

# Mitochondrial phylogeography of *Testudo graeca* in the Western Mediterranean: Old complex divergence in North Africa and recent arrival in Europe

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**Abstract.** We investigated the mitochondrial phylogeography of spur-thighed tortoises (*Testudo graeca*) in the Western Mediterranean. In North Africa, four major lineages (A-D) occur that together constitute a well-supported clade corresponding to one of the six major clades within *T. graeca*; the North African clade is sister to a Caucasian clade representing the subspecies *T. g. armeniaca*. Phylogenetic relationships between the North African lineages are badly resolved. Lineage A is distributed in Tunisia and adjacent Algeria, lineage B in Algeria and northern Morocco, lineage C in the Libyan Cyrenaica Peninsula, and lineage D north of the High Atlas Mts. and in the Souss Valley (southern Morocco). Lineage B is subdivided into two subgroups, B<sub>1</sub> (eastern Morocco and Algeria) and B<sub>2</sub> (north-western Morocco). Italian tortoises harbour haplotypes of lineage A, Spanish tortoises of subgroup B<sub>1</sub>. Based on a relaxed molecular clock calibrated with fossil evidence, the six major mtDNA clades of *T. graeca* are estimated to have diverged approximately 4.2-1.8 Ma ago; the split between the clades representing the eastern subspecies *T. g. iberica* and *T. g. terrestris* is younger than the split between Western Mediterranean tortoises and *T. g. armeniaca*. The Western Mediterranean lineages A-D were dated to have diverged at least 1.4-1.1 Ma ago; B<sub>1</sub> and B<sub>2</sub> split approximately 0.7 Ma ago. Our results suggest that Italian and Spanish tortoises were either introduced or originated from trans-oceanic dispersal in historic or prehistoric times. Spur-thighed tortoises invaded North Africa probably across Near Eastern landbridges that emerged in the Late Tertiary. Their diversification in North Africa seems to be correlated with habitat aridization cycles during the Pleistocene. The ranges of the Western Mediterranean lineages largely correspond to the distribution of morphologically defined subspecies in North Africa, with exception of *T. g. graeca* and *T. g. whitei*, and of *T. g. lamberti* and *T. g. marokkensis*, which are not differentiated. We propose to lump the first two subspecies under the name of *T. g. graeca* and the latter under the name of *T. g. marokkensis*. The complex differentiation of spur-thighed tortoises in North Africa implies that the model of a bipartite east-west differentiation, as proposed for other Maghrebian amphibians and reptiles, may be too simplistic, reflecting incomplete locality sampling rather than actual phylogeographic differentiation.

**Keywords:** Algeria, cytochrome *b* gene, Italy, Libya, Morocco, Spain, Testudinidae, Tunisia.

## Introduction

Spur-thighed tortoises (*Testudo graeca*) represent the most widely distributed species of tortoise in the Western Palaearctic. Their range covers part of three continents (Africa, Europe, Asia) and extends approximately 6500 km in an east-west direction from easternmost Iran to the Moroccan Atlantic coast and about 1600 km in a north-south direction from the Danube Delta to the Libyan Cyrenaica Peninsula. A recent investigation provided evidence for a deep phy-

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logeographic structure, reflecting most likely Upper Tertiary dispersal and vicariance events. Five distinctive mitochondrial lineages occur in the east of the range and at least one additional lineage in the Western Mediterranean, being sister of a Caucasian lineage (Fritz et al., 2007). However, being based on only seven samples from the Western Mediterranean, that study does not allow conclusions about phylogeographic differentiation there. In the eastern part of the range, highly discordant patterns were found between morphological and genetic variation, leading to a revised subspecies delimitation (Fritz et al., 2007).

In the Western Mediterranean, spur-thighed tortoises occur in several isolated regions of north-western Africa and southern Spain; a few populations are also known from Mallorca, Sardinia, and Sicily (fig. 1). Recent taxonomic work based on external morphology resulted in up to seven recognized subspecies for North Africa (Highfield, 1990; Pieh, 2001; Pieh and Perälä, 2002, 2004; see also van der Kuyl et al., 2002, 2005). This suggests considerable phylogeographic differentiation. However, few data have been available for the Western Mediterranean. Some previous molecular investigations focussing on the western part of the species' range used either a geographically restricted sampling (Spain, northern Morocco: Álvarez et al., 2000; Morocco: Harris et al., 2003) or individuals of uncertain geographic provenance (van der Kuyl et al., 2002, 2005; see criticism in Harris et al., 2003; Fritz et al., 2007 and Discussion). Comparing partial cytochrome *b* and 12S rRNA sequences, Álvarez et al. (2000) found southern Spanish and eastern Moroccan tortoises to be indistinguishable, while tortoises from eastern and north-western Morocco were slightly distinct. Álvarez et al. (2000) thought that this variation reflects subspecific differentiation and concluded that Spanish tortoises were either introduced by man or the result of recent oversea dispersal. However, using the 12S rRNA data set of Álvarez et al. (2000) and additional sequences from southern Morocco, Harris

et al. (2003) found so little variation that they concluded that all Moroccan tortoises represent only a single taxonomic unit. The status of the Italian populations was never assessed before using molecular markers.

The present paper is based for the first time on a range-wide sampling for the Western Mediterranean. We use sequence variation of the phylogeographically highly informative cytochrome *b* (cyt *b*) gene and compare our data with previously published sequences of spur-thighed tortoises from the Near and Middle East and southeast Europe (Fritz et al., 2007) to address the following questions: (i) does the phylogeographic structure of Western Mediterranean *T. graeca* correspond to the six or seven recently proposed subspecies in the region, (ii) when did the mitochondrial lineages of *T. graeca* in the Western Mediterranean, the Near and Middle East and southeast Europe diverge, and (iii) are Italian and Spanish tortoises phylogeographically distinct from North African populations?

