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Phenotypic plasticity leads to incongruence between morphology-based taxonomy and genetic differentiation in western Palaearctic tortoises (*Testudo graeca* complex; Testudines, Testudinidae)

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Abstract. Tortoises of the *Testudo graeca* complex inhabit a patchy range that covers part of three continents (Africa, Europe, Asia). It extends approximately 6500 km in an east-west direction from eastern Iran to the Moroccan Atlantic coast and about 1600 km in a north-south direction from the Danube Delta to the Libyan Cyrenaica Peninsula. Recent years have seen a rapid increase of recognized taxa. Based on morphological investigations, it was suggested that this group consists of as many as 20 distinct species and is paraphyletic with respect to *T. kleinmanni* sensu lato and *T. marginata*. Based on samples from representative localities of the entire range, we sequenced the mitochondrial cytochrome *b* gene and conducted nuclear genomic fingerprinting with ISSR PCR. The *T. graeca* complex is monophyletic and sister to a taxon consisting of *T. kleinmanni* sensu lato and *T. marginata*. The *T. graeca* complex comprises six well-supported mtDNA clades (A-F). Highest diversity is found in the Caucasian Region, where four clades occur in close neighbourhood. This suggests, in agreement with the fossil record, the Caucasian Region as a radiation centre. Clade A corresponds to haplotypes from the East Caucasus. It is the sister group of another clade (B) from North Africa and western Mediterranean islands. Clade C includes haplotypes from western Asia Minor, the southeastern Balkans and the western and central Caucasus Region. Its sister group is a fourth, widely distributed clade (D) from southern and eastern Asia Minor and the Levantine Region (Near East). Two further clades are distributed in Iran (E, northwestern and central Iran; F, eastern Iran). Distinctness of these six clades and sister group relationships of (A + B) and (C + D) are well-supported; however, the phylogeny of the resulting four clades (A + B), (C + D), E and F is poorly resolved. While in a previous study (Fritz et al., 2005a) all traditionally recognized *Testudo* species were highly distinct using mtDNA sequences and ISSR fingerprints, we detected within the *T. graeca* complex no nuclear genomic differentiation paralleling mtDNA clades. We conclude that all studied populations of the *T. graeca* complex are conspecific under the Biological Species Concept. There is major incongruence between mtDNA clades and morphologically defined taxa. Morphologically well-defined taxa, like *T. g. armeniaca* or *T. g. floweri*, nest within clades comprising also geographically neighbouring, but morphologically distinctive populations of other taxa (clade A: *T. g. armeniaca*, *T. g. ibera*, *T. g. pallasi*; clade D: *T. g. anamurensis*, *T. g. antakyensis*, *T. g. floweri*, *T. g. ibera*, *T. g. terrestris*), while sequences of morphologically similar tortoises of the same subspecies (*T. g. ibera* sensu stricto or *T. g. ibera* sensu lato) scatter over two or three genetically distinct clades (A, C or A, C, D, respectively). This implies that pronounced morphological plasticity, resulting in phenotypes shaped by environmental pressure, masks genetic differentiation. To achieve a more realistic taxonomic arrangement reflecting mtDNA clades, we propose reducing the number of *T. graeca* subspecies considerably and regard in the eastern part of the range five subspecies as valid (*T. g. armeniaca*, *T. g. buxtoni*, *T. g. ibera*, *T. g. terrestris*, *T. g. zarudnyi*). As not all North African taxa were included in the present study, we refrain from synonymizing North African taxa with *T. g. graeca* (mtDNA clade B) that represents a further valid subspecies.

Introduction

Tortoises of the genus *Testudo* Linnaeus, 1758 are distributed over most of the southwestern

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Palaearctic Region (Ernst et al., 2000; Fritz and Cheylan, 2001). The greatest portion of this area is inhabited by representatives of the *Testudo graeca* complex. These chelonians are also known as spur-thighed tortoises, a name referring to two prominent horny thigh tubercles present in most individuals. The patchy range of spur-thighed tortoises covers part of three continents (Africa, Europe, Asia) and extends approximately 6500 km in an east-west direction from eastern Iran to the Moroccan Atlantic coast and about 1600 km in a north-south direction from the Danube Delta to the Libyan Cyrenaica Peninsula (fig. 1). Spur-thighed tortoises occur under very different climatic and environmental conditions, ranging from a Mediterranean climate with mild, frost-free winters to an extreme continental steppe climate with severe winter frost.

While *T. graeca* Linnaeus, 1758 was long accepted as single polytypic species with four to seven subspecies (Mertens, 1946; Wermuth, 1958; Wermuth and Mertens, 1961, 1977; Anderson, 1979; Ernst and Barbour, 1989; Gasperetti et al., 1993; Fritz et al., 1996; Ernst et al., 2000; Buskirk et al., 2001; Fritz and Cheylan, 2001), its taxonomy has fluctuated greatly in recent years. Based on morphology, many species and subspecies were described or resurrected, in part in grey literature (Chkhikvadze and Tuniyev, 1986; Weissinger, 1987; Highfield and Martin, 1989a, b, c; Highfield, 1990; Chkhikvadze and Bakradze, 1991, 2002; Perälä, 1996, 2002a; Pieh, 2001; Pieh and Perälä, 2002, 2004; van der Kuyl et al., 2002; table 1), and some authors suggested that as many as 20 distinct species were traditionally lumped under *T. graeca* (Bour, 1989; Highfield and Martin, 1989a, b, c; Highfield, 1990; Gmira, 1993a, b, 1995; David, 1994; Pieh, 2001; Perälä, 2002a, b; Pieh and Perälä, 2002, 2004; see also Guyot-Jackson, 2004). In contrast, Fritz et al. (1996) argued that most taxa within the *T. graeca* complex are badly defined and that two distinct lineages exist, one including the populations of small to moderately sized tortoises with light coloration occurring in Spain, North Africa and along the Levantine coast, and a second lineage consisting of the populations with larger and darker coloured tortoises from the rest of the range. Gmira (1993a, b, 1995) and Perälä (2002b) proposed that species of the *T. graeca* complex are paraphyletic with respect to two other traditional *Testudo* species that were never considered part of the *T. graeca* complex (*T. kleinmanni* Lortet, 1883 sensu lato, *T. marginata* Schoepff, 1792). As a consequence, systematics and taxonomy in these tortoises became a much debated field (Gasperetti et al., 1993; Ernst et al., 2000; Buskirk et al., 2001; Fritz and Cheylan, 2001; van der Kuyl et al., 2002, 2005; Harris et al., 2003; Guyot-Jackson, 2004; Perälä, 2004a; Semyenova et al., 2004; Carretero et al., 2005; Korsunenko et al., 2005). In this study we treat all taxa within the *T. graeca* complex provisionally as subspecies of *T. graeca*.

Some recent studies on mtDNA sequence variation of *T. graeca*, mainly in the western Mediterranean (Álvarez et al., 2000; van der Kuyl et al., 2002, 2005; Harris et al., 2003), used the slowly evolving mitochondrial 12S rRNA gene and in part 426 bp of the cytochrome *b* gene (Álvarez et al., 2000) and 411 bp of the D-loop (van der Kuyl et al., 2005). These studies suggested distinctly less taxonomic differentiation than morphological investigations. In contrast, Semyenova et al. (2004) and Korsunenko et al. (2005) found four eastern subspecies (*T. g. ibera*, *T. g. nikolskii*, *T. g. pallasi*, *T. g. terrestris*) distinct using nuclear genomic fingerprinting (Randomly Amplified Polymorphic DNA = RAPDs). If it is considered that RAPDs may reflect population-specific, and not taxon-specific, differentiation (e.g. Haig et al., 1994, 1997; Prior et al., 1997; Tassanakajon et al., 1997; Gomes et al., 1998; Vanlerberghe-Masutti and Chavigny, 1998; Palkovacs et al., 2003), these contradictory findings are not surprising.

Here we present for the first time a range-wide phylogeography for the *T. graeca* complex that includes most nominal taxa (table 1).

Figure 1. Range of the *Testudo graeca* complex (shaded) and collection sites of samples. Neighbouring localities combined; for exact localities see table 2. Symbols correspond to distinct mtDNA clades. Range according to Gasperetti et al. (1993) and Buskirk et al. (2001); some records of introduced tortoises omitted.

We used the mitochondrial cytochrome *b* gene that in testudinids is phylogenetically more informative than the 12S rRNA gene and a powerful tool for revealing their matrilineal differentiation (Caccone et al., 1999; Palkovacs et al., 2002; Austin et al., 2003; Fritz et al., 2005a, 2006). To find out whether gene flow takes place between populations harbouring different matrilineages, we applied ISSR (Inter-Simple Sequence Repeat) nuclear genomic fingerprinting, a technique that has been shown to be useful for *Testudo* species (Fritz et al., 2005a) and other chelonians as well (Wink et al., 2001; Guicking et al., 2002; Schilde et al., 2004; Fritz et al., 2005b). ISSR PCR produces species-specific nuclear fingerprints in a wide range of organisms (e.g. Gupta et al., 1994; Zietkiewicz et al., 1994; Wink et al., 1998; Bornet and Branchard, 2001; Fritz et al., 2005a, b; Hundsdörfer and Wink, 2005), allowing identification of interspecific hybrids (Wink et al., 2001; Schilde et al., 2004), and of gene flow and introgression (Wolfe et al., 1998; Nagy et al., 2003; Fritz et al., 2005b).

Materials and methods

Sampling

We studied 94 samples from localities covering most of the distribution range of the *Testudo graeca* complex (fig. 1; table 2). Blood samples were obtained by coccygeal vein puncture of wild or captive tortoises. Alternatively, muscle tissue was extracted from thighs of frozen carcasses prior to alcohol preservation. Samples were either preserved in an EDTA buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) or in ethanol, and stored at −20°C until processing. Remaining blood, tissue and DNA samples are permanently kept at −80°C in the blood and tissue sample collection of the Museum of Zoology, Dresden.

