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Environmentally caused dwarfism or a valid species—Is *Testudo weissingeri* Bour, 1996 a distinct evolutionary lineage? New evidence from mitochondrial and nuclear genomic markers

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Abstract

We examine the evolutionary relationships of the five traditionally recognized species of the western Palearctic tortoise genus *Testudo* (*T. graeca*, *T. hermanni*, *T. horsfieldii*, *T. kleinmanni*, and *T. marginata*) and the newly described dwarfed species *T. weissingeri* by using sequence data of the mitochondrial cytochrome *b* gene and nuclear genomic fingerprints with inter-simple sequence repeats (ISSR). *Testudo weissingeri* differs from *T. marginata* mainly by its smaller size and some color-pattern characteristics. *T. weissingeri* lives in the driest, poorest and hottest part of the distributional range of *T. marginata*. While both data sets demonstrated phylogenetic distinctness of the five traditionally recognized species of *Testudo*, some subspecies and even some local populations, we detected no differentiation between *T. marginata* and *T. weissingeri*. We conclude that *T. weissingeri* is not a distinct evolutionary unit. We suggest that its small size is the result of suboptimal environmental conditions with limited resources and synonymize it with *T. marginata*. *T. marginata* and *T. kleinmanni* form a clade that is supported both by our mtDNA and nuclear genomic data sets. According to mtDNA data, this clade is the sister taxon to the *T. graeca* complex. A sister group relationship of *T. hermanni* and ((*T. marginata* + *T. kleinmanni*) + *T. graeca*) is moderately to weakly supported by mtDNA data; *T. horsfieldii* is the sister taxon to a clade comprising all other *Testudo* species.

Keywords: Testudinidae; *Testudo*; *Testudo marginata*; *Testudo weissingeri*; Molecular phylogenetics; Mitochondrial DNA; Nuclear genomic fingerprinting; ISSR

1. Introduction

For decades it was accepted that the western Palearctic tortoise genus *Testudo* contains four to five, in part polytypic species (Ernst and Barbour, 1989; Fritz and Cheylan, 2001; Loveridge and Williams, 1957). In recent years, a large number of subspecies and species have been described or revived, in part in "gray literature" (Bour, 1996; Chkhikvadze, 1988; Chkhikvadze et al., 1990; Chkhikvadze and Bakradze, 1991, 2002; Chkhikvadze and Tuniyev, 1986; Highfield, 1990; Highfield and Martin, 1989a,b,c; Mayer, 1992; Perälä, 1996, 2001, 2002a; Pieh, 2001; Pieh and Perälä, 2002, 2004; Weissinger, 1987). Moreover, it has been suggested many subspecies be elevated to species level (Perälä, 2002a,b,c),

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resulting in as many as 27 instead of five species within *Testudo* (Guyot-Jackson, 2004; Pieh and Perälä, 2004). All of these investigations were based exclusively on more or less pronounced morphological differences. Until now, only two investigations applied mtDNA data to assess inter- and intraspecific diversity of *Testudo*. Álvarez et al. (2000) used a 426 bp fragment of the cytochrome *b* gene and a 397 bp fragment of the 12S rRNA gene to study variation within *T. graeca* on both sides of the Strait of Gibraltar. van der Kuyl et al. (2002) investigated phylogenetic relationships within *Testudo* using the 12S rRNA gene. In this study, we present new data on variation between and within all five traditionally recognized *Testudo* species, based on sequence data of the mitochondrial cytochrome *b* gene and nuclear fingerprinting with ISSR PCR. In particular, we focus on testing the validity of the two species of Marginated Tortoises, *Testudo marginata* Schoepff, 1792 and the recently described *T. weissingeri* Bour, 1996.

Testudo marginata is the largest representative of its genus, reaching a maximum shell length of approximately 40 cm. Its natural distribution is nearly entirely confined to peninsular Greece, the Peloponnese, Evvia, and a few Aegean islands. Outside of Greece, only a few records are known from Albania along the Greek border. In addition, an introduced population occurs in Sardinia (Bringsøe et al., 2001). A few years ago, a dwarfed population of Marginated Tortoises from the Mani Peninsula (Peloponnese) was described as a new species (*T. weissingeri* Bour, 1996). Soon after the description of *T. weissingeri*, concerns about its validity were raised (Artner, 1996, 2000). *T. weissingeri* lives in the hottest, driest and poorest part of the range of *T. marginata*. Thus, the small size and related characters of *T. weissingeri* could simply reflect suboptimal habitat conditions (Bringsøe et al., 2001). Therefore, Bringsøe et al. (2001) speculated that *T. weissingeri* could be synonymous with *T. marginata* and tentatively suggested treating *T. weissingeri* as a subspecies of *T. marginata*. However, based on a morphological comparison of 21 *T. weissingeri* with 14 *T. marginata* specimens, Perälä (2002b) concluded that *T. weissingeri* is "fully diagnosable in multivariate space" and hence deserves recognition as a full species. In contrast, van der Kuyl et al. (2002) found no difference in the slowly evolving mitochondrial 12S rRNA gene when comparing two *T. marginata* and two *T. weissingeri* sequences and assumed that both taxa were synonymous.