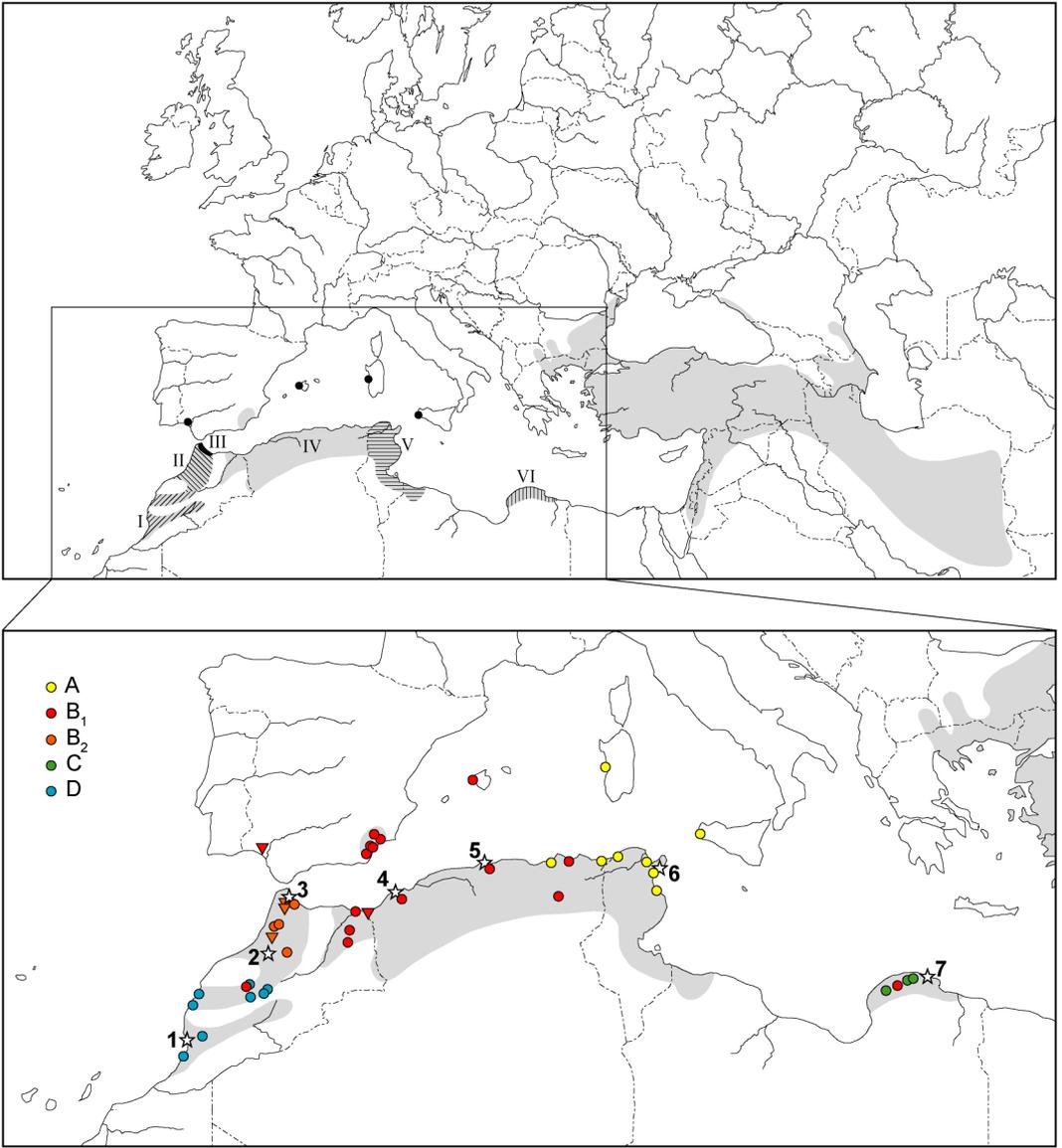
## Materials and methods

### Sampling

Blood, tissue or saliva samples of *Testudo graeca* were collected from localities covering nearly the entire Western Mediterranean part of the species' range and all currently recognized subspecies in that region. We took no samples in the Doñana National Park (south-western Spain) because published information on mitochondrial haplotypes is available (Álvarez et al., 2000; see below). Besides field-collected samples, we also used some captive tortoises or tortoises confiscated at German airports, in so far as their geographic origin was clear, resulting in a total number of 90 studied samples. Remaining DNA and samples are stored in the tissue sample collection of the Museum of Zoology Dresden (fig. 1; Appendix).

### DNA extraction, PCR and sequencing

Total genomic DNA from blood or salivary samples was extracted by overnight incubation at 55°C in lysis buffer (6% DTAB, 1.125 M NaCl, 75 mM Tris-HCl, 37.5 mM EDTA, pH 8.0) including 0.5 mg of proteinase K (Merck, Whitehouse Station, NJ) and subsequent purification following the DTAB method (Gustincich et al., 1991). DNA was precipitated from the supernatant with 0.2 volumes of 4 M LiCl



**Figure 1.** Distribution of *Testudo graeca* (shaded; combined from Loveridge and Williams, 1957; Bannikov et al., 1977; Anderson, 1979; Iverson, 1992; Bons and Geniez, 1996; Buskirk et al., 2001; Disi et al., 2001). Ranges of currently recognized Western Mediterranean subspecies indicated on the top, type localities (asterisks) and sampling sites (circles) on the bottom. Subspecies ranges: I – *Testudo graeca soussensis* Pieh, 2001; II – *T. g. marokkensis* Pieh and Perälä, 2004; III – *T. g. lamberti* Pieh and Perälä, 2004; IV – *T. g. graeca* Linnaeus, 1758; V – *T. g. nabeulensis* (Highfield, 1990); VI – *T. g. cyrenaica* Pieh and Perälä, 2002. Type localities: 1 – *T. g. soussensis*; 2 – *T. g. marokkensis*; 3 – *T. g. lamberti*; 4 – *T. g. graeca*; 5 – *T. g. whitei* Bennett in White, 1836 (designated by Highfield and Martin, 1989; see Discussion); 6 – *T. g. nabeulensis*; 7 – *T. g. cyrenaica*. Colours correspond to figs 2, 3 and indicate Western Mediterranean clades of mtDNA haplotypes. Triangles indicate haplotypes identified by Álvarez et al. (2000), see Results and Discussion. For additional Tunisian samples without precise locality data, see Appendix.

and 0.8 volumes of isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Total genomic DNA from tissue samples was isolated with the peqGOLD Tissue DNA mini-kit (Peqlab, Erlangen, Germany).

Polymerase chain reaction (PCR) was used to amplify an mtDNA fragment containing the complete *cyt b* gene and approximately 20 bp of the adjacent tRNA-Thr gene. Amplification, PCR product purification and sequencing fol-

lowed Praschag et al. (2007); for PCR and sequencing the primers CytbG (Spinks et al., 2004), mt-c-For2, mt-f-na3, and mt-E-Rev2 (Praschag et al., 2007) were used. Sequencing was performed on an ABI 3130 (Applied Biosystems, Foster City, CA). None of the sequences contained internal stop codons, and nucleotide frequencies corresponded to those of coding mtDNA; therefore we conclude that we have amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

#### Phylogenetic and population genealogy analyses

We added to our data set previously published *cyt b* sequences of seven Western Mediterranean *Testudo graeca* and of 87 representatives of eastern *T. graeca* subspecies (for GenBank accession numbers see Fritz et al., 2005, 2007). Using TCS 1.21 (Clement et al., 2000), these 184 sequences were collapsed into haplotypes, resulting in 28 haplotypes for Western Mediterranean *T. graeca* ( $n$  = number of sequences, 97), eight haplotypes for *T. g. armeniaca* ( $n$  = 14), five for *T. g. buxtoni* ( $n$  = 9), 11 for *T. g. iberica* ( $n$  = 20), 24 for *T. g. terrestris* ( $n$  = 37) and four for *T. g. zarudnyi* ( $n$  = 7). Uncorrected  $p$  distances were computed for all haplotypes using PAUP\*4.0b10 (Swofford, 2002) and an AMOVA for the Western Mediterranean sequences was calculated using Arlequin 3.11 (Excoffier et al., 2005). The alignment for calculation of uncorrected  $p$  distances comprised 1058 bp to match the length of sequences of eastern *T. graeca* subspecies.

Our data set of haplotypes was also compared with published *cyt b* sequences (Álvarez et al., 2000) of southern Spanish and northern Moroccan tortoises, but due to the short lengths of these sequences (426 bp), their haplotypes could not be unambiguously determined (see Results and Discussion). Accession numbers of Western Mediterranean haplotypes identified in this study are FM162020-FM162043; the previously published sequences AM230971, AM230972, AJ888343, and AM230965 correspond to unique haplotypes (A1, A5, A9, and B17, respectively).