DNA extraction, marker gene amplification and sequencing

Total genomic DNA was isolated by an overnight incubation at 37°C in lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl, 1% SDS) including 1 mg of proteinase K (Roth or Merck), followed by purification with either the DTAB method (Gustincich et al., 1991) or a phenol-chloroform protocol (Sambrook et al., 1989). The primers mt-A1 5′-CCC CCT ACC AAC ATC TCA GCA TGA TGA AAC TTC G-3′ or mt-a-neu2 5′-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC-3′ and mtf-na 5′-AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC CAA TG-3′ or mt-Fr 5′-CTA AGA AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA-3′ were used for amplification of an approximately 1150 bp long mtDNA

Table 1. Taxa of the *Testudo graeca* complex. Several authors believe that further taxa exist but assign no names (two additional Levantine taxa within *T. g. antakyensis*: Perälä, 2002a, b; further taxa within *T. g. ibera*: Perälä, 2002b; Pieh et al., 2002; a further taxon in Turkmenia: Pieh and Perälä, 2001). It is debated whether the name *Testudo whitei* Bennett in White, 1836 is applicable to any North African population or not (Pieh and Perälä, 2002, 2004; van der Kuyl et al., 2002, 2005; Perälä, 2004a). Pieh and Perälä (2002, 2004) treat *T. whitei* as doubtful name that cannot be allocated to a certain population, while van der Kuyl et al. (2002, 2005) use *T. g. whitei* for several North African populations from within the range of other taxa. Moreover, van der Kuyl et al. (2002) proposed that Sardinian *T. graeca* might represent an additional subspecies although they did not report morphological or genetic distinction. Validity of *Testudo flavominimaralis* Highfield and Martin, 1989 is generally not accepted (Ernst et al., 2000; Buskirk et al., 2001; Pieh and Perälä, 2002, 2004; van der Kuyl et al., 2002, 2005). (+) Samples of respective taxon studied for present paper, (−) not studied, (?) unclear, see above. MSL = maximum shell length. MSL for *Testudo graeca graeca* from Pieh and Perälä (2004), for *T. g. ibera* sensu lato from Buskirk et al. (2001), for *T. g. lamberti* and *T. g. marokkensis* from Pieh and Perälä (2004), and for *T. g. whitei* from Ernst et al. (2000); other data from Guyot-Jackson (2004). Distribution ranges as defined in original descriptions, if not otherwise stated.

	Taxon	MSL (mm)	MtDNA clade	Type locality	Approximate range
+	*Testudo graeca anamurensis* Weissinger, 1987	267	D	Anamurum, southern Turkey	Central Mediterranean coast of Turkey
+	*Testudo graeca antakyensis* Perälä, 1996	250	D	Antakya, southeastern Turkey	Levant without central and southern coastal part (Perälä, 2002a)
+	*Testudo graeca armeniaca* Chkhikvadze and Bakradze, 1991	258	A	Megri, southeastern Armenia	Araxes Valley and adjacent regions in Turkey, Armenia, Azerbaijan, and Iran (Pieh et al., 2002)
+	*Testudo graeca buxtoni* Boulenger, 1921	267	E	Manjil, between Resht and Kaswin, Iran	Southwestern corner of Caspian Sea, Iran (Perälä, 2004b)
+	*Testudo graeca cyrenaica* Pieh and Perälä, 2002	205	B	Darnah, eastern Libya	Cyrenaica Peninsula, Libya
+	*Testudo graeca floweri* Bodenheimer, 1935	154	D	Negev, probably vicinity of Gaza, Palestine (restricted by Bour, 1989, but see Perälä and Shacham, 2004)	Coastal plains of Gaza, Israel and Lebanon (Perälä, 2002a; Perälä and Shacham, 2004)
+	*Testudo graeca graeca* Linnaeus, 1758	below 214	B	Santa Cruz near Oran, Algeria (restricted by Mertens and Müller, 1928)	Northeastern Morocco (eastern part of Mediterranean coast and adjacent inland regions), Algeria (Pieh and Perälä, 2004)
+	*Testudo graeca ibera* Pallas, 1814	sensu stricto: 260 sensu lato: 358	sensu stricto: A, C sensu lato: A, C, D	Tbilisi, Georgia (designated by Bour, 1987)	Sensu stricto: Kura River Basin, Caucasus (Perälä, 2002a; Danilov and Milto, 2004); *ibera* sensu lato includes also southeastern Europe and vast parts of Asia Minor (Buskirk et al., 2001)
−	*Testudo graeca lamberti* Pieh and Perälä, 2004	214	?	22 km north of Tétouan, Morocco	Northern Morocco (western part of Mediterranean coast)
−	*Testudo graeca marokkensis* Pieh and Perälä, 2004	237	?	Tarmilete, Morocco	Northwestern Morocco (Atlantic coast and adjacent inland region)

Table 1. (Continued).

	Taxon	MSL (mm)	MtDNA clade	Type locality	Approximate range
+	*Testudo graeca nabeulensis* (Highfield, 1990)	below 180	B	Forest 7-8 km northwest of Nabeul, in direction of Grombalia, Tunisia (restricted by Pieh and Perälä, 2004)	Tunisia, adjacent Libya (Pieh and Perälä, 2002)
+	*Testudo graeca nikolskii* Chkhikvadze and Tuniyev, 1986	297	C	Nebug Settlement, Tuapse County, Krasnodar District, Russia	Northern Black Sea coast of Russia and Georgia (Buskirk et al., 2001)
+	*Testudo graeca pallasi* Chkhikvadze and Bakradze, 2002	247	A	Gilyary-Dag Settlement, Dagestan, Russia	Caspian Sea coastal area in Dagestan and adjacent Azerbaijan (Danilov et al., 2004)
+	*Testudo graeca perses* Perälä, 2002	240	E, F	3 miles W Lālābād Village, 25 miles NW Kermānshāh, Kermānshāhān Province, western central Iran	Zagros Mountains (Iran) and adjacent mountain chains
−	*Testudo graeca soussensis* Pieh, 2001	249	?	Agadir, Morocco	Southwestern Morocco, region of Marrakech and region southwest of High Atlas Mountains (Pieh and Perälä, 2004)
+	*Testudo graeca terrestris* Forsskål, 1775	254	D	Aleppo, Syria (by neotype designation; Perälä and Bour, 2004)	Northern Mesopotamia (Bour and Perälä, 2004; Perälä and Bour, 2004)
?	*Testudo graeca whitei* Bennett in White, 1836	292	?	Algiers and its environs, Algeria (designated by Highfield and Martin, 1989b, but see remarks)	See remarks
+	*Testudo graeca zarudnyi* Nikolsky, 1896	275	F	Bīrjand, Khorāsān Province, northeastern Iran (restricted by Perälä, 2002a)	Eastern Iran (Perälä, 2002a)

Table 2. Studied samples of the *Testudo graeca* complex and outgroups. Question marks denote uncertain taxonomic allocations due to collection sites along range borders (for Adiyaman, Turkey, see Türkozan et al., 2003). MTD D numbers refer to complete voucher specimens, MTD T numbers to blood, tissue or DNA samples in the collection of the Museum of Zoology, Dresden. Provisional HD numbers of samples studied by Fritz et al. (2005a) replaced by permanent MTD numbers. Accession numbers refer to mtDNA sequences.

Sample	Taxon	Locality	MTD	Accession number	ISSR $(GAA)_5$	ISSR $(GACA)_4$	ISSR L18
ANAMURENSIS 1	*Testudo graeca anamurensis*	Turkey: Anamurum; 36°03′N 32°51′E	**T 257**	**AJ888347**			
ANAMURENSIS 2	*Testudo graeca anamurensis*	Turkey: Gazipaşa; 36°17′N 32°18′E	**T 259**	**AJ888348**			
ANAMURENSIS 3	*Testudo graeca anamurensis*	Turkey: Gazipaşa; 36°17′N 32°18′E	**T 258**	**AM230946**			
ANAMURENSIS 4	*Testudo graeca anamurensis*	Turkey: Sahayi Sitige S Serik and Aspendos; 36°50′02″N 31°09′01″E	**T 151**	**AJ888356**	+	+	+
ANAMURENSIS 5	*Testudo graeca anamurensis*	Turkey: Side; 36°46′52″N 31°23′56″E	**T 610**	**AM231002**	+	+	+
ANAMURENSIS 6	*Testudo graeca anamurensis*	Turkey: Side; 36°47′18″N 31°24′25″E	**T 609**	**AM230948**	+	+	+
ANAMURENSIS 7	*Testudo graeca anamurensis*	Turkey: W Side; 36°49′N 31°21′E	**T 253**	**AM231003**	+	+	
ANAMURENSIS 8	*Testudo graeca anamurensis*	Turkey: W Side; 36°49′N 31°21′E	**T 254**	**AM231004**	+	+	+
? ANAMURENSIS 9	*Testudo graeca anamurensis?*	Turkey: Mersin; 36°49′N 34°39′E	**T 260**	**AM231005**			
ANTAKYENSIS 1	*Testudo graeca antakyensis*	Israel: Tiberias; 32°48′N 35°31′E	**T 1644**	**AM230947**			
ANTAKYENSIS 2	*Testudo graeca antakyensis*	Israel: Tiberias; 32°48′N 35°31′E	**T 1376**	**AJ888344**			
ANTAKYENSIS 3	*Testudo graeca antakyensis*	Israel: Tiberias; 32°48′N 35°31′E	**T 1377**	**AJ888345**			
ANTAKYENSIS 4	*Testudo graeca antakyensis*	Jordan: Jarash; 32°11′N 35°51′E	**T 1380**	**AJ888346**			
ANTAKYENSIS 5	*Testudo graeca antakyensis*	Syria: Anti Lebanon Mts. (Jabal esh Sharqi): Ma'lūlā; 33°51′N 36°33′E	**T 2179**	**AM230949**			
ANTAKYENSIS 6	*Testudo graeca antakyensis*	Syria: Anti Lebanon Mts. (Jabal esh Sharqi): Şaydnāyā; 33°42′N 36°22′E	**T 2174**	**AM230950**			
ANTAKYENSIS 7	*Testudo graeca antakyensis*	Syria: Jabal al Nuşayrīyah: Ayn al Baydāh; 34°59′N 36°20′E	**T 2183**	**AM230951**	+	+	+
ANTAKYENSIS 8	*Testudo graeca antakyensis*	Syria: Jabal al Nuşayrīyah: Jourine (at Qalat Merza); 35°39′N 36°16′E	**T 2188**	**AM230952**			
ANTAKYENSIS 9	*Testudo graeca antakyensis*	Syria: Jabal al Nuşayrīyah: Maşyāf; 35°04′N 36°21′E	**T 2181**	**AM230953**	+	+	+
ANTAKYENSIS 10	*Testudo graeca antakyensis*	Syria: Jabal Durūz: Al Kafr; 32°39′N 36°38′E	**T 2169**	**AM230954**			
ANTAKYENSIS 11	*Testudo graeca antakyensis*	Syria: Jabal Durūz: As Suwaydā'; 32°43′N 36°35′E	**T 2162**	**AM230955**			
ANTAKYENSIS 12	*Testudo graeca antakyensis*	Syria: Jabal Durūz: half-way As Suwaydā' to Saleh; 32°41′N 36°41′E	**T 2163**	**AM230956**			
ARMENIACA	*Testudo graeca armeniaca*	Turkey: Araxes River Valley: Vilayet Iğdır: Melekli; 39°57N' 44°06′E	**T 2095**	**AM230957**	+	+	+
BUXTONI 1	*Testudo graeca buxtoni*	Iran: Nowshar near Manjil; 36°44′03″N 49°25′12″E	**T 2260**	**AM230958**		+	
BUXTONI 2	*Testudo graeca buxtoni*	Iran: S Resht: between Saravan and Rostamabad; 36°57′N 49°33′E	**T 2265**	**AM230959**	+	+	+
BUXTONI 3	*Testudo graeca buxtoni*	Iran: S Resht: between Saravan and Rostamabad; 36°57′N 49°33′E	**T 2267**	**AM230960**			
BUXTONI 4	*Testudo graeca buxtoni*	Iran: S Resht: between Saravan and Rostamabad; 36°57′N 49°33′E	**T 2268**	**AM230961**	+	+	+
BUXTONI 5	*Testudo graeca buxtoni*	Iran: Sefid Rud; 37°22′20″N 48°08′10″E	**T 1999**	**AM230962**	+	+	+
CYRENAICA	*Testudo graeca cyrenaica*	Libya: Cyrenaica, approx. 32°25′N 21°20′E	**D 42819**	**AJ888341**	+	+	+

