *Testudo graeca*, Fritz et al. 2009b; Graciá et al. 2013; *Macroprotodon brevis*, Carranza et al. 2004; *Malpolon monspessulanus*, Carranza et al. 2006). This suggests that the Straits of Gibraltar constitutes no significant biogeographic barrier for these taxa, and this may be especially true for *E. orbicularis*, as indicated by the weak differentiation of pond turtles from southern Spain (Doñana National Park) and Morocco (Fig. 4).

Taxonomic conclusions

Currently, several subspecies are recognized for *Emys orbicularis*. Most of these taxa correspond to distinct mtDNA lineages (Velo-Antón et al. 2008; Fritz et al. 2009a; Pedall et al. 2011; van Dijk et al. 2012). Exceptions are *E. o. occidentalis* from North Africa, thought to include populations from Morocco, Algeria and Tunisia (Fritz 1993), and *E. o. fritzjuergenobsti* from the Iberian Peninsula (Fig. 3).

As shown in the present study, all Moroccan pond turtles belong to the same mitochondrial lineage as Iberian conspecifics (lineage VI), while eastern Algerian and Tunisian turtles represent the distinct mtDNA lineage IX. This differentiation pattern is also supported by microsatellite data. Moreover, concerning mtDNA sequences, Moroccan *E. o. occidentalis* are paraphyletic with respect to *E. o. fritzjuergenobsti* from the Iberian Peninsula.

We therefore conclude that Iberian and Moroccan pond turtles should be better regarded as representing one and the same subspecies. According to the First Reviser Principle (ICZN 1999: Article 24.2), we give the name *Emys orbicularis occidentalis* Fritz 1993 priority over *Emys orbicularis fritzjuergenobsti* Fritz 1993. Morphological differences that were originally thought to differentiate these two subspecies (Fritz 1993) can now be understood as the result of genetic influx from other taxa along the Mediterranean coast of Spain (Pedall et al. 2011).

Eastern Algerian and Tunisian pond turtles cannot be identified with *E. o. occidentalis* and represent a distinct taxon. Considering the degree of differentiation (Figs. 2, 3, 4), eastern Algerian and Tunisian populations should be regarded as a distinct new subspecies for which no name is available (Fritz and Havaš 2007). Unfortunately, our knowledge of external morphology of eastern Algerian and Tunisian pond turtles is deficient. There are only five adult museum specimens known (Fritz 1993) that can be identified with this subspecies. This

situation does not allow a sound morphological comparison of the new subspecies with other taxa. As a consequence, we abstain from its formal description here, acknowledging that this situation is counterproductive for conservational purposes because only named taxa are covered by legislation.

Conservation

Emys orbicularis is a seriously imperiled species in North Africa (Fritz 2003; Fahd et al. 2009; Pleguezuelos et al. 2010). Notwithstanding the generally low individual densities, there is no evidence for a decline in genetic diversity (Table S9), suggesting that conservation measures for the current relicts may still preserve viable populations.

In Morocco, the populations along the Atlantic coast are either extinct or close to extinction because of massive habitat alteration (Fahd et al. 2009), and all remaining populations in other regions are threatened for the same reason. Our sampling sites in the Moroccan Rif Mountains correspond to two disjunct regions, one in the western and the other in the eastern region of this mountain chain (Fig. 1). With respect to mitochondrial haplotypes and microsatellites, we found both isolates not differentiated. By contrast, the only two individuals we studied from Kenitra Province share their mitochondrial haplotypes not with the geographically closer western Rif population, but with the pond turtles from the Middle Atlas Mountains, to which the Kenitra turtles were also assigned using microsatellite data (Figs. 2 and 4). This has important implications for conservation measures, for instance if in future an exchange of individuals between different local populations should be considered.

Our results suggest that the relict populations in the Rif Mountains and from the Middle Atlas Mountains plus Kenitra Province should be treated as two distinct Management Units (Moritz 1994). The pond turtles in Kenitra Province perhaps represent the last survivors of the populations that were formerly widely distributed in the Gharb plain (Fritz 1993; Bons and Geniez 1996; Fahd et al. 2009). Consequently, if habitat improvements and later reintroductions should be ever planned for the Gharb plain, pond turtles from the Rif Mountains should not be used for that purpose.

In Tunisia, we could confirm the presence of a few individuals at only two of the many sites listed in Loveridge and Williams (1957), despite intensive fieldwork. In neighboring Algeria, the El Kala National Park still supports a substantial population, while the situation outside the park is completely unclear. As Algerian and Tunisian pond turtles represent an endemic taxon, we recommend surveying their population status throughout the historical range (Fig. 1) to establish a basis for conservation measures. This could also shed light on the possible survival and taxonomic status of the isolated populations around Algiers (Fritz 1994, 2003).

Acknowledgements Most of the laboratory work was done by Anke Müller. Special thanks go to Martin Päckert for the exploratory molecular clock calculations. Alessandra Sicilia assisted during fieldwork. Oscar Arribas, Massimo Delfino and Aitor Valdeón helped with references for fossils. Guillermo Velo-Antón's research was supported by the Fundação para a Ciência e Tecnologia (SFRH/BPD/74834/2010).

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