Phylogenetic analyses were run using a 1164-bp-long alignment including all 28 Western Mediterranean haplotypes, the two most distinct haplotypes of each eastern *T. graeca* subspecies as well as sequences of all other *Testudo* species (*T. g. armeniaca*: accession numbers AM230957, AM230982; *T. g. buxtoni*: AM230961, AM230985; *T. g. iberica*: AJ888351, AM231010; *T. g. terrestris*: AM230964, AM230996; *T. g. zarudnyi*: AM230986, AM230988; *T. hermanni hermanni*: AM230507, AM230515; *T. h. boettgeri*: AM230525, AM230555; *T. horsfieldii kazachstanica*: AJ888365; *T. h. rustamovi*: AJ888366; *T. kleinmanni*: AJ888370, AM398193; *T. marginata*: AJ888318, AJ888332). The distantly related tortoise species *Gopherus agassizii* (AY434562) and *Stigmochelys pardalis* (DQ497301) served as outgroups. Data were analysed under the optimality criteria Maximum Parsimony (MP; equal weighting, command: hs add = cl) and Maximum Likelihood (ML) using PAUP\*4.0b10 and under Bayesian inference of phylogeny (BA) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003; settings: ngen = 1000000 nchains = 4

nrun = 1 sample = 500 temp = 0.2 mcmcdiag = yes diagnfreq = 1000 swapfreq = 1 nswaps = 1 printfreq = 500 saveblens = yes startingtree = random; burn-in set to sample only the plateau of most likely trees). The best evolutionary model was selected by the Akaike information criterion (AIC) using Modeltest 3.06 (K81uf+I+G; Posada and Crandall, 1998). Under parsimony, 787 of 1164 characters were constant and 273 were variable and parsimony-informative; 104 variable characters were parsimony-uninformative. For *T. graeca* only, 979 characters were constant, 118 variable characters were parsimony-informative, and 67 variable characters were singletons. The MP analysis resulted in 216 equally parsimonious trees (tree length = 765; CI = 0.5987, RI = 0.8240) and the ML analysis in six equally likely trees ( $-\ln L$  = 5410.49). Bootstrap support values were calculated with PAUP\*4.0b10 for MP (nreps = 1000) and with GARLI 0.95 for ML (Zwickl, 2006; settings: bootstrapreps = 100 genthreshfortopterm = 5000 ratematrix = 6rate statefrequencies = estimate ratehetmodel = gamma numratecats = 4 invariantsites = estimate).

As intraspecific gene genealogies cannot necessarily be reflected by software enforcing dichotomies (Posada and Crandall, 2001), we also calculated for all haplotypes of Western Mediterranean *T. graeca* a parsimony network using TCS 1.21. Subnets were not connected when a 95% probability threshold was applied, but were connected using a 90% threshold.

#### Molecular clock estimates

Divergence times were estimated with the relaxed Bayesian molecular clock approach as implemented in the Multidistribute package (<http://statgen.ncsu.edu/thorne/multidivtime.html>; Thorne et al., 1998; Thorne and Kishino, 2002; individual programs mentioned below). This method does not require the assumption of constant evolutionary rates among lineages, allowing the use of prior constraints on divergence times. Fossil evidence was used for inferring minimum ages of three nodes within the *Testudo graeca* + (*T. kleinmanni* + *T. marginata*) clade (table 1), assuming that a split event dates back at least to a given fossil age, and no additional speculative periods were added. For instance, the first fossil record of the *T. graeca* group in North Africa dates back to the Upper Pliocene (2.5–2.0 Ma; de Lapparent de Broin, 2000). Hence, the split between North African *T. graeca* and their Caucasian sister taxon *T. g. armeniaca* is at least 2.5–2.0 Ma old.

Two fossil Caucasian tortoise species, *T. burtschaki* and *T. eldarica*, are candidates for the first record of the *T. graeca* group (Danilov, 2005). *Testudo burtschaki* is known from the “Upper Sarmatian” (= Upper Khersonian; see Harzhauser and Piller, 2007) to the Lower Maeotian of eastern Georgia and adjacent Azerbaijan (Chkhikvadze, 2006, 2007), corresponding to an age of 10.0–8.0 Ma (Harzhauser and Piller, 2007). The second species, *T. eldarica*, originates from slightly younger strata (Maeotian, 9.0–6.0 Ma; Gabashvili et al., 2000; Chkhikvadze, 2006, 2007; for dating see Harzhauser and Piller, 2007). While it is generally accepted that *T. eldarica* is closely allied to the extant *T.*

**Table 1.** Calibration points for estimating divergence times within *Testudo graeca*.

Taxon	Group/Remarks	Node	Age (Ma)	Reference
<i>Testudo burtschaki</i>	<i>Testudo graeca</i> group	1 – Split between <i>T. graeca</i> and ( <i>T. kleinmanni</i> + <i>T. marginata</i> )	≥10.0-8.0	See text
<i>Testudo marmorum</i>	<i>Testudo marginata</i> group	2 – Split between <i>T. marginata</i> and <i>T. kleinmanni</i>	≥6.7-6.6	de Lapparent de Broin (2001); for dating Koufos (2003)
<i>Testudo aff. kenitrensis</i>	<i>Testudo graeca</i> group in North Africa	3 – Split between North African <i>T. graeca</i> and <i>T. g. armeniaca</i>	≥2.5-2.0	de Lapparent de Broin (2000)

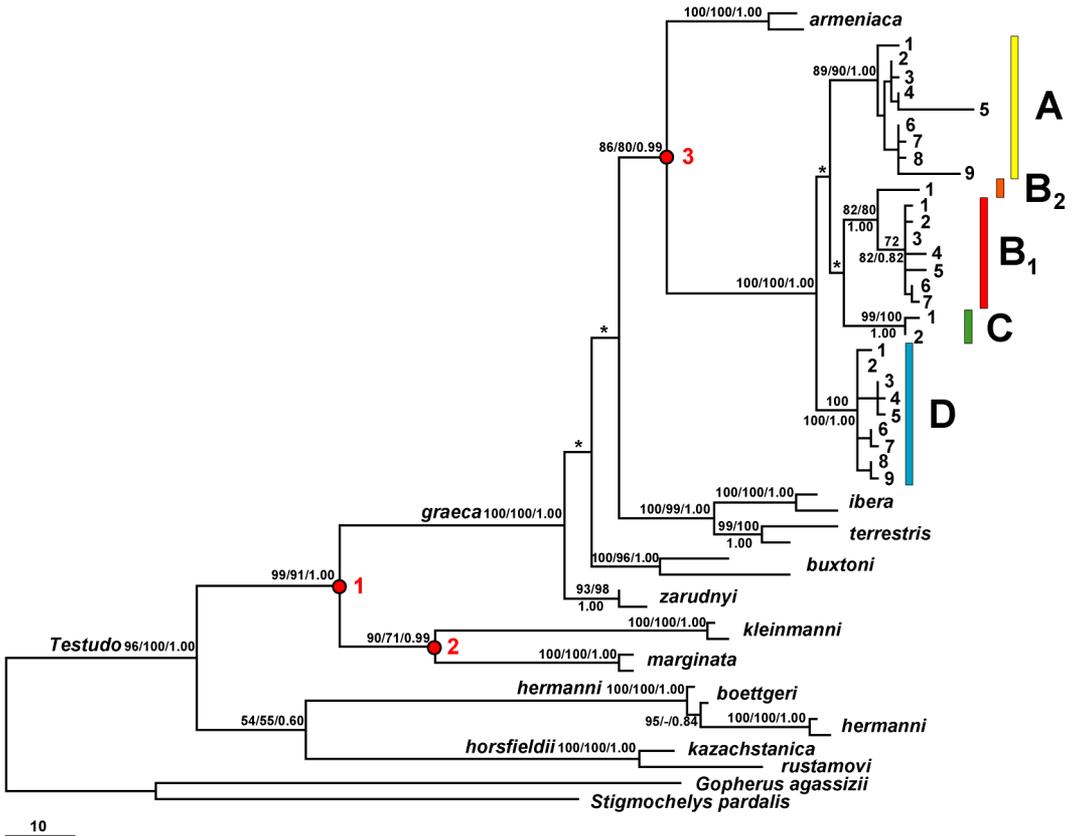
*graeca*, Gabashvili et al. (2000) proposed that *T. burtschaki* represents the ancestor of the extant North-east African and Near Eastern species *T. kleinmanni*, a point of view not shared by de Lapparent de Broin (2001), who believes that *T. kleinmanni* originated in North Africa. Moreover, *T. eldarica* replaces *T. burtschaki* in temporally successive strata in several Caucasian sites (Gabashvili et al., 2000), corroborating that *T. burtschaki* is ancestral to *T. eldarica* and *T. graeca*. Consequently, we treated *T. burtschaki* as representative of the *T. graeca* group and used it for dating the basal split between *T. graeca* and (*T. kleinmanni* + *T. marginata*).