Table 2. (Continued).

Sample	Taxon	Locality	MTD	Accession number	ISSR $(GAA)_5$	ISSR $(GACA)_4$	ISSR L18
FLOWERI 1	*Testudo graeca floweri*	Israel: Bet Zevi, Karmel Mts.; 32°43′N 34°58′E	**T 1374**	**AM230963**			
FLOWERI 2	*Testudo graeca floweri*	Israel: Bet Zevi, Karmel Mts.; 32°43′N 34°58′E	**T 1375**	**AM231006**			
FLOWERI 3	*Testudo graeca floweri*	Israel: Rosh Ha'Ayin; 32°07′N 34°58′E	**T 1378**	**AM230964**	+	+	+
FLOWERI 4	*Testudo graeca floweri*	Israel: Rosh Ha'Ayin; 32°07′N 34°58′E	**T 1379**	**AM231007**	+		+
GRAECA	*Testudo graeca graeca*	Morocco: NE Otat Oulad el Hajj; 33°37′20″N 03°06′39″W	**T 2294**	**AM230965**	+	+	+
MALLORCA	*Testudo graeca graeca* s. l.	Spain: Mallorca: N Calvià; 39°36′N 02°31′E	**D 42822**	**AJ888342**			
SARDINIA	*Testudo graeca graeca* s. l.	Italy: Sardinia: Sinis Peninsula; approx. 40°00′N 08°25′E	**T 1113**	**AJ888343**			
SICILY 1	*Testudo graeca graeca* s. l.	Italy: Sicily: Marsala; 37°48′N 12°27′E	**T 2230**	**AM230966**			
IBERA 1	*Testudo graeca ibera*	Azerbaijan: Katekh; 41°39′N 46°34′E	**T 1461**	**AM231008**	+		+
IBERA 2	*Testudo graeca ibera*	Azerbaijan: Katekh; 41°39′N 46°34′E	**T 1462**	**AM231009**	+	+	+
IBERA 3	*Testudo graeca ibera*	Azerbaijan: SW Beylaqan, near Dashburun; 39°43′N 47°34′E	**T 1446**	**AM230969**	+	+	+
IBERA 4	*Testudo graeca ibera*	Azerbaijan: SW Beylaqan, near Dashburun; 39°43′N 47°34′E	**T 1447**	**AM230967**	+	+	
IBERA 5	*Testudo graeca ibera*	Azerbaijan: SW Beylaqan, near Dashburun; 39°43′N 47°34′E	**T 1448**	**AM230968**	+	+	+
IBERA 6	*Testudo graeca ibera*	Bulgaria: Albena; 43°22′27″N 28°05′00″E	**T 724**	**AM231010**			
IBERA 7	*Testudo graeca ibera*	Bulgaria: Albena; 43°22′53″N 28°04′97″E	**T 725**	**AJ888349**			
IBERA 8	*Testudo graeca ibera*	Bulgaria: Albena; 43°22′37″N 28°05′14″E	**T 727**	**AJ888350**			
IBERA 9	*Testudo graeca ibera*	Bulgaria: Pirin; 41°33′N 23°34′E	**T 1365**	**AM231011**			
IBERA 10	*Testudo graeca ibera*	Bulgaria: Železino; 41°28′N 25°56′E	**T 2332**	**AM231012**			
IBERA 11	*Testudo graeca ibera*	Georgia: Mtskheta; 41°50′N 44°43′E	**D 40654**	**AM231013**	+		+
IBERA 12	*Testudo graeca ibera*	Greece: 15 km E Alexandroúpolis; 40°50′N 26°00′E	**T 1615**	**AM231014**	+	+	+
IBERA 13	*Testudo graeca ibera*	Greece: between Dráma and Xanthi; 41°11′N 24°37′E	**T 1614**	**AM231015**	+	+	+
IBERA 14	*Testudo graeca ibera*	Greece: Evros Delta, approx. 25 km E Alexandroúpolis; 40°46′N 26°05′E	**T 1616**	**AM231016**			
IBERA 15	*Testudo graeca ibera*	Greece: Kos Island; 36°52′37″N 27°17′76″E	**T 821**	**AJ888351**			
IBERA 16	*Testudo graeca ibera*	Greece: Kos Island; 36°53′36″N 27°17′49″E	**T 823**	**AJ888352**			
IBERA 17	*Testudo graeca ibera*	Republic of Macedonia: Skopje; 42°00′N 21°28′E	**T 1975**	**AM231017**			
IBERA 18	*Testudo graeca ibera*	Romania: Histria; 44°35′N 28°42′E	**T 1362**	**AM231018**	+	+	+
IBERA 19	*Testudo graeca ibera*	Romania: Histria; 44°35′N 28°42′E	**T 1399**	**AM231019**	+	+	+
IBERA 20	*Testudo graeca ibera*	Romania: Histria; 44°35′N 28°42′E	**T 1634**	**AM231020**			
IBERA 21	*Testudo graeca ibera*	Turkey: Menemen; 38°37′N 27°04′E	**T 268**	**AM231021**			
IBERA 22	*Testudo graeca ibera*	Turkey: Seydişehir; 37°25′19″N 31°50′05″E	**T 150**	**AJ888355**	+	+	+

Table 2. (Continued).

Sample	Taxon	Locality	MTD	Accession number	ISSR $(GAA)_5$	ISSR $(GACA)_4$	ISSR L18
IBERA 23	*Testudo graeca ibera*	Turkey: Lake Van: 10 km N Van; 38°34′N 43°24′E	**T 382**	**AJ888354**	+		+
IBERA 24	*Testudo graeca ibera*	Turkey: Lake Van: Budakli; 38°23′56″N 42°37′56″E	**T 381**	**AM231022**	+		
IBERA 25	*Testudo graeca ibera*	Turkey: Lake Van: Küçüksu; 38°27′N 42°20′E	**T 380**	**AM231023**	+		+
IBERA 26	*Testudo graeca ibera*	Turkey: Lake Van: Erciş; 39°02′N 43°23′E	**T 2721**	**AJ888353**	+	+	+
SICILY 2	*Testudo graeca ibera*	Italy: Sicily: Campobello di Mazara; 37°38′N 12°45′E	**T 2142**	**AM230970**			
NABEULENSIS 1	*Testudo graeca nabeulensis*	Tunisia: Sousse (Susah); 35°50′N 10°39′E	**T 149**	**AM230971**			
NABEULENSIS 2	*Testudo graeca nabeulensis*	Tunisia	**D 42893**	**AM230972**			
NIKOLSKII 1	*Testudo graeca nikolskii*	Russia: N Tuapse; 44°11′N 39°06′E	**T 1808**	**AM230973**		+	+
NIKOLSKII 2	*Testudo graeca nikolskii*	Russia: Sukko, SE Anapa; 44°47′N 37°23′E	**T 1806**	**AM230974**		+	
PALLASI 1	*Testudo graeca pallasi*	Azerbaijan: Kolani; 41°11′N 49°08′E	**T 1450**	**AM230975**	+	+	+
PALLASI 2	*Testudo graeca pallasi*	Azerbaijan: Kolani; 41°11′N 49°08′E	**T 1451**	**AM230976**	+	+	
PALLASI 3	*Testudo graeca pallasi*	Azerbaijan: Kolani; 41°11′N 49°08′E	**T 1452**	**AM230977**	+		+
PALLASI 4	*Testudo graeca pallasi*	Russia: Dagestan: Dagestanskie Ogni; 42°07′N 48°11′E	**T 2388**	**AM230978**	+		+
PALLASI 5	*Testudo graeca pallasi*	Russia: Dagestan: Dagestanskie Ogni; 42°07′N 48°11′E	**T 2389**	**AM230979**	+	+	+
PALLASI 6	*Testudo graeca pallasi*	Russia: Dagestan: Dagestanskie Ogni; 42°07′N 48°11′E	**T 2390**	**AM230980**	+	+	
PALLASI 7	*Testudo graeca pallasi*	Russia: Dagestan: Zelenomorsk; 42°45′N 47°42′E	**T 873**	**AM231000**	+	+	+
PALLASI 8	*Testudo graeca pallasi*	Russia: Dagestan: Zelenomorsk; 42°45′N 47°42′E	**T 874**	**AM231001**	+	+	+
? *PALLASI* 9	*Testudo graeca pallasi?*	Azerbaijan: Absheron Peninsula: Novkhani; 40°32′N 49°47′E	**T 1453**	**AM230981**	+	+	+
? *PALLASI* 10	*Testudo graeca pallasi?*	Azerbaijan: Absheron Peninsula: Novkhani; 40°32′N 49°47′E	**T 1454**	**AM230982**	+	+	+
PERSES 1	*Testudo graeca perses*	Iran: Anjīr Āvand; 32°30′N 54°26′E	**T 2283**	**AM230983**			
PERSES 2	*Testudo graeca perses*	Iran: 6 km NE Meshgīn Shahr; 38°26′N 47°42′E	**T 1427**	**AM230984**	+	+	
PERSES 3	*Testudo graeca perses*	Iran: 6 km NE Meshgīn Shahr; 38°26′N 47°42′E	**T 1428**	**AM230985**	+		+
PERSES 4	*Testudo graeca perses*	Iran: Neyrīz; 29°12′N 54°19′E	**T 2282**	**AM230986**	+	+	+
PERSES 5	*Testudo graeca perses*	Iran: Shahr-e Babāk, Maymand; approx. 30°07′N 55°07′E	**T 2284**	**AM230987**	+	+	+
PERSES 6	*Testudo graeca perses*	Iran: Shahr-e Babāk, Maymand; approx. 30°07′N 55°07′E	**T 2285**	**AM230988**	+	+	+
PERSES 7	*Testudo graeca perses*	Iran: E Esfahān: Kūhpāyeh; 32°43′N 52°26′E	**T 2269**	**AM230989**	+	+	+
PERSES 8	*Testudo graeca perses*	Iran: Nīr; 31°30′N 54°08′E	**T 2272**	**AM230990**		+	+
? *PERSES* 9	*Testudo graeca perses?*	Iran: between Germī and Razay-Amīr Abād (Amīr Kandī); 38°53′N 48°00′E	**T 2286**	**AM230991**	+	+	+

Table 2. (Continued).