Calculations were based on the tree topology obtained from ML analysis. In order to determine the appropriate nucleotide substitution model parameters, we used the program BaseML (in PAML 3.13; Yang, 1997). Subsequently, branch lengths and their variance-covariance matrix were estimated with the program Estbranches. Markov chains in the application Multidivtime were run with numamps = 10000, sampfreq = 100 and a burn-in of  $1 \times 10^5$  (three independent runs to test the stability of the result; bigtime = 100). The prior for the mean of the ingroup root age (rttm) was set to 9.0 Ma, corresponding to the first record of the *T. graeca* group dated to 10.0-8.0 Ma, with a standard deviation (SD: rttmsd) of also 9.0 Ma to avoid violation of the definition of a prior (San Mauro et al., 2005); mean and standard deviation of the rate of molecular evolution at the ingroup root node (rtrate and rtratesd) corresponded to 0.0099 substitutions per site in 1 Ma with 1 time unit = 1 Ma (calculated with the mean of the branch lengths from Estbranches); mean and standard deviation of the Brownian motion constant (brownmean and brownstd) were set to 0.2 according to the recommendations in the manual. Following the approach of Noonan and Chippindale (2006), we examined the influence of the specified prior for the age of the ingroup root node on posterior estimates of divergence times by conducting additional analyses by increasing or decreasing the prior constraint of the ingroup root node (rttm and also rttmsd) by 25% and adapted the other parameters accordingly (+25%: rttm and rttmsd = 1.125 with 1 time unit = 10 Ma; rtrate and rtratesd = 0.0796; brownmean = 1.30 and -25%: rttm and rttmsd = 6.75 with 1 time unit = 1 Ma; rtrate and rtratesd = 0.0133; brownmean = 0.25).

## Results

### *Phylogenetic and population genealogy analyses*

All tree-building methods yielded an identical topology for *Testudo graeca* (fig. 2); differences occurred only with respect to the position of terminal branches within the major Western Mediterranean lineages. The 28 Western Mediterranean haplotypes form a perfectly supported clade (under all methods bootstrap and posterior probability support of 100%) being sister of the eastern Caucasian subspecies *T. g. armeniaca*. Within the Western Mediterranean clade, four moderately to well-supported lineages were revealed, one of which is further subdivided into two subordinate lineages (named A, B<sub>1</sub>, B<sub>2</sub>, C, and D in figs 1-3). The branching pattern of the lineages remains largely unresolved; only for the sister group relation of lineage B<sub>1</sub> and the distinct haplotype representing lineage B<sub>2</sub> is moderate to high support obtained. With exception of the closely allied haplotypes of B<sub>1</sub> and B<sub>2</sub>, all lineages appear as well-defined haplotype clusters in the parsimony network (fig. 3). The clusters are connected via alternative pathways, reflecting the weak resolution achieved in phylogenetic analyses, and reticulations also occur within lineage B<sub>1</sub>. Lineages B<sub>1</sub> and B<sub>2</sub> are separated by a minimum of 10 mutational steps; the other lineages differ from one another by at least 13-32 steps (table 2). According to the AMOVA, 86.8% of sequence variation occurs between and 13.2% within the lineages ( $F_{st} = 0.868$ ,  $p < 0.001$ ). Average uncorrected  $p$  distances between the lineages range

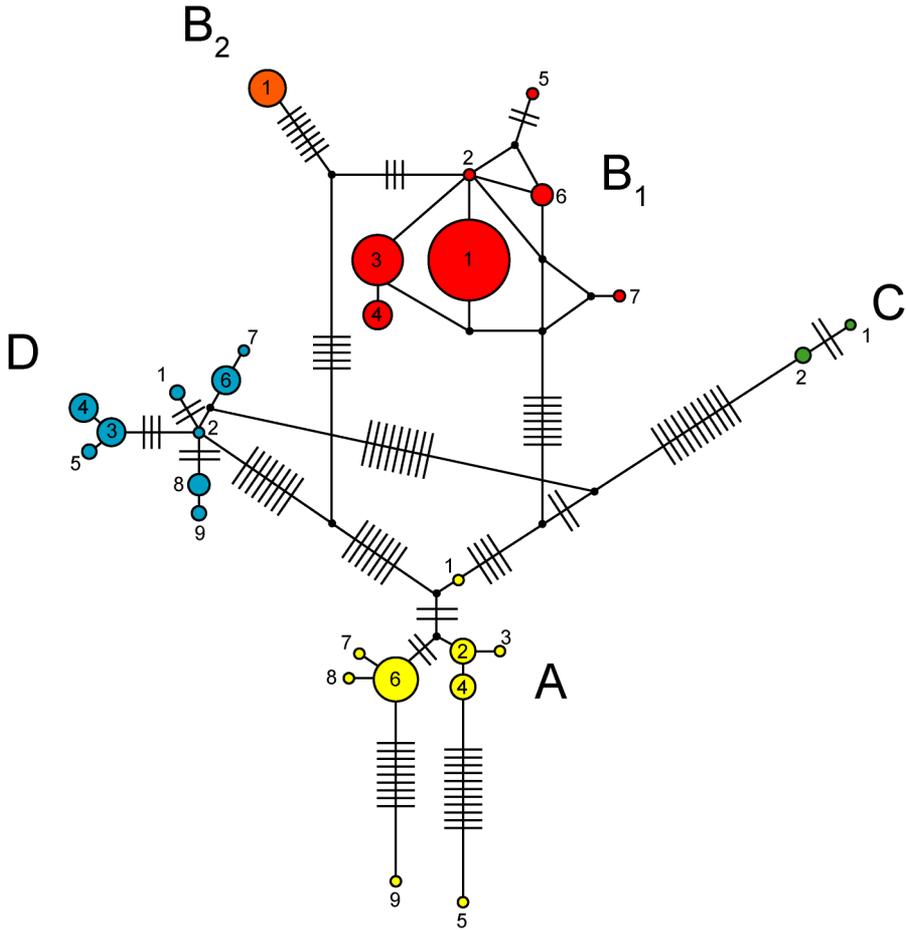


**Figure 2.** One of six obtained ML trees for *Testudo* based on a 1164-bp-long mtDNA fragment comprising the complete cytochrome *b* gene and part of the adjacent tRNA-Thr gene (21 bp), rooted with *Gopherus agassizii* and *Stigmochelys pardalis*. The other five ML trees differed only by the arrangement of the short terminal branches within clade B<sub>1</sub>. Species names of polytypic *Testudo* species are given along branches; terminal names correspond with subspecies or monotypic species. For Western Mediterranean *T. graeca*, haplotype clades A-D are indicated on the right (colours correspond to figs 1 and 3); individual haplotypes of each clade are consecutively numbered. Red circles and numbers at nodes are calibration points (table 1), black numbers along branches, ML and MP bootstrap values and Bayesian posterior probabilities. Asterisks indicate nodes with ML bootstrap support < 50%; these nodes do not occur in 50% majority rule consensus trees of MP and BA. A dash indicates a node within *T. hermanni* not found under MP (see Fritz et al., 2006).

from 1.04%-2.34%, within the lineages from 0.18%-0.76% (table 3). If the average uncorrected *p* distances of Western Mediterranean lineages are compared with eastern *T. graeca* subspecies, it is obvious that the observed variation within all Western Mediterranean tortoises ( $A + B + C + D = 0.50\%$ ) falls into the range observed within individual eastern subspecies (0.20%-1.79%); the Western Mediterranean lineages differ from the eastern subspecies by 4.01%-5.72%, while 2.71%-5.04% results when the eastern subspecies are compared (tables 3, 4).

#### *Distribution of mitochondrial lineages and haplotypes*

Geographically, lineage A occurs in eastern Algeria, Tunisia, Sardinia and Sicily. Lineage B<sub>1</sub> is mainly distributed in Algeria, the neighbouring eastern Morocco, in south-eastern Spain and Mallorca. In eastern Algeria, lineage A and B<sub>1</sub> haplotypes occur in close proximity. Two entirely isolated records of lineage B<sub>1</sub> haplotypes, being associated with haplotypes of other Western Mediterranean lineages, originate from the Libyan Cyrenaica Peninsula and the region



**Figure 3.** Parsimony network for mtDNA haplotypes of Western Mediterranean *Testudo graeca* (1164 bp, *cyt b* and partial tRNA-Thr genes). Haplotype colour corresponds to figs 1 and 2; symbol size, approximate haplotype frequency ( $n = 97$ ; see Appendix). Missing node haplotypes, black. Each line joining haplotypes indicates one nucleotide substitution except when hatches across lines are present; then each hatch indicates one step. Haplotypes identified only from European tortoises are: A9, B11, B12, B14; haplotypes identified from North African and European tortoises: A2, A6, B13, B16. All other haplotypes occur only in North Africa.

of Marrakech (Morocco). Lineage B<sub>2</sub>, represented by only one haplotype, occurs in Morocco northeast and southwest of the Rif Mountains. Lineage C is confined to the Cyrenaica Peninsula and lineage D is distributed in southern Morocco (north of the High Atlas Mts. and in the Souss Valley). The partial *cyt b* sequences published by Álvarez et al. (2000) represent our lineages B<sub>1</sub> and B<sub>2</sub>; their geographic origin matches our findings. The four *cyt b* sequences from the Doñana National Park published by Álvarez et al. (2000) represent lineage B<sub>1</sub> (fig. 1).

Our 26 samples from south-eastern Spain, from at least five distinct populations, correspond to four haplotypes (B<sub>1</sub>1–B<sub>1</sub>4; see Appendix). Our only sample from Mallorca contained haplotype B<sub>1</sub>6. Haplotypes B<sub>1</sub>1, B<sub>1</sub>2 and B<sub>1</sub>4 were only identified from tortoises originating in south-eastern Spain. However, their supposed absence from North Africa could be a bias caused by the small sample of North African tortoises harbouring B<sub>1</sub> haplotypes. Haplotype B<sub>1</sub>3 was also found in samples from some Algerian localities as well as in one sample each from south-western Morocco (central

**Table 2.** Number of mutational steps within and between clusters of Western Mediterranean haplotypes of *Testudo graeca* in parsimony network analysis. On the diagonal, the number of mutational steps within the respective cluster is given in bold; below the diagonal, minimum number of mutational steps between the clusters; *n*, number of haplotypes.

	A	B <sub>1</sub>	B <sub>2</sub>	C	D
A ( <i>n</i> = 9)	<b>1-16</b>				
B <sub>1</sub> ( <i>n</i> = 7)	13	<b>1-6</b>			
B <sub>2</sub> ( <i>n</i> = 1)	20	10	–		
C ( <i>n</i> = 2)	17	22	32	<b>2</b>	
D ( <i>n</i> = 9)	16	16	20	21	<b>1-7</b>

**Table 3.** Uncorrected *p* distances of haplotypes of Western Mediterranean *Testudo graeca* based on a 1058-bp-long alignment of *cyt b* (percentages; mean, minimum and maximum values). Letters indicate haplotype lineages or groups as revealed by phylogenetic and network analyses; on the diagonal, within-lineage distances in bold. Note that haplotypes may differ in positions not included into this alignment; *n* denotes number of identified haplotypes.

	A	B <sub>1</sub>	B <sub>2</sub>	B = B <sub>1</sub> + B <sub>2</sub>	C	D
A ( <i>n</i> = 9)	<b>0.76 (0.09-2.09)</b>					
B <sub>1</sub> ( <i>n</i> = 7)	1.76 (1.41-2.66)	<b>0.18 (0-0.47)</b>				
B <sub>2</sub> ( <i>n</i> = 1)	2.34 (1.98-3.25)	1.04 (0.94-1.23)	–			
B = B <sub>1</sub> + B <sub>2</sub> ( <i>n</i> = 8)	1.83 (1.41-3.25)	–	–	<b>0.40 (0-1.20)</b>		
C ( <i>n</i> = 2)	2.10 (1.79-3.01)	1.89 (1.70-2.17)	1.98 (1.89-2.08)	1.90 (1.70-2.17)	<b>0.19 (–)</b>	
D ( <i>n</i> = 9)	2.03 (1.51-3.12)	1.76 (1.51-1.98)	1.97 (1.79-2.08)	1.78 (1.51-2.08)	1.97 (1.79-2.08)	<b>0.30 (0-0.47)</b>

Jbilet Mountains) and Libya. Haplotype B<sub>1</sub>6 occurred not only in the Mallorcan tortoise, but also in two samples from the mouth of the Moulouya River, Morocco.

Three of our four Sardinian samples yielded haplotype A6; the fourth, A9 (see Appendix). Haplotype A6 also occurred in five Tunisian and one eastern Algerian tortoise, while the distinctive haplotype A9 was only found in the Sardinian sample. The haplotype of our Sicilian sample is A2, also identified from three Tunisian tortoises.

### Molecular clock estimates

All calculations with different prior constraints of the ingroup root age produced very similar results, corroborating the reliability of the estimates so obtained. In the three runs using the correct prior age of root node, the minimum divergence times of eastern *Testudo graeca* subspecies were estimated to date back from 4.10-4.20 ± 0.61-0.71 Ma to 1.79-1.80 ± 0.59-0.60 Ma, with the split between *T. g. iberica* and *T. g. terrestris* being the youngest. The subspecies *T. g. buxtoni* and *T. g. zarudnyi* were estimated

to have branched off 3.61-3.63 ± 0.61-0.62 Ma and 4.10-4.20 ± 0.61-0.71 Ma ago, respectively. The branching event between *T. g. iberica* and *T. g. terrestris* is younger than the split of Western Mediterranean tortoises and their Caucasian sister taxon *T. g. armeniaca* used as calibration point (fig. 2; table 1). For the Western Mediterranean, it was estimated that lineages B<sub>1</sub> and B<sub>2</sub> split 0.66-0.71 ± 0.32-0.34 Ma ago; the divergences of lineages A, B<sub>1</sub> + B<sub>2</sub>, C and D were dated to range from 1.37-1.42 ± 0.42-0.43 Ma to 1.03-1.09 ± 0.37-0.38 Ma.

### Discussion

#### *Are Italian and Spanish spur-thighed tortoises native?*

According to our data, Italian and Spanish *Testudo graeca* are not clearly differentiated from the North African lineages A and B<sub>1</sub>, respectively. For southern Spain, this is in line with the findings by Álvarez et al. (2000). Based on 12S rRNA and partial cytochrome *b* sequences of 19 tortoises from southern Spain (Doñana, Murcia) and northern Morocco (east and west of



Moulouya River), these authors identified only four mitochondrial haplotypes, one of which (haplotype I) was shared between all Spanish and some north-eastern Moroccan tortoises. This haplotype I was found in north-eastern Morocco together with a similar haplotype (II), differing in one nucleotide of its *cyt b* fragment. North-western Moroccan tortoises had the distinct haplotypes III and IV. Even though Álvarez et al. (2000) sequenced only 426 bp of the *cyt b* gene, their haplotype II is identifiable with our haplotype B<sub>16</sub> or an unknown related haplotype differing in the lacking part of the sequence; their haplotype I (eastern Morocco and Spain) could be identical with one of our haplotypes B<sub>12</sub>, B<sub>13</sub>, B<sub>14</sub> or B<sub>15</sub>, all of which are identical in the fragment sequenced by Álvarez et al. (2000). Their haplotypes III and IV differ only in the 12S rRNA fragment but not in the *cyt b* sequence; the latter being identical with our haplotype B<sub>21</sub>.