Sample	Taxon	Locality	MTD	Accession number	ISSR $(GAA)_5$	ISSR $(GACA)_4$	ISSR L18
TERRESTRIS 1	*Testudo graeca terrestris*	Syria: hills NW Aleppo: Dar Ta'izzah; 36°17′N 36°51′E	**T 2193**	**AM230992**	+	+	+
TERRESTRIS 2	*Testudo graeca terrestris*	Syria: hills NW Aleppo: Qalat Samān; 36°22′N 36°51′E	**T 2194**	**AM230993**			
TERRESTRIS 3	*Testudo graeca terrestris*	Turkey: Mardin; 37°19′N 40°47′N	**T 267**	**AM230994**			
TERRESTRIS 4	*Testudo graeca terrestris*	Turkey: Street E-99 between Hilvan and Çaylarbasi; 37°39′N 39°06′E	**T 364**	**AM230995**			
? *TERRESTRIS* 5	*Testudo graeca terrestris?*	Turkey: Adiyaman; 37°46′09″N 38°21′35″E	**T 378**	**AM230996**			
? *TERRESTRIS* 6	*Testudo graeca terrestris?*	Turkey: Adiyaman; 37°46′N 38°16′N	**T 264**	**AM230997**			
ZARUDNYI 1	*Testudo graeca zarudnyi*	Iran: Sāghand, southern border of Kavīr Desert; 32°33′N 55°13′E	**T 2280**	**AM230998**	+	+	+
ZARUDNYI 2	*Testudo graeca zarudnyi*	Iran: Tabas, southern border of Kavīr Desert; 33°35′N 56°56′E	**T 2273**	**AM230999**	+	+	+
OUTGROUPS:							
HERMANNI HERMANNI 1	*Testudo hermanni hermanni*	Italy: Roccatederighi; 43°02′N 11°05′E	**D 41590**	**AJ888362**			
HERMANNI HERMANNI 2	*Testudo hermanni hermanni*	South Italy	**D 42972**	**AJ888364**			
HORSFIELDII KAZACHSTANICA	*Testudo horsfieldii kazachstanica*	Kazakhstan	**D 44201**	**AJ888365**			
HORSFIELDII RUSTAMOVI	*Testudo horsfieldii rustamovi*	Iran: Gorgan; 36°50′N 54°26′E	**T 1419**	**AJ888366**			
KLEINMANNI 1	*Testudo kleinmanni*	Unknown	**D 44284**	**AJ888370**			
KLEINMANNI 2	*Testudo kleinmanni*	Unknown	**D 44285**	**AJ888371**			
MARGINATA 1	*Testudo marginata*	Greece: Peloponnese: Dídimi; 37°28′N 23°07′E	**T 388**	**AJ888318**			
MARGINATA 2	*Testudo marginata*	Italy: Sardinia: Pittulongo; 40°59′N 09°35′E	**T 1117**	**AJ888332**			

fragment (cytochrome *b* gene and adjacent portion of tRNA-Thr gene). PCR was performed in a 50 μl volume (Bioron PCR buffer or 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of *Taq* DNA polymerase (Bioron), 10 pmol dNTPs and 5 or 10 pmol of each primer. The PCR program consisted of a 5 min initial denaturation at 94°C, followed by 35 cycles of 45 s at 94°C, 52 s at 55-60°C and 80 s at 72°C with a final elongation step of 10 min at 72°C, or alternatively, a 4 min initial denaturation at 94°C, then 30 cycles of 45 s at 94°C, 60 s at 52°C and 120 s at 72°C with a 10 min final elongation step at 72°C. PCR products were sequenced directly on both strands on ABI or MegaBace 1000 (Amersham Biosciences) sequencers using the internal primers mt-c2 5′-TGA GGA CAA ATA TCA TTC TGA GG 3′ or mt-c-For2 5′-TGA GGV CAR ATA TCA TTY TGA G-3′ and mt-E-Rev 5′-GCA AAT AGG AAG TAT CAT TCT GG-3′ or mt-E-Rev2 5′-GCR AAT ARR AAG TAT CAT TCT GG-3′.

Sequence alignment, phylogenetic analyses and genetic distances

The corresponding sequences from both strands were combined with the software DNAsis 7.00 (copyright Hitachi Software Engineering Company, 1991) for every sample with the resulting consensus sequences being aligned manually using BioEdit 7.0.5.2 (Hall, 1999). Sixteen sequences (**AJ888341-56**) were already published in a previous study (Fritz et al., 2005a); sequences of all other *Testudo* species from this paper served as outgroups for our phylogenetic analyses (table 2). Data were analyzed using Bayesian inference of phylogeny as implemented in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), maximum parsimony (MP; equal weighting) and neighbour joining (NJ; with model-corrected maximum likelihood distances) as implemented in PAUP* 4.0b10 (Swofford, 2002). Bayesian analysis was performed using four chains of 1 000 000 generations sampling every 100 generations and with the first 1000 generations discarded as burn-in (with which only the plateau of the most likely trees was sampled). The best evolutionary model for the data (also included in the analysis) was established by hierarchical likelihood testing using Modeltest 3.06 (Posada and Crandall, 1998). For the ingroup species, 934 of 1134 aligned sites were constant, 74 characters were variable but parsimony-uninformative, and 126 variable characters were parsimony-informative. Due to the protracted computation times for MP analyses, the number of equally most parsimonious solutions that were saved needed to be limited using the maxtrees option. However, to determine whether or not this constraint was limiting the search unduly, maxtrees was set to 10 000, 50 000 and 100 000 in three separate analyses and the resolution and topologies of the resulting strict consensus trees were compared. All calculations resulted in MP trees of 603 steps (CI = 0.6385, RI = 0.9135). To test the robustness of obtained branching patterns, bootstrap permutations (Felsenstein, 1985) were run under both MP (setting maxtrees = 1000, nreps = 1000) and NJ (ML-distances; nreps = 1000). Genetic distances (uncorrected *p* distances) were calculated with PAUP* 4.0b10; previously published sequences (Fritz et al., 2005a) were used for comparison with other *Testudo* species.

Nuclear genomic fingerprinting and analysis

ISSR employs a single PCR primer, binding to di- or trinucleotide repeat motifs (microsatellites), which are abundant in eukaryotic genomes (Tautz and Renz, 1984; Condit and Hubbell, 1991). Since sequences of microsatellites are conserved over a wide range of organisms, universal primers can be applied. Amplified regions correspond to the nucleotide sequence between two inverted simple sequence repeat (SSR) priming sites (Wolfe et al., 1998; Bornet and Branchard, 2001). SSR regions appear to be scattered evenly throughout the genome (Tautz and Renz, 1984; Condit and Hubbell, 1991), resulting in a large number of polymorphic bands. ISSR markers are inherited in a dominant or codominant Mendelian fashion (Gupta et al., 1994; Tsumura et al., 1996). They are interpreted as dominant markers scored as diallelic with 'band present' or 'band absent'. The absence of a band is interpreted as primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement (Wolfe and Liston, 1998; Wolfe et al., 1998). ISSR fingerprints are usually diagnostic for species-level taxa (e.g. Gupta et al., 1994; Zietkiewicz et al., 1994; Wink et al., 1998; Bornet and Branchard, 2001; Fritz et al., 2005a, b; Hundsdörfer and Wink, 2005). Individuals from contact zones of distinct subspecies as well as interspecific hybrids share with both parental taxa diagnostic bands. Thus, limited or non-existing gene flow is reflected by distinct banding patterns for reproductively isolated taxa, while a high percentage of shared bands is indicative of gene flow or incomplete differentiation (Wolfe et al., 1998; Nagy et al., 2003; Fritz et al., 2005b).

We used two non-anchored primers that yielded species-diagnostic banding patterns for *Testudo* in a previous study (Fritz et al., 2005a), $(GAA)_5$, annealing temperature 40°C, and $(GACA)_4$, annealing temperature 55°C. In addition, we applied the short anchored primer L18 (CTC GGG AAG GGA), annealing temperature 45°C, that was useful in another chelonian genus, *Emys* (Fritz et al., 2005b). Each PCR was performed with approximately 60 ng template DNA in a 25 μl volume [10 pmol of the primer and 0.625 nmol of each dNTP, except dATP: 0.28 nmol cold dATP plus 0.1 μl radioactive α-^{33}P-dATP solution (370 MBq/ml, Amersham Biosciences), 0.75 units of *Taq* polymerase (SIGMA) and water, buffered with 10 mM Tris-HCl, 50 mM KCl, 0.5% Triton X-100, 1.5 mM $MgCl_2$] covered by two drops of mineral oil. Thermo-cycling was performed with a Trio Thermo block TB1 (Biometra, Göttingen). Following an initial 5 min denaturation at 94°C, the program consisted of 30 cycles of 45 s at 94°C, 60 s at the respective annealing temperature, 120 s at 72°C and 5 min at 72°C for final elongation. DNA fragments were separated by PAGE in a vertical apparatus (Base Acer Sequencer, Stratagene) for 2.5-4 h at 65 W. The denaturing gel [6 M Urea, 100 ml Long Ranger Solution, Biozym (PA), 100 ml TBE buffer (10x: 1 M Tris, 0.83 M Boric Acid, 10 mM EDTA, pH 8.6)] had a size of 45 × 30 cm and a thickness of 0.25 mm. After drying, an

X-ray film (Hyperfilm-MP, Amersham) was exposed to the gel for at least 12 h and developed.