Although there is a good fossil record for the extant *T. hermanni* and allied extinct taxa in the European part of the Western Mediterranean (e.g., Hervet, 2000, 2001; de Lapparent de Broin, 2001; Delfino, 2002), there is not a single fossil specimen known from this area that resembles the extant *T. graeca* (Bailon, 2001; Abbazzi et al., 2008). In contrast, a number of fossil *T. graeca* and related extinct species were excavated in North Africa (Gmira, 1993; de Lapparent de Broin, 2000). Considering the genetic similarity of Italian, Spanish and North African *T. graeca*, this situation suggests that the European populations were either introduced or originated from trans-oceanic dispersal in historic or prehistoric times. Álvarez et al. (2000) came to the same conclusion for the Spanish populations of *T. graeca*. That trans-oceanic dispersal may happen in tortoises is not only evinced by the presence of giant tortoises (*Chelonoidis nigra*) on the volcanic Galápagos Islands (Caccone et al., 1999a), but also by a recently documented case of a live Aldabra tortoise (*Aldabrachelys gigantea*) washed ashore on the East African coast (Gerlach, 2006). How-

ever, with respect to the Doñana National Park (south-western Spain) oversea dispersal seems highly unlikely because distinct haplotypes occur on the opposite North African seacoast. Parenthetically it may be noted that the observation that Spanish *T. graeca* are never infested by ticks (E. Graciá Martínez and A. Giménez Casalduero, unpubl.) fits into this picture. Normally, wild-living spur-thighed tortoises bear a considerable load of ticks (*Hyalomma aegyptium*; Cheylan, 2001; Široký et al., 2006). Captive tortoises lose their ticks within a short span of time (U. Fritz and P. Široký, unpubl.) and ticks most probably die in seawater.

### Phylogeography

According to our molecular clock estimates, the eastern subspecies of *Testudo graeca* and Western Mediterranean spur-thighed tortoises diverged 4.2-1.8 Ma ago. During this phase landbridges emerged in the Near East, leading to the closure of the Tethyan seaway and the formation of the Mediterranean Basin (Popov et al., 2004). Considering the sister group relationship of North African tortoises and the Caucasian *T. g. armeniaca* as well as the extant high diversity of *T. graeca* in the Caucasus (Fritz et al., 2007), it seems likely that the ancestors of the North African tortoises originated in the Caucasus region and spread over the emerging Middle and Late Pliocene landbridges to Africa.

Genetic differentiation of extant *T. graeca* is pronounced and complex in North Africa. Four to five parapatrically or allopatrically distributed mitochondrial lineages occur that are estimated to have originated during the Early and Middle Pleistocene (approx. 1.4-0.7 Ma ago). This diversification was probably driven by Pleistocene climatic oscillations (deMenocal, 2004; Tzedakis, 2007), especially with respect to increasing aridity connected to North Atlantic cold events (Bout-Roumazeilles et al., 2007) that should have caused repeated range fragmentation by contracting vegetation zones suitable for tortoises.

For many other widespread North African amphibians and reptiles an east-west differentiation has become evident in recent years, coinciding with a deep genetic divergence of Moroccan and Tunisian populations (see review in Barata et al., 2008). Our findings in *T. graeca* suggest that the model of such a bipartite east-west differentiation could be definitely too simplistic, reflecting incomplete locality sampling rather than real geographic differentiation, and that the actual pattern could be more complex in other taxa as well. This is also supported by the recently revealed, complex phylogeography of the tree-frog *Hyla meridionalis* in North Africa (Recuero et al., 2007).

While the mitochondrial lineages of *T. graeca* exhibit a largely mutually exclusive distribution in North Africa, we found a few tortoises with haplotypes not matching the general picture (fig. 1). One interpretation could be that this is the result of ancestral polymorphism or incomplete sorting. Alternatively, introgression or translocation by man could be responsible. One tortoise harbouring a lineage B<sub>1</sub> haplotype was recorded far away from the range of lineage B<sub>1</sub> in the Cyrenaica Peninsula (Libya), where otherwise only lineage C was found. Moreover, the range of lineage B<sub>1</sub> is separated by a distribution gap and the range of lineage A from lineage C, suggesting that this tortoise is not native. It is well-known that *T. graeca* was collected and shipped in enormous numbers for more than a century until the tortoise trade stopped with the implementation of CITES in the 1980s; most of the tortoises were collected in Algeria and Morocco (Groombridge, 1982; Lambert, 1983), where lineage B<sub>1</sub> occurs. At least since the late 19<sup>th</sup> Century spur-thighed tortoises were also sold in Egypt (Anderson, 1898), a country in which *T. graeca* does not occur (Buskirk, 1996; Baha el Din, 2006). These tortoises most probably arrived in Egypt via Libya and some might have been abandoned on their way eastward. Two other cases, the record of a lineage A tortoise within the range of lineage B<sub>1</sub> in eastern Algeria, and the record of a B<sub>1</sub> haplotype within

the range of lineage D, could represent either similarly translocated tortoises or natural introgression along the Mediterranean coast or the Moulouya River Valley.

It should be noted that our finding of a complex phylogeographic differentiation of *T. graeca* in the Western Mediterranean does not contradict the results of Harris et al. (2003), who found little variation when comparing 12S rRNA sequences from northern Morocco and southern Spain, because the 12S rRNA gene is known to be more conserved than *cyt b* in tortoises (e.g., Caccone et al., 1999b; Palkovacs et al., 2002).

#### Taxonomic conclusions

In a previous study, nuclear genomic markers supported the conspecificity of Western Mediterranean spur-thighed tortoises with the eastern taxa of the *Testudo graeca* complex (Fritz et al., 2007). In accordance with the Biological Species Concept (e.g., Mayr, 1963; see also Coyne and Orr, 2004), we understand subspecies as genetically distinct, geographically vicariant groups of populations between which extensive gene flow occurs along contact zones or, in the case of allopatric taxa, potentially may occur. Subspecies constitute evolutionarily significant units and reflect an early stage of speciation in which reproductive isolation is not yet achieved. Well-supported geographic patterns of mtDNA differentiation, like those in *T. graeca*, may provide strong inference about subspecies borders and their evolutionary history (e.g., Avise, 2000; Fritz et al., 2007; Zink and Barrowclough, 2008).

In the Western Mediterranean, six subspecies of *T. graeca* are currently recognized (fig. 1; Fritz and Havaš, 2007). Our present investigation is the first range-wide assessment of their validity using a genetic marker. Previous molecular studies focused on the situation in Morocco and southern Spain (Álvarez et al., 2000; Harris et al., 2003) or used tortoises with imprecise or unknown locality data (van der Kuyl et al., 2002, 2005). By doing so, van der Kuyl

et al. (2002, 2005) considerably contributed to confusion as extensively discussed and clarified in Harris et al. (2003), Perälä (2004) and Fritz et al. (2007). Though we do not intend to repeat this discussion here, it must be pointed out that van der Kuyl et al. (2002, 2005) recognized, in contrast to the other authors, the subspecies *T. g. whitei* (designated type locality: vicinity of Algiers; Highfield and Martin, 1989). In their first study, van der Kuyl et al. (2002) assigned samples from “Algeria” and “Morocco” to *T. g. whitei*, while in their second paper (van der Kuyl et al., 2005) samples of *T. g. whitei* were either lacking locality data or should originate from “Larache [sic], western Morocco”; other Moroccan and Algerian samples were credited to *T. g. graeca* then. It must be noted that Larache (north-western Morocco; approx. 35°12'N 6°09'W) lies within the range of *T. g. marokkensis* (fig. 1). We conclude that the identity of the tortoises labelled as *T. g. whitei* in van der Kuyl et al. (2002, 2005) is doubtful, like that of all their other samples. We studied a sample from the type locality of *T. g. whitei*; it yielded the second most frequent haplotype (B<sub>1</sub>3; see Appendix) of the lineage corresponding with *T. g. graeca*, which is why we propose to place *T. g. whitei* Bennett in White, 1836 into the synonymy of *T. g. graeca* Linnaeus, 1758.