Fragment patterns were analyzed manually. Unmistakably identifiable bands were transferred into a presence/absence matrix scoring each particular fragment. $(GAA)_5$ yielded 33 fragments for 50 samples, $(GACA)_4$ 29 fragments for 45 samples, and L18 resulted in 89 fragments for 46 samples. To enable comparison of variation within *T. graeca* and other organisms, we report average Dice and Jaccard distance values (Jaccard, 1901, 1908; Dice, 1945; Nei and Li, 1979), calculated with RAPDistance 1.04 (Armstrong et al., 1996), following the appeal for standardization in ISSR PCR analyses by Hundsdörfer and Wink (2005). Based on the presence/absence matrix, cluster analyses (NJ trees) as implemented in PAUP* 4.0b10 (Swofford, 2002) were calculated for banding patterns of each primer and for a combined dataset for all three primers (151 characters for 36 samples). Robustness of trees was tested by bootstrapping (2000 replicates) under the 50% consensus criterion.

Results

MtDNA sequence variation and phylogeny

Monophyly of the *Testudo graeca* complex was confirmed by high posterior probability and bootstrap values under all tree building methods; its sister taxa are *T. kleinmanni* and *T. marginata* (fig. 2). All phylogenetic analyses resulted in six well-supported major clades (A-F) within the *T. graeca* complex, and the MP topology obtained was corroborated by all maxtrees settings.

Average uncorrected *p* distances between the *T. graeca* complex and other *Testudo* species range between 8.870% and 12.662%. Within the *T. graeca* complex, a mean of 3.346% was observed. This value clearly exceeds sequence divergences within other *Testudo* species, and within-divergences of clades B and E correspond approximately to that within the polytypic *T. hermanni* (table 3).

Within the *T. graeca* complex, clade A comprises haplotypes distributed in the East Caucasus. It is sister to another clade (B) that corresponds to haplotypes from the western Mediterranean islands of Mallorca, Sardinia and Sicily (which are thought to be inhabited by allochthonous tortoise populations; Buskirk et al., 2001) and from North Africa. Clade C includes haplotypes from western Asia Minor, the southeastern Balkans and the western and central Caucasus Region. Its sister group is a fourth, widely distributed clade (D) from southern and eastern Asia Minor and the Levantine Region (Near East). Two further clades (E, F) are distributed in Iran (one clade in northwestern and central Iran; the other in eastern Iran).

While the distinctness of each of these six clades and the sister group relationships between (A + B) and between (C + D) were unequivocally supported, the phylogeny of the resulting four clades (A + B), (C + D), E and F was poorly resolved (small cladograms in fig. 2). Under Bayesian analysis, there was weak support for a sister group relationship of the two major western clades, i.e. ((A + B) + (C + D)), whereas MP and NJ resulted in a sister group relation between a weakly supported clade ((A + B) + E, F) and (C + D).

Nuclear genomic fingerprinting

In contrast to obvious geographical sequence differentiation of the mitochondrial genome, we found a quite homogenous pattern in nuclear ge-

Figure 2. Bayesian phylogram of mtDNA haplotypes for spur-thighed tortoises (*Testudo graeca* complex), rooted with all other *Testudo* species. Taxon names within *T. graeca* complex are assigned according to morphology and geographic provenance and follow Chkhikvadze and Tuniyev (1986), Weissinger (1987), Chkhikvadze and Bakradze (1991, 2002), Perälä (2002a, b, 2004b), Pieh et al. (2002) and Pieh and Perälä (2002, 2004). Question marks denote uncertain taxonomic allocations due to localities along range borders. Locality names (Mallorca, Sardinia, Sicily) instead of taxon names indicate introduced or allochthonous tortoises from the western Mediterranean. Numbers following taxon or locality names refer to table 2, where exact localities are given. Symbols correspond to distinct mtDNA lineages and to figure 1. Branch lengths are Bayesian estimates and proportional to the scale bar with the unit being a mean number of nucleotide changes per site. Numbers at crucial nodes represent posterior probabilities and MP and NJ bootstrap values (1000 replicates) greater than 50; NJ using ML distances. Dashes indicate that the respective branch was not supported. Small cladograms show alternative topologies for major groups within *T. graeca* complex. For clade D from southern and eastern Asia Minor and the Levant, see also figure 7.

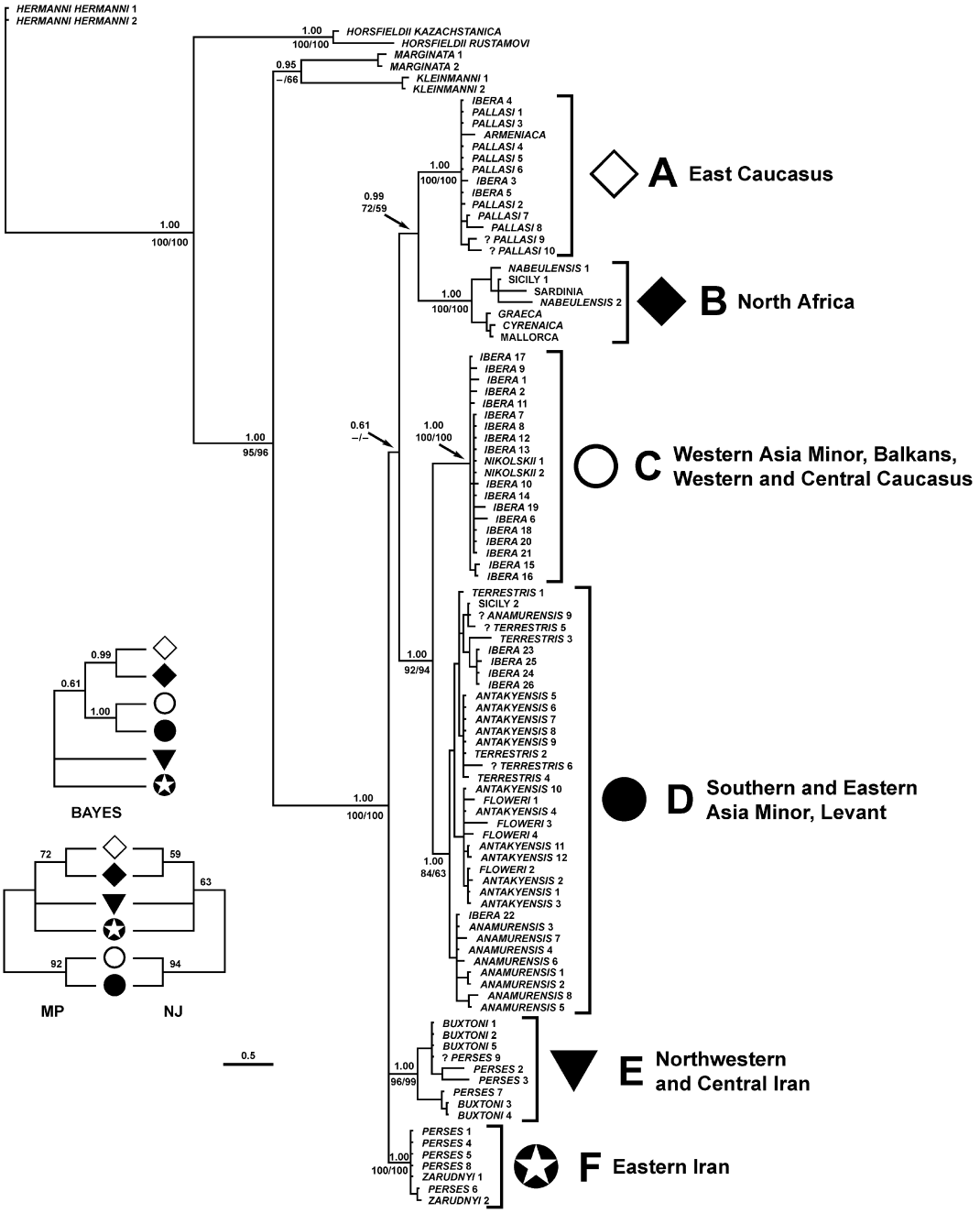
HERMANNI HERMANNI 1
HERMANNI HERMANNI 2
HORSFIELDII KAZACHSTANICA
HORSFIELDII RUSTAMOVI
MARGINATA 1
MARGINATA 2
KLEINMANNI 1
KLEINMANNI 2
IBERA 4
PALLASI 1
PALLASI 3
ARMENIACA
PALLASI 4
PALLASI 5
PALLASI 6
IBERA 3
IBERA 5
PALLASI 2
PALLASI 7
PALLASI 8
? PALLASI 9
? PALLASI 10
A East Caucasus
NABEULENSIS 1
SICILY 1
SARDINIA
NABEULENSIS 2
GRAECA
CYRENAICA
MALLORCA
B North Africa
IBERA 17
IBERA 9
IBERA 1
IBERA 2
IBERA 11
IBERA 7
IBERA 8
IBERA 12
IBERA 13
NIKOLSKII 1
NIKOLSKII 2
IBERA 10
IBERA 14
IBERA 19
IBERA 6
IBERA 18
IBERA 20
IBERA 21
IBERA 15
IBERA 16
C Western Asia Minor, Balkans, Western and Central Caucasus
TERRESTRIS 1
SICILY 2
? ANAMURENSIS 9
? TERRESTRIS 5
TERRESTRIS 3
IBERA 23
IBERA 25
IBERA 24
IBERA 26
ANTAKYENSIS 5
ANTAKYENSIS 6
ANTAKYENSIS 7
ANTAKYENSIS 8
ANTAKYENSIS 9
TERRESTRIS 2
? TERRESTRIS 6
TERRESTRIS 4
ANTAKYENSIS 10
FLOWERI 1
ANTAKYENSIS 4
FLOWERI 3
FLOWERI 4
ANTAKYENSIS 11
ANTAKYENSIS 12
FLOWERI 2
ANTAKYENSIS 2
ANTAKYENSIS 1
ANTAKYENSIS 3
IBERA 22
ANAMURENSIS 3
ANAMURENSIS 7
ANAMURENSIS 4
ANAMURENSIS 6
ANAMURENSIS 1
ANAMURENSIS 2
ANAMURENSIS 8
ANAMURENSIS 5
D Southern and Eastern Asia Minor, Levant
BUXTONI 1
BUXTONI 2
BUXTONI 5
? PERSES 9
PERSES 2
PERSES 3
PERSES 7
BUXTONI 3
BUXTONI 4
E Northwestern and Central Iran
PERSES 1
PERSES 4
PERSES 5
PERSES 8
ZARUDNYI 1
PERSES 6
ZARUDNYI 2
F Eastern Iran
0.5
BAYES
MP
NJ