In a previous phylogeographic study on the eastern part of the species' range, we found a major conflict between morphologically defined subspecies and mitochondrial and nuclear genomic markers, necessitating abandoning many taxa (Fritz et al., 2007). In contrast, in North Africa the geographic distribution of the mitochondrial lineages corresponds strikingly well to the putative ranges of most of the recently described subspecies (fig. 1). In the eastern part of the range, habitat type and morphology are closely correlated, for instance with small and light-coloured tortoises being associated with arid habitats and large dark-coloured tortoises with more humid environments. This led to a confusing taxonomic situation because population-specific, ecologically

shaped variation and subspecies characteristics were mixed-up. The better agreement of morphologically delineated subspecies and genetic differentiation in North Africa could be related to a smaller diversity of habitats when compared to the much larger eastern part of the species' range, comprising a large variety of distinct environments in the Near and Middle East and southeast Europe (e.g., frost-free Mediterranean habitats and winter-cold extreme steppe habitats). However, that similar selective forces also may play a certain role in North Africa is suggested by the fact that all the tortoises assigned to '*T. g. whitei*' by some authors (Highfield and Martin, 1989; Highfield, 1990) are extraordinarily large and dark.

As for North Africa, the distribution of lineage A matches the range of *T. g. nabeulensis*; lineage B<sub>1</sub>, the range of *T. g. graeca*; lineage C, the range of *T. g. cyrenaica*; and lineage D, the range of *T. g. soussensis* (fig. 1; Appendix). The subspecies *T. g. lamberti* and *T. g. marokkensis*, however, not only share the same mitochondrial lineage but also the same individual haplotype (B<sub>2</sub>1; see below). Compared to the eastern subspecies of *T. graeca*, the level of differentiation of the Western Mediterranean lineages clearly is lower (tables 3, 4) and the North African radiation took place later than the formation of the five eastern subspecies (approx. 1.4-0.7 Ma vs. 4.2-1.8 Ma). Our phylogenetic and molecular clock analyses suggest that all North African lineages together approximately correspond to the degree of differentiation occurring in each of the five eastern subspecies. Thus, it could be argued that all should be lumped in one subspecies to achieve a classification matching the eastern taxa. Nevertheless, we do not recommend synonymizing all Western Mediterranean subspecies. In lineages A, B<sub>1</sub>, B<sub>2</sub>, C and D, complete or at least nearly complete lineage sorting is already accomplished, indicating a comparatively young, but advanced degree of differentiation. Therefore, we propose to recognize those subspecies as valid whose ranges correspond

with the distribution of one of the mitochondrial lineages, necessitating only synonymizing *T. g. lamberti* and *T. g. marokkensis*. There are also other good reasons for synonymizing these two taxa. Their descriptions (Pieh and Perälä, 2004) were based on small sample sizes, suggesting that the minute morphological differences used for subspecies delineation reflect individual (or population-specific) characters. Furthermore, in contrast to the distribution map in Pieh and Perälä (2004) showing for *T. g. lamberti* and *T. g. marokkensis* two entirely allopatric ranges northeast and southwest of the Rif Mts., there is no distribution gap, but a continuous distribution of tortoises across the Rif Mts. (compare the maps with individual locality records in Lambert, 1983 and Bons and Geniez, 1996; S. Fahd, unpubl.). Considering this situation, we propose to lump these two simultaneously described subspecies under the name of *T. g. marokkensis* Pieh and Perälä, 2004, according to the First Reviser Principle of the International Code of Zoological Nomenclature (ICZN, 1999; Art. 24.2).

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**Appendix.** Samples of Western Mediterranean *Testudo graeca* and their haplotypes. MTD D numbers refer to complete voucher specimens, MTD T to tissue, saliva or DNA samples in the collection of the Museum of Zoology Dresden. Subspecies names of North African tortoises were assigned according to Pieh and Perälä (2002, 2004). Asterisks indicate samples with conflicting haplotypes.

Subspecies	Locality	Haplotype	MTD
<i>Testudo graeca cyrenaica</i> *	Libya: Cyrenaica	B <sub>1</sub> 3	D 42819
<i>Testudo graeca cyrenaica</i>	Libya: Cyrenaica: Al-Kouf	C1	T 4496
<i>Testudo graeca cyrenaica</i>	Libya: Slonta, 32°40.340N 21°47.580E	C2	T 5046
<i>Testudo graeca cyrenaica</i>	Libya: Taknis, 32°17.400N 21°04.220E	C2	T 5047
<i>Testudo graeca graeca</i>	Algeria: Algiers (type locality of <i>T. g. whitei</i> )	B <sub>1</sub> 3	T 4104
<i>Testudo graeca graeca</i>	Algeria: Annaba	B <sub>1</sub> 3	T 4106
<i>Testudo graeca graeca</i> *	Algeria: El Kala	A7	T 3250
<i>Testudo graeca graeca</i>	Algeria: Khenchela	B <sub>1</sub> 3	T 4101
<i>Testudo graeca graeca</i>	Algeria: Khenchela	B <sub>1</sub> 3	T 4102
<i>Testudo graeca graeca</i>	Algeria: Oran (type locality of <i>T. g. graeca</i> )	B <sub>1</sub> 3	T 5144
<i>Testudo graeca graeca</i> *	Algeria: Skikda	A6	T 5145
<i>Testudo graeca graeca</i>	Morocco: NE Outat Oulad el Hajj, 33°37.191N 3°06.390W; 1325.8 m	B <sub>1</sub> 7	T 2294
<i>Testudo graeca graeca</i>	South-eastern Spain	B <sub>1</sub> 2	D 46394
<i>Testudo graeca graeca</i>	Spain: Almería: Centinares	B <sub>1</sub> 4	T 5207
<i>Testudo graeca graeca</i>	Spain: Almería: Centinares	B <sub>1</sub> 4	T 5208
<i>Testudo graeca graeca</i>	Spain: Almería: Centinares	B <sub>1</sub> 4	T 5209
<i>Testudo graeca graeca</i>	Spain: Almería: Centinares	B <sub>1</sub> 4	T 5210
<i>Testudo graeca graeca</i>	Spain: Almería: Centinares	B <sub>1</sub> 4	T 5211
<i>Testudo graeca graeca</i>	Spain: Almería: Malacate	B <sub>1</sub> 1	T 5202
<i>Testudo graeca graeca</i>	Spain: Almería: Malacate	B <sub>1</sub> 1	T 5203
<i>Testudo graeca graeca</i>	Spain: Almería: Malacate	B <sub>1</sub> 1	T 5204
<i>Testudo graeca graeca</i>	Spain: Almería: Malacate	B <sub>1</sub> 1	T 5205
<i>Testudo graeca graeca</i>	Spain: Almería: Malacate	B <sub>1</sub> 1	T 5206
<i>Testudo graeca graeca</i>	Spain: Almería: Palas	B <sub>1</sub> 3	T 5197
<i>Testudo graeca graeca</i>	Spain: Almería: Palas	B <sub>1</sub> 1	T 5198
<i>Testudo graeca graeca</i>	Spain: Almería: Palas	B <sub>1</sub> 1	T 5199
<i>Testudo graeca graeca</i>	Spain: Almería: Palas	B <sub>1</sub> 1	T 5200
<i>Testudo graeca graeca</i>	Spain: Almería: Palas	B <sub>1</sub> 1	T 5201
<i>Testudo graeca graeca</i>	Spain: Murcia: Bas Sur	B <sub>1</sub> 1	T 5187
<i>Testudo graeca graeca</i>	Spain: Murcia: Bas Sur	B <sub>1</sub> 1	T 5188
<i>Testudo graeca graeca</i>	Spain: Murcia: Bas Sur	B <sub>1</sub> 1	T 5189
<i>Testudo graeca graeca</i>	Spain: Murcia: Bas Sur	B <sub>1</sub> 1	T 5190
<i>Testudo graeca graeca</i>	Spain: Murcia: Bas Sur	B <sub>1</sub> 3	T 5191
<i>Testudo graeca graeca</i>	Spain: Murcia: Galera	B <sub>1</sub> 1	T 5192
<i>Testudo graeca graeca</i>	Spain: Murcia: Galera	B <sub>1</sub> 1	T 5193
<i>Testudo graeca graeca</i>	Spain: Murcia: Galera	B <sub>1</sub> 3	T 5194
<i>Testudo graeca graeca</i>	Spain: Murcia: Galera	B <sub>1</sub> 1	T 5195
<i>Testudo graeca graeca</i>	Spain: Murcia: Galera	B <sub>1</sub> 1	T 5196
<i>Testudo graeca graeca</i>	Spain: Mallorca: N Calvià	B <sub>1</sub> 6	D 42822
<i>Testudo graeca graeca</i>	Morocco: Debdou	B <sub>1</sub> 5	T 4451
<i>Testudo graeca graeca</i>	Morocco: Moulouya River Mouth	B <sub>1</sub> 6	T 4447
<i>Testudo graeca graeca</i>	Morocco: Moulouya River Mouth	B <sub>1</sub> 6	T 4448
<i>Testudo graeca lamberti</i> *	Morocco: Ouadrass, S Tétouan (close to type locality of <i>T. g. lamberti</i> )	B <sub>2</sub> 1	T 4182
<i>Testudo graeca lamberti</i> *	Morocco: Ouadrass, S Tétouan (close to type locality of <i>T. g. lamberti</i> )	B <sub>2</sub> 1	T 4183
<i>Testudo graeca lamberti</i> *	Morocco: Ouadrass, S Tétouan (close to type locality of <i>T. g. lamberti</i> )	B <sub>2</sub> 1	T 4184
<i>Testudo graeca marokkensis</i> *	Morocco: Arbaoua, 34°55.375N 5°48.300W; 104 m	B <sub>2</sub> 1	T 5235
<i>Testudo graeca marokkensis</i> *	Morocco: Arbaoua, 34°55.375N 5°48.300W; 104 m	B <sub>2</sub> 1	T 5236