Table 3. Uncorrected *p* distances (percentages) within and between clades of the *Testudo graeca* complex and other *Testudo* species, based on a dataset of 1000 bp (mtDNA, cytochrome *b* gene). Mean distances between species or clades are given below, ranges (in italics) above the diagonal. The within-taxon sequence divergence is given in bold on the diagonal (mean and range). Polytypic species asterisked. Sequences from Fritz et al. (2005a): *T. hermanni boettgeri* **AJ888357-60**, *T. hermanni hermanni* **AJ888361-64**, *T. horsfieldii kazachstanica* **AJ888365**, *T. horsfieldii rustamovi* **AJ888366**, *T. kleinmanni* **AJ888370-71**, *T. marginata* **AJ888308-40**.

	n	*Testudo graeca** – all	Clade A	Clade B	Clade C	Clade D	Clade E	Clade F	*Testudo hermanni**	*Testudo horsfieldii**	*Testudo kleinmanni*	*Testudo marginata*
*Testudo graeca** – all	94	**3.346 (0.000-7.953)**	–	–	–	–	–	–	*10.562-15.087*	*9.845-14.609*	*8.057-10.928*	*7.256-10.665*
Clade A	14	–	**0.304 (0.000-1.148)**	*3.908-6.149*	*4.208-5.423*	*4.008-5.834*	*3.707-5.732*	*3.307-4.020*	*12.094-14.050*	*11.186-13.060*	*10.020-10.551*	*8.868-10.256*
Clade B	7	–	4.664	**1.515 (0.000-2.440)**	*5.206-7.953*	*4.868-7.429*	*4.709-7.447*	*3.707-4.990*	*11.208-13.314*	*11.025-13.608*	*8.994-10.454*	*8.870-10.382*
Clade C	20	–	4.677	5.990	**0.243 (0.000-0.921)**	*2.092-4.081*	*3.974-6.212*	*3.707-4.509*	*12.020-15.087*	*10.587-14.609*	*9.197-10.928*	*7.658-9.844*
Clade D	37	–	4.486	5.591	2.612	**0.789 (0.000-2.509)**	*3.607-6.939*	*3.009-4.883*	*11.049-13.692*	*9.845-12.544*	*8.057-9.703*	*7.288-9.514*
Clade E	9	–	4.274	5.419	4.858	4.409	**1.343 (0.000-3.046)**	*2.505-4.309*	*11.228-14.028*	*10.374-13.916*	*8.918-10.922*	*7.894-10.665*
Clade F	7	–	3.545	4.061	3.954	3.510	3.081	**0.150 (0.000-0.401)**	*10.562-12.224*	*10.090-12.314*	*8.460-9.218*	*7.256-8.717*
*Testudo hermanni**	8	12.662	13.203	12.404	13.154	12.453	12.605	11.615	**1.183 (0.000-2.752)**	*9.931-13.057*	*10.673-11.907*	*9.871-11.864*
*Testudo horsfieldii**	2	11.737	11.994	12.328	12.308	11.243	11.930	11.420	11.622	**2.461 (–)**	*9.990-11.333*	*10.072-11.788*
Testudo kleinmanni	2	9.410	10.184	9.601	9.872	8.828	9.746	8.995	11.454	10.662	**0.000 (–)**	*6.452-7.301*
Testudo marginata	33	8.870	9.433	9.703	9.207	8.481	8.973	7.884	11.086	10.949	6.847	**0.220 (0.000-1.101)**

nomic fingerprints. All studied samples shared a high percentage of bands. The primer $(GACA)_4$ resulted in average distances of 0.30 (Dice; Nei and Li) and 0.44 (Jaccard), $(GAA)_5$ in 0.35 (Dice; Nei and Li) and 0.50 (Jaccard), and L18, which produced the most variable fingerprints, in average distances of 0.23 (Dice; Nei and Li) and 0.37 (Jaccard).

Using NJ cluster analyses, the datasets for the three single primers resulted in unresolved polytomies for most samples under the 50% bootstrap consensus criterion, and those samples that clustered with weak to high bootstrap support often belong to different mtDNA lineages and different taxa. The NJ tree of the $(GAA)_5$ dataset serves as example (fig. 3, left). The combined dataset of 151 characters from the three primers resulted in fewer polytomies (fig. 3, right); however, also in this tree none of the major clusters corresponds with taxon limits or mtDNA lineages.

Systematics, zoogeography and discussion

Incongruence of morphology, taxa and genetic data

Populations of spur-thighed tortoises comprise in some regions (Spain, North Africa, Levant) small to medium-sized, mainly yellow-coloured tortoises, while in other parts of the range larger sized, dark individuals occur. However, ontogenetic variation of shell coloration and proportions is considerable. Different age classes of Bulgarian *Testudo graeca* display nearly the entire range of shell coloration, with young, small adults being light-coloured and aged, large adults being dark-coloured. Also shell proportions are known to change much during growth (Fritz et al., 1996).

Nevertheless, such characters (shell shape and proportions, colour pattern, in part also scutellation characters) were repeatedly used for taxon delineation, leading to recent descriptions or resurrections of several morphologically diagnosable taxa (Pieh, 2001; Perälä, 2002a; Pieh and Perälä, 2002, 2004). For Moroccan *T. graeca* it was demonstrated, however, that such variation corresponds rather to population-specific differentiation than to taxonomic distinctness (Harris et al., 2003; Carretero et al., 2005).

Using ISSR fingerprinting we found no support for any taxonomic differentiation and none of our six mtDNA clades within the *T. graeca* complex is congruent with any morphologically defined taxon. Also a distinction between a North African-Levantine lineage, comprising mainly yellow-coloured tortoises of small to moderate size and a second lineage of larger and darker tortoises, occurring in Southeast Europe and the Near and Middle East, as suggested by Fritz et al. (1996), is not corroborated by our data (figs 1-2). While all studied North African tortoises fall into one clade (B), the sister group of this clade is not formed by Levantine haplotypes but by haplotypes from the East Caucasus (clade A), representing populations with large and dark tortoises.

According to our mtDNA data, this East Caucasian clade A is composed of haplotypes from tortoises that belong to *T. g. armeniaca*, *T. g. ibera* and *T. g. pallasi*. The North African clade B contains haplotypes of the nominal taxa *T. g. graeca*, *T. g. cyrenaica* and *T. g. nabeulensis* as well as most specimens from the western Mediterranean islands. Clade C, found in the Balkans, in western Asia Minor, and in the western and central Caucasus, comprises sequences representing *T. g. ibera* and *T. g. nikolskii*. Clade D from southern and eastern Asia Minor and the Levant is composed of haplotypes of *T. g. anamurensis*, *T. g. antakyensis*, *T. g. floweri*, *T. g. ibera*, *T. g. terrestris* and one (allochthonous) tortoise from Sicily. Clade E from northwestern and central Iran consists of haplotypes from *T. g. buxtoni* and *T. g. perses*, and the eastern Iranian clade F contains haplotypes assigned to *T. g. perses* and *T. g. zarudnyi*.

From a morphological point of view, clades A, C, E and F contain populations with dark-coloured tortoises of medium to large size.

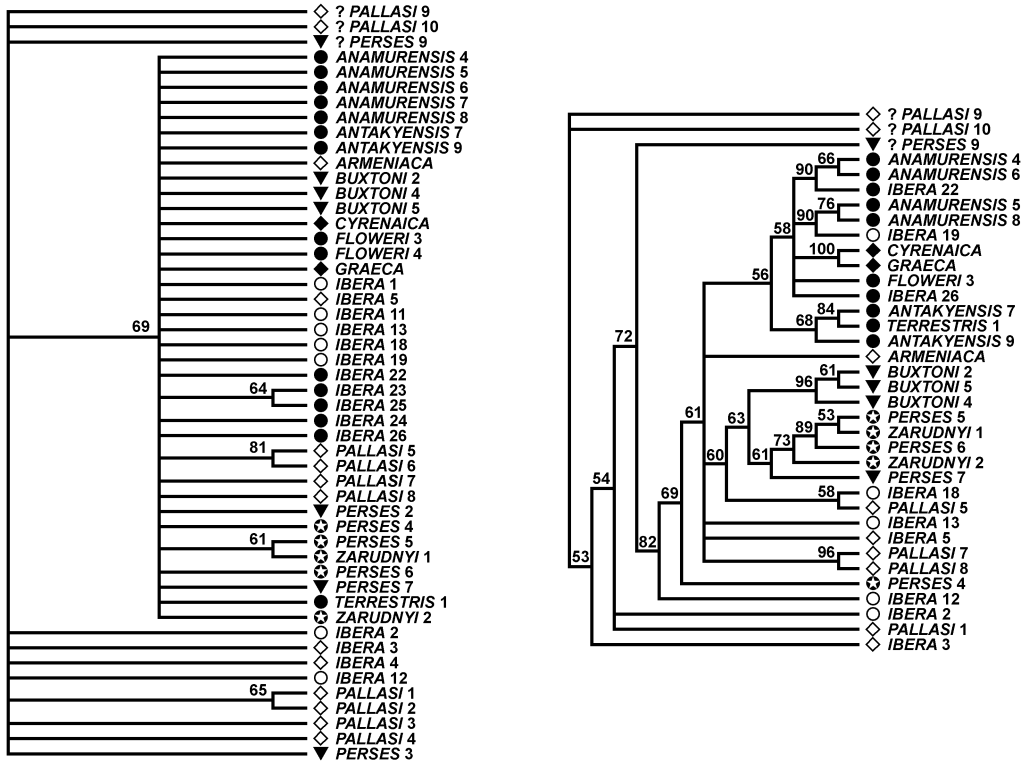

Figure 3. Unrooted NJ bootstrap 50% majority-rule consensus trees of ISSR fingerprints for samples of the *Testudo graeca* complex. Left, primer $(GAA)_5$, based on 50 samples and 33 fragments. Right, combined dataset of primers $(GAA)_5$, $(GACA)_4$ and L18, based on 36 samples and 151 fragments. Numbers along branches represent bootstrap values (2000 replicates) greater than 50. For further explanations see figure 2.