**Appendix.** (Continued).

Subspecies	Locality	Haplotype	MTD
<i>Testudo graeca marokkensis</i> *	Morocco: Lalla Mimouna, 34°51.858N 6°02.996W; 92 m	B <sub>2</sub> 1	T 5237
<i>Testudo graeca marokkensis</i> *	Morocco: Balcon d'Ito, S El Hajeb	B <sub>2</sub> 1	T 4446
<i>Testudo graeca nabeulensis</i>	Tunisia	A4	D 41852
<i>Testudo graeca nabeulensis</i>	Tunisia	A2	D 42883
<i>Testudo graeca nabeulensis</i>	Tunisia	A4	D 42893
<i>Testudo graeca nabeulensis</i>	Tunisia	A5	D 42903
<i>Testudo graeca nabeulensis</i>	Tunisia	A6	D 44857
<i>Testudo graeca nabeulensis</i>	Tunisia	A4	D 44864
<i>Testudo graeca nabeulensis</i>	Tunisia	A2	D 44865
<i>Testudo graeca nabeulensis</i>	Tunisia	A3	D 46397
<i>Testudo graeca nabeulensis</i>	Tunisia	A6	D 46588
<i>Testudo graeca nabeulensis</i>	Tunisia: Djebel Bou Kornine near Hamam Lif, 36°43.590N 10°19.297E; 136 m	A2	T 4151
<i>Testudo graeca nabeulensis</i>	Tunisia: Nabeul (type locality of <i>T. g. nabeulensis</i> )	A4	T 800
<i>Testudo graeca nabeulensis</i>	Tunisia: Sousse	A1	T 149
<i>Testudo graeca nabeulensis</i>	Tunisia: Tabarka	A6	T 5002
<i>Testudo graeca nabeulensis</i>	Tunisia: Tabarka	A6	T 5003
<i>Testudo graeca nabeulensis</i>	Tunisia: Tabarka	A8	T 5004
<i>Testudo graeca nabeulensis</i>	Tunisia: Tabarka	A6	T 5005
<i>Testudo graeca soussensis</i>	Morocco: foothills of Jbel Amsitene, 31°09.263N 9°42.431W; 440 m	D3	T 3815
<i>Testudo graeca soussensis</i>	Morocco: region of Demnate: Iminifri	D5	T 4449
<i>Testudo graeca soussensis</i>	Morocco: region of Demnate: Iminifri	D5	T 4450
<i>Testudo graeca soussensis</i>	Morocco: Sidi Toual, 30°16.060N 9°37.800W	D8	T 5036
<i>Testudo graeca soussensis</i>	Morocco: approx. 25 km N Marrakech, 31°53.131N 7°56.793W; 579 m	D3	T 3817
<i>Testudo graeca soussensis</i>	Morocco: approx. 25 km N Marrakech, 31°53.131N 7°56.793W; 579 m	D1	T 3818
<i>Testudo graeca soussensis</i>	Morocco: central Jbilet Mountains	D6	T 4441
<i>Testudo graeca soussensis</i>	Morocco: central Jbilet Mountains	D1	T 4442
<i>Testudo graeca soussensis</i>	Morocco: central Jbilet Mountains	D6	T 4443
<i>Testudo graeca soussensis</i> *	Morocco: central Jbilet Mountains	B <sub>1</sub> 3	T 4444
<i>Testudo graeca soussensis</i>	Morocco: central Jbilet Mountains	D6	T 4445
<i>Testudo graeca soussensis</i>	Morocco: Essaouira	D4	T 4431
<i>Testudo graeca soussensis</i>	Morocco: Essaouira	D4	T 4432
<i>Testudo graeca soussensis</i>	Morocco: Essaouira	D4	T 4433
<i>Testudo graeca soussensis</i>	Morocco: Essaouira	D4	T 4434
<i>Testudo graeca soussensis</i>	Morocco: Essaouira	D9	T 4435
<i>Testudo graeca soussensis</i>	Morocco: Souss Valley: Admine Forest	D4	T 4436
<i>Testudo graeca soussensis</i>	Morocco: Souss Valley: Admine Forest	D9	T 4437
<i>Testudo graeca soussensis</i>	Morocco: Souss Valley: Admine Forest	D8	T 4438
<i>Testudo graeca soussensis</i>	Morocco: Souss Valley: Admine Forest	D2	T 4439
<i>Testudo graeca soussensis</i>	Morocco: Souss Valley: Admine Forest	D8	T 4440
<i>Testudo graeca soussensis</i>	Morocco: Tafarayate (Tafroute), 31°36.670N 7°30.000W	D3	T 4426
<i>Testudo graeca soussensis</i>	Morocco: Tafarayate (Tafroute), 31°36.670N 7°30.000W	D3	T 4427
<i>Testudo graeca soussensis</i>	Morocco: Tafarayate (Tafroute), 31°36.670N 7°30.000W	D7	T 4428
<i>Testudo graeca soussensis</i>	Morocco: Tafarayate (Tafroute), 31°36.670N 7°30.000W	D3	T 4429
<i>Testudo graeca soussensis</i>	Morocco: Tafarayate (Tafroute), 31°36.670N 7°30.000W	D6	T 4430

**Appendix.** (Continued).

Subspecies	Locality	Haplotype	MTD
<i>Testudo graeca soussensis</i>	Morocco: Ait-Ouir, 31°26.582N 7°46.660W	D6	T 3826
<i>Testudo graeca</i> subspecies uncertain	Italy: Sardinia: Sinis Peninsula	A6	T 5044
<i>Testudo graeca</i> subspecies uncertain	Italy: Sardinia: Sinis Peninsula	A6	T 5045
<i>Testudo graeca</i> subspecies uncertain	Italy: Sardinia: Sinis Peninsula: 5 km NW S. Giovanni di Sinis	A6	T 1112
<i>Testudo graeca</i> subspecies uncertain	Italy: Sardinia: Sinis Peninsula: Putzu Idu	A9	T 1113
<i>Testudo graeca</i> subspecies uncertain	Italy: Sicily: Marsala	A2	T 2230