Taxa in these clades differ mainly in shell shape and in part in colour pattern. Tortoises of clade B are generally lighter coloured and small- to moderately-sized (table 1). Most variation is found in clade D that comprises small- to medium-sized, mainly yellow-coloured tortoises (*T. g. antakyensis*, *T. g. floweri*, *T. g. terrestris*) as well as huge, dark brownish individuals, either with flat, elongated shells (*T. g. anamurensis*) or distinctly domed shells (*T. g. ibera* from Lake Van Region, Turkey).

However, even within populations attributed to *T. g. antakyensis* by Perälä (2002a) considerable morphological differences are known to occur, reflecting the same general pattern. Within Syria, morphological variation was shown to follow Gloger's Rule in that tortoises from arid regions are distinctly lighter coloured (and smaller) than tortoises from more humid regions. Further, tortoises living on dark basaltic soil, as in the Jabal Durūz Region, are significantly darker coloured than tortoises from other regions with light substrate (figs 4-6; Fritz et al., 1996). We observed the same pattern also in other regions of the Near East during field work. This suggests stabilizing selection by environmental pressure as a source of morphological variation. Similarity of general body and substrate coloration is a well-known phenomenon also in other animal species ('substrate races'; Mayr, 1963).

Haplotypes within clade D, that contains among others all *T. g. antakyensis* sequences, group into three weakly to well-supported subclades. These subclades rather correspond to sequences from neighbouring regions than to taxonomic segregation or morphological similarity (fig. 7). This is most obvious in the

well-supported subclade from Israel, Jordan and southern Syria, which consists of sequences from the nominal taxa *T. g. antakyensis* and *T. g. floweri*. While the sequences of the four *T. g. floweri* and a *T. g. antakyensis* from Jordan (*antakyensis* 4) represent small-sized, mainly yellow tortoises from arid regions, other sequences from *T. g. antakyensis* originate from larger and darker tortoises that live in moister environments (*antakyensis* 1-3: Tiberias, Israel). The three Syrian *T. g. antakyensis* sequences in this subclade (*antakyensis* 10-12) are from dark-coloured tortoises from the basaltic Jabal Durūz Region.

Testudo graeca armeniaca is a further prominent example for incongruence of morphological and genetic differentiation. This taxon is characterized by a depressed shell and flattened forearms. In general appearance it resembles *T. horsfieldii* Gray, 1844 (Chkhikvadze and Bakradze, 1991; Pieh et al., 2002), a species that is not closely related to the *T. graeca* complex and allocated to the genus *Agrionemys* by some authors (Fritz and Cheylan, 2001; Parham et al., 2006). The mtDNA haplotype of a *T. g. armeniaca* is nested within the East Caucasian clade A together with other, morphologically distinctive tortoises with domed shell from the same region (fig. 2; table 2). Both *T. g. armeniaca* and *T. horsfieldii* occur in regions with strictly continental climate with severe, cold winters. Farther, both are steppicolous tortoises that dig and inhabit deep burrows, suggesting that environmental pressure and the similar mode of life results via selection in similar phenotypes and masks phylogenetic relationships.

Also in another *Testudo* species, *T. marginata*, environmental pressure was shown to result in considerable phenotypic variation (size reduction, persisting juvenile characteristics in a poor region of the range; Fritz et al., 2005a), suggestive of considerable phenotypic plasticity in *Testudo* species.

How many species?

Based on morphology and zoogeographic considerations, it was repeatedly suggested that *Testudo graeca* is composed of several distinct species (Bour, 1989; Gmira, 1993a, b, 1995; David, 1994; Pieh, 2001; Perälä, 2002a, b; Pieh and Perälä, 2002, 2004). In contrast to Gmira (1993a, b, 1995) and Perälä (2002b), whose morphological datasets argued for the paraphyly of the *T. graeca* complex with respect to other *Testudo* species (*T. kleinmanni* sensu lato, *T. marginata*), our mtDNA data confirmed a monophyletic *T. graeca* complex and its sister group relationship to *T. kleinmanni* and *T. marginata*. This agrees with other recent studies employing genetic markers (mtDNA data: van der Kuyl et al., 2002; Fritz et al., 2005a; Parham et al., 2006; nuclear genomic fingerprinting: Fritz et al., 2005a).

Obviously, species delineation depends on the adopted species concept, and there is an ongoing debate about the benefits and shortcomings of different competing species concepts (e.g. Ereshefsky, 1992; Wheeler and Meier, 2000; Agapow et al., 2004; Coyne and Orr, 2004). There is no doubt that the six mtDNA lineages within the *T. graeca* complex could be recognized as representing full species under the Evolutionary (e.g. Wiley and Mayden, 2000) or Phylogenetic Species Concepts (e.g. Cracraft, 1983, 1987; Mishler and Theriot, 2000; Wheeler and Platnick, 2000; see also the review in Coyne and Orr, 2004), although morphological characterization of these species would be difficult (see below). However, the most restrictive species concept for bisexual animals, the Biological Species Concept of Mayr (e.g. 1942, 1963, 2000), defines species as reproductively (and thus genetically) isolated groups of populations. Under this species concept, mitochondrial genomic markers alone are not conclusive, mainly due to their matrilineal inheritance and the different effective population sizes of mitochondrial and nuclear genomes, resulting in different patterns for incomplete lineage sorting and intro-

gression (Funk and Omland, 2003; Ballard and Whitlock, 2004).

ISSR PCR provides an ideal tool for obtaining information about nuclear genomic differentiation and gene flow. In distinct 'biological species', differentiation of nuclear and mitochondrial genomes is typically congruent and ISSR profiles differ clearly (e.g. for chelonians: Wink et al., 2001; Guicking et al., 2002; Schilde et al., 2004; Fritz et al., 2005a, b). In such cases, introgression and hybridization are indicated by shared bands in otherwise distinct banding patterns. In contrast, if populations still form a genetic continuum, ISSR profiles do not differ markedly. ISSR also allows identification of taxa in which introgression of mtDNA might mask nuclear genomic differentiation (Gupta et al., 1994; Zietkiewicz et al., 1994; Wink et al., 1998, 2001; Wolfe and Liston, 1998; Wolfe et al., 1998; Guicking et al., 2002; Nagy et al., 2003; Schilde et al., 2004; Fritz et al., 2005a, b).

In a previous study, ISSR profiles of all five traditionally recognized *Testudo* species were shown to be significantly distinct (in NJ cluster analysis bootstrap support values of 100 for each species; Fritz et al., 2005a). Using the same primers, $(GAA)_5$ and $(GACA)_4$, plus one additional primer (L18) shown to be useful in other chelonians (*Emys*; Fritz et al., 2005b), we found little differentiation within the *T. graeca* complex, despite extensive mtDNA differentiation. As we can exclude homoplasy due to the excellent discrimination of the other *Testudo* species, the similarity of fingerprints within the *T. graeca* complex indicates either a high level of gene flow or a low level of differentiation (including incomplete lineage sorting). We conclude that *T. graeca* represents a single polytypic species under the Biological Species Concept.

How many subspecies?

Based on a combined data set of 393-394 bp of the 12S rRNA gene and 411 bp of the D-loop, van der Kuyl et al. (2005) found among 22 spurthighed tortoises only two well-supported haplotype clades, corresponding to tortoises from (i) North Africa and (ii) Turkey (one sample) and the Near East and presumed that these represent only two subspecies, *Testudo graeca graeca* and *T. g. ibera*, while genetic evidence for the existence of other subspecies was thought to be weak. According to locality data, the '*ibera* clade' of van der Kuyl et al. (2005) could be identical with our clade D. However, a problem of the papers by van der Kuyl et al. (2002, 2005) was that many tortoises of unknown provenance were studied. Such specimens cannot be used when assessing geographic variation (Harris et al., 2003). Moreover, subspecies names assigned by van der Kuyl et al. (2002, 2005) often did not fulfil nomenclatural requirements and locality data in part did not match the ranges of the respective taxa (Perälä, 2004a). For example, van der Kuyl et al. (2005) assign tortoises from Bulgaria in part to *T. g. terrestris*; this taxon does not occur in Bulgaria (Bour and Perälä, 2004; Perälä and Bour, 2004; see also table 1).

In accordance with the Biological Species Concept, we understand subspecies as genetically distinct, geographically vicariant groups of populations between which gene flow occurs. Distinct mitochondrial matrilineages often, but not always, mirror such subspecific differentiation (Avise, 2000; but see Funk and Omland, 2003).

Using the mitochondrial cytochrome *b* gene, we discovered distinctly more variation than previous studies based on the 12S rRNA gene (van der Kuyl et al., 2002, 2005; Harris et al., 2003). This is in line with other investigations that found in testudinids the cytochrome *b* gene to be more variable than the 12S rRNA gene (Caccone et al., 1999; Palkovacs et al., 2002; Fritz et al., 2005a, 2006). Neither the four subspecies that were originally thought to compose *T. graeca* (*T. g. graeca* from Spain and northwestern Africa; *T. g. terrestris* from northeastern Africa, southeastern Turkey and the Levantine Region; *T. g. ibera* from Southeast Europe, Asia Minor, the Caucasus Region, west-

Figure 4. Light coloured spur-thighed tortoise from arid region in Syria (Ṣaydnāyā, Anti Lebanon Mts.; maximum weight 990 g, $n = 17$). Note healed serious injury on top of carapace.

Figure 5. Dark coloured spur-thighed tortoise from more humid environment in Syria (Qalat Samān, hills NW Aleppo, maximum weight 1900 g, $n = 15$).

Figure 6. Dark coloured spur-thighed tortoise from basaltic region in Syria (Jabal Durūz, maximum weight 1320 g, $n = 28$).

ern and central Iran; *T. g. zarudnyi* from eastern Iran; Mertens, 1946; Wermuth, 1958; Wermuth and Mertens, 1961, 1977), nor the many taxa that were later described or resurrected (table 1) correspond perfectly to any of our six major mtDNA clades. This could be indicative of inaccurate taxon limits.

Several authors agree that *T. g. ibera*, as currently understood, might be a conglomerate of distinct taxa, and that the name *ibera* should be used only for tortoises from the Caucasian Kura River Basin, the area around the type locality (Perälä, 2002a; Pieh et al., 2002; Danilov and Milto, 2004). However, the disagreement of subspecies delineation and mtDNA clades would not be resolved by using a restricted subspecies concept for *T. g. ibera* (table 1). Even our sequences of *T. g. ibera* sensu stricto from the Kura River Basin are scattered over two clades (A, C). The sequences from central Caucasian *ibera* localities, one from Mtskheta only 15 km away from the type locality of *ibera* (Tbilisi, Georgia), belong to clade C, which is otherwise found along the northeastern Black Sea coast (=*T. g. nikolskii*), in the Balkans and western Asia Minor (=*T. g. ibera* sensu lato), while the *ibera* sequences from the more eastern Caucasian locality Dashburun (Azerbaijan) appear in the same clade (A) as *T. g. pallasi* and the morphologically very distinctive *T. g. armeniaca*, so that a taxonomic break must be assumed within the Kura River Basin.

Until now, taxa within the *T. graeca* complex were defined exclusively by morphology. Our data imply that genetic differentiation is masked by pronounced morphological plasticity. This results in the unexpected nesting of morphologically well-defined taxa, like *T. g. armeniaca* or *T. g. floweri*, within clades comprising also geographically neighbouring, but morphologically distinctive populations of other taxa (clade A: *T. g. armeniaca*, *T. g. ibera*, *T. g. pallasi*; clade D: *T. g. anamurensis*, *T. g. antakyensis*, *T. g. floweri*, *T. g. ibera*, *T. g. terrestris*), or the scattering of sequences of tortoises of the same subspecies (*T. g. ibera* sensu stricto or *T. g. ibera* sensu lato)

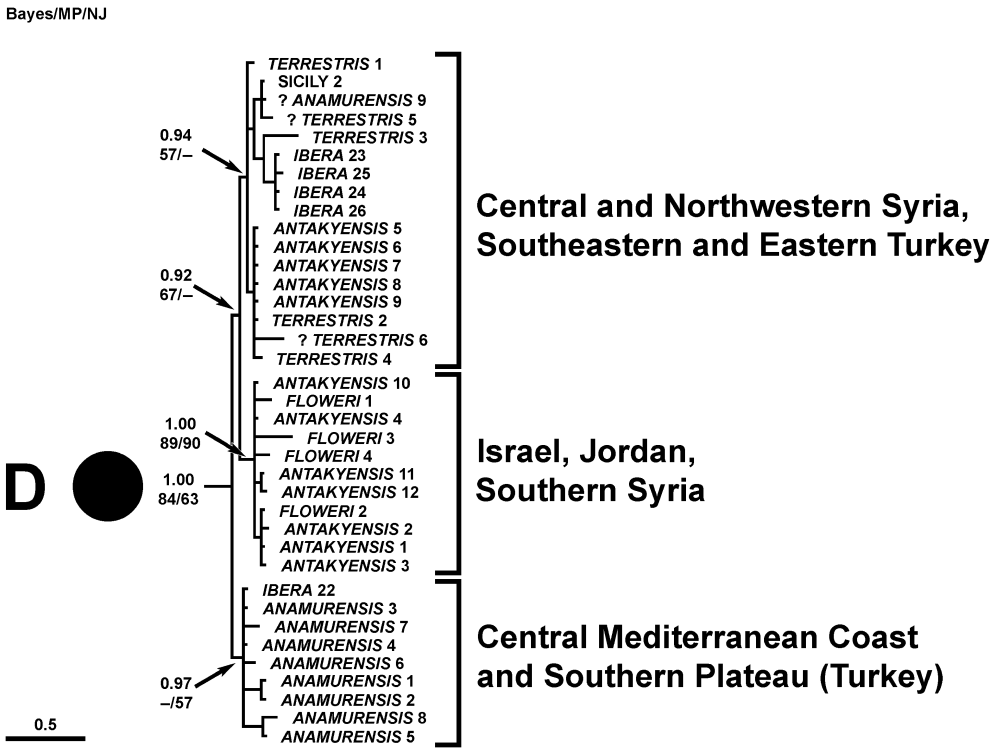

Figure 7. Detail of Bayesian tree of mtDNA haplotypes for spur-thighed tortoises from southern and eastern Asia Minor and the Levant. For further explanations see figure 2.

over two or three genetically distinct clades (A, C or A, C, D, respectively; fig. 2). Future research is needed to find out whether any morphological differences exist paralleling the six mtDNA clades.

In any case, it is obvious that the suggested existence of up to 20 distinct taxa within the *T. graeca* complex (Highfield and Martin, 1989a, b, c; Highfield, 1990; Pieh, 2001; Perälä, 2002a, b; Pieh and Perälä, 2002, 2004) is exaggerated and does not match genetic differentiation. To achieve a more realistic taxonomic arrangement reflecting mtDNA clades, we propose usage of the taxonomic arrangement in table 4, which is also most parsimonious with respect to nomenclatural changes of the formerly widely accepted subspecies delineation of Mertens (1946), Wermuth (1958) and Wermuth and Mertens (1961, 1977). As not all North African taxa were included in the present study, we refrain from synonymizing North African taxa with *T. g. graeca* Linnaeus, 1758 (mtDNA clade B) that represents a further valid subspecies. We admit that our subspecies delineation may be imprecise along range borders due to introgression of mitochondrial genomes.

Historic zoogeography

The distribution of the six major mtDNA clades within the *Testudo graeca* complex allows some zoogeographical insights. Like in many other organisms (Myers et al., 2000), a remarkably high diversity is found in the Caucasus Region, where four of the six mtDNA clades occur (A, C, D, E; fig. 1). The high diversity of the Caucasus Region is often explained by an admixture of old endemics and taxa of different geographic origin (e.g. Satunin, 1910; Darevskii, 1967; Tuniyev, 1990, 1995), highlighting the role of this mountain range as centre of endemism, refuge area and crossroads between the Mediterranean,

Table 4. Proposed new subspecies delineation for the eastern part of the range of *Testudo graeca* acknowledging mtDNA clades. For most type localities see table 1. Type localities of taxa that have not been regarded as valid in past decades are: *Testudo ibera racovitzai* Calinescu, 1931 – Tutrakan, Bulgaria; *Testudo ibera* var. *bicaudalis* Venzmer, 1920 – Taurus Mts., Cilicia, Turkey (Buskirk et al., 2001). We refrain from assigning the doubtful name *Testudo ecaudata* Pallas, 1814 (see Buskirk et al., 2001; Pieh et al., 2002) to clade E although its type locality (forested parts of Persia at the Caspian Sea) suggests identity with *T. g. buxtoni*.

MtDNA clade	Subspecies	Junior synonyms	Range
A	*Testudo graeca armeniaca* Chkhikvadze and Bakradze, 1991	*Testudo graeca pallasi* Chkhikvadze and Bakradze, 2002	Central west coast of Caspian Sea; eastern Caucasus Region and parts of central Caucasus Region, including Araks River Valley (Armenia, Turkey)
C	*Testudo graeca ibera* Pallas, 1814	*Testudo ibera racovitzai* Calinescu, 1931 *Testudo graeca nikolskii* Chkhikvadze and Tuniyev, 1986	Southeast Europe, western Asia Minor, Russian and Georgian Black Sea coast, central Caucasus Region (Georgia, adjacent Azerbaijan)
D	*Testudo graeca terrestris* Forsskål, 1775	*Testudo ibera* var. *bicaudalis* Venzmer, 1920 *Testudo floweri* Bodenheimer, 1935 *Testudo graeca anamurensis* Weissinger, 1987 *Testudo antakyensis* Perälä, 1996	Southern and eastern Asia Minor, Levant
E	*Testudo graeca buxtoni* Boulenger, 1921	*Testudo perses* Perälä, 2002	Northwestern and central Iran, in the eastern Caucasus Region probably also adjacent parts of other countries
F	*Testudo graeca zarudnyi* Nikolsky, 1896	–	East Iran

Europe, and Central Asia. The complicated geological history of the Caucasus Region and adjacent East Anatolia includes repeated Oligocene and Miocene episodes of isolation during marine transgressions and reconnections with European and Asiatic landmasses, in part opening corridors to Africa via Arabia (Rögl, 1998, 1999), resulting in several vicariance events for biota.

Not only the current high diversity in the Caucasus Region, but also the fossil record suggests a Caucasian origin for the *Testudo graeca* complex. Fossils referred to the extinct species *T. burtschaki* and *T. eldarica* from the Medial to Upper Miocene of Azerbaijan and eastern Georgia (Chkhikvadze, 1983, 1989; Danilov, 2005) are the oldest representatives of this group. In the Pliocene, tortoises resembling *T. graeca* were already widely distributed, with records in the central and northern Caucasus Region, Ukraine, Moldavia, Romania (*T. cernovi*, *T. kucurganica*; Chkhikvadze, 1983, 1989; Danilov, 2005) and Morocco (*T*. aff. *kenitrensis*; de Lapparent de Broin, 2000), indicating rapid dispersal.

The fossil record also provides evidence that the current patchy distribution of clade C (fig. 1) is a consequence of secondary range interruption. Besides the above-mentioned Pliocene fossils, there are many Pleistocene tortoise findings from the northern Caucasus and Black Sea Regions that are in part referred to *T. graeca* already (Chkhikvadze, 1983, 1989). Thus, the current gaps in the range of clade C tortoises are most probably the result of glacial extinction.

All tree building methods favour a sister group relationship of the East Caucasian and North African clades A and B, although both are fully allopatric today and separated by a large distribution gap (figs 1-2). From Central and West Europe there are no tortoise fossils known resembling unambiguously the *T. graeca* complex, despite an excellent fossil record (Bailón, 2001; Buskirk et al., 2001; de Lapparent de Broin, 2001). This suggests that the founder individuals of the current Spanish (and Italian)

populations were introduced. If the East Caucasian and the North African clades A and B are sister groups indeed and an invasion of North Africa via West Europe is to be excluded, a colonization route via the Levantine Region must be hypothesized. However, along the Levant another clade (D) is distributed today, so that at least two colonization waves must have reached this region.

A first dispersal event from the Caucasus Region to North Africa could have occurred in the Middle to Late Miocene, when intermittent land bridges connected North Africa with the Near and Middle East due to the northeastward movement of the Arabian Plate, leading later to complete closure of the Tethys Sea (Rögl, 1998, 1999). An invasion of North Africa via such a temporary land bridge is an attractive scenario, explaining later extermination and replacement of geographically connecting populations through clade D, now distributed in the Levantine Region (fig. 1).

Our investigation confirms that most of the introduced spur-thighed tortoise populations on western Mediterranean islands originated in North Africa, as suggested by the previous allocation of these populations to the subspecies *T. g. graeca* (Wermuth and Mertens, 1961, 1977; Buskirk et al., 2001). However, the record of a Sicilian tortoise belonging to the eastern clade D instead of the North African clade B provides evidence for multiple introductions from different regions, and that some individuals definitely did not come from North Africa.

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