We compare *T. marginata*, *T. weissingeri*, and representatives of other *Testudo* species using mtDNA sequence data of the faster evolving cytochrome *b* gene (cyt *b*) and nuclear genomic fingerprinting with inter-simple sequence repeats (ISSR). Cyt *b* is known to have in other testudinid species a higher percentage of phylogenetically informative sites than the 12S rRNA gene (Caccone et al., 1999; Palkovacs et al., 2002). ISSR PCR produces species-specific nuclear fingerprints (Bornet and Branchard, 2001; Gupta et al., 1994; Wink et al., 1998; Zietkiewicz et al., 1994), allowing the identification of interspecific hybrids (Schilde et al., 2004; Wink et al., 2001; Wolfe et al., 1998) or of introgression (Nagy et al., 2003). This combined approach should enable us to decide whether *T. weissingeri* and *T. marginata* are distinct, even if mtDNA sequence data is not conclusive.

2. Materials and methods

2.1. Sampling

We studied 25 *Testudo marginata* samples from different parts of the species' range and seven samples of *T. weissingeri*, including the type locality Kardamíli. In addition, a sample from Kalámata was analyzed, from where Bour (1996) reported both taxa and intermediate individuals (Fig. 1). To get a measure of the cyt *b* variation among *Testudo* species, we studied eight samples of both subspecies of *T. hermanni*; 16 samples of *T. graeca* sensu lato, representing populations from the West Mediterranean, the Balkans, Asia Minor, and the Middle East; two samples of *T. kleinmanni*; and two samples of *T. horsfieldii* (Table 1). Blood samples were obtained by coccygeal vein puncture of wild or captive individuals. Samples were either preserved in an EDTA buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, and 0.1% thymol) or in ethanol (Wink, 1998) and stored at −20 °C until processing. Remaining blood and DNA samples are permanently kept at −80 °C in the blood sample and tissue collection of the Museum of Zoology Dresden.

2.2. DNA isolation

Total genomic DNA was extracted from the blood and tissue samples by an overnight incubation at 37 °C in lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl, and 1% SDS) including 1 mg of proteinase K (Merck, Darmstadt), followed by a standard phenol/chloroform protein extraction (Sambrook et al., 1989). DNA was precipitated from the supernatant with 0.8 volumes of cold isopropanol, centrifuged, washed, dried, and resuspended in TE buffer.

2.3. mtDNA

Our target sequence is the mitochondrial cytochrome *b* gene (cyt *b*), which is phylogenetically highly informative in testudinoid chelonians (Barth et al., 2004; Caccone et al., 1999; Feldman and Parham, 2002; Lenk et al., 1999; Palkovacs et al., 2002; Stephens and Wiens, 2003). DNA was amplified using the PCR primers MTa 5′-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC T-3′ and MT-f-na 5′-AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC

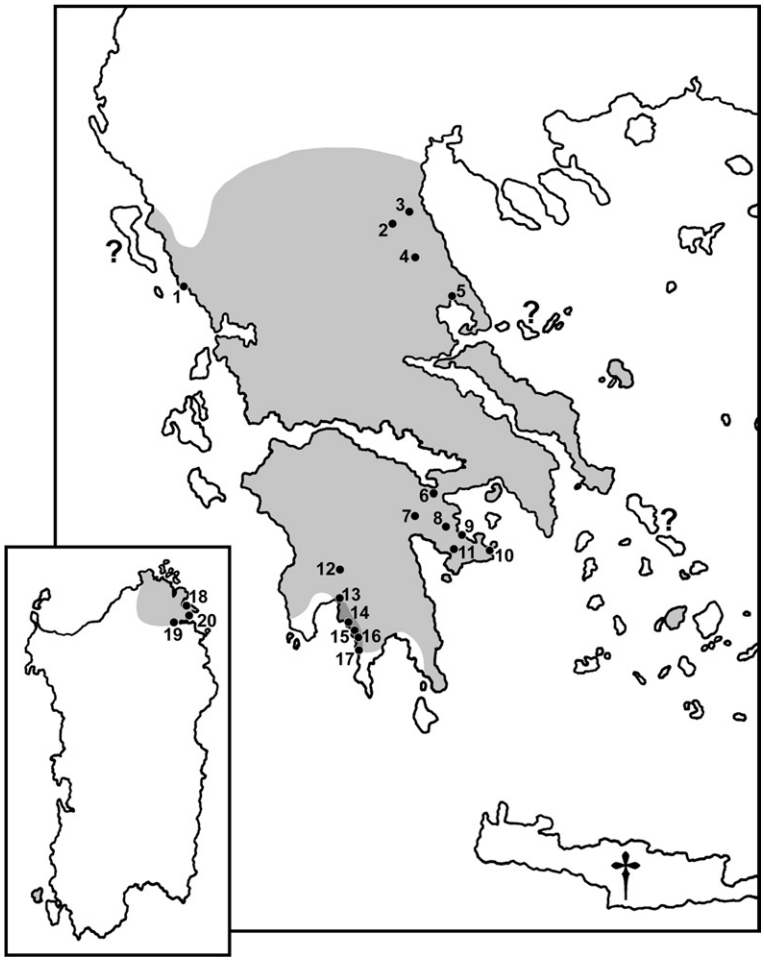

Fig. 1. Localities of *Testudo marginata* and *T. weissingeri* samples studied. Greece: 1, Parga; 2, Elassón; 3, Mt. Olymp; 4, Lárisa; 5, Volos; 6, Kórinthos; 7, Mykene; 8, Kapanitza; 9, between Áno Fanárion and Áyía Elení; 10, Poros Town; 11, Dídimi; 12, Loutro Valley near Fília; 13, Kalámata; 14, Kardamíli (type locality of *T. weissingeri*); 15, Áyios Nikólaos; 16, Stoupa; 17, Ithilo and Neo Ithilo; Sardinia (Italy): 18, Porto Rotondo; 19, near Olbia; and 20, Pittulongo. Range of *Testudo marginata* shaded; putative range (Bour, 1996) of *T. weissingeri* dark gray. Range of *T. marginata* on Sardinia superimposed. ? indicates doubtful records for *T. marginata*. The (†) cross on Crete indicates a Pleistocene occurrence (*T. marginata cretensis*; Bachmayer et al., 1976; Kotsakis, 1978; Mangili, 1980; Mayhew, 1977). Range of *T. marginata* according to Bringsøe et al. (2001).

CAA TG-3′. PCR was performed in a 50 µl volume containing 1 U of Amersham-Pharmacia Biotech *Taq* DNA polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl, pH 9. After an initial denaturing step for 5 min at 94 °C, 31 cycles were performed with annealing 52 s at 60 °C, primer extension 80 s at 72 °C, and denaturing 45 s at 94 °C. PCR products were purified by precipitation under the following conditions: 1 volume PCR product (30 µl), 1 volume 4 M NH_4Ac (30 µl) and 12 volumes EtOH (100%; 360 µl). DNA was pelleted by centrifugation (15 min at 13,000 rpm) and the pellet washed with 70% ethanol. The pellet was dissolved in 20 µl H_2O. Sequencing was performed under the following conditions: Sequencing primers were MT-a 5′-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC-3′, MT-c-emys 5′-CCG GAT CAA ACA AYC CAA CAG G-3′, and MT-E 5′-GCA AAT AGG AAG TAT CAT TCT GG-3′. Sequencing solution: 1 µl primer (5 pM/µl), 1–3 µl PCR product, 1 µl premix and H_2O to give a final volume of 5 µl. The premix was supplied by DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences). PCR cycle: 26 × 20 s at 95 °C, 15 s at 50 °C, and 2 min at 60 °C. Sequencing products were purified on sephadex G50 columns in Multiscreen MAHVN 4510 well plates (Millipore). Purified sequencing products were sequenced with a MegaBace 1000 instrument (Amersham Biosciences) equipped with 96 capillaries filled with MegaBace Long Read Matrix (Amersham Biosciences). Sequence data of almost the complete cyt *b* gene were obtained directly from the sequencer. Raw data were analyzed with Sequence Analyzer Version 3.0 (Professional Edition; Amersham Biosciences). Because no internal stop codons were found and nucleotide frequencies corresponded to those known for coding mtDNA, we conclude that we amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes. Nucleotide composition was as follows: T = 27.4%, C = 30.0%, A = 31.0%, and G = 11.6%.

Table 1
Studied *Testudo* samples and outgroups

Taxon	Locality	cyt *b*	EMBL Accession No.	ISSR	MTD T/HD
Testudo marginata	Greece: Epirus: Parga	+	AJ888308	+	25660
Testudo marginata	Greece: Thessalia: Elassón	+	AJ888309		22069
Testudo marginata	Greece: Thessalia: Lárisa	+	AJ888310		22068
Testudo marginata	Greece: Thessalia: Mt. Olymp	+	AJ888311		22046
Testudo marginata	Greece: Thessalia: Volos			+	25656
Testudo marginata	Greece: Thessalia: Volos	+	AJ888312	+	25657
Testudo marginata	Greece: Thessalia: Volos	+	AJ888313	+	25658
Testudo marginata	Greece: Thessalia: Volos	+	AJ888314		25771
Testudo marginata	Greece: Thessalia: Volos	+	AJ888315		25768
Testudo marginata	Greece: Thessalia: Volos	+	AJ888316		25770
Testudo marginata	Greece: Peloponnes: between Áno Fanárion and Áyía Elení 37°34′09N, 23°14′36E	+	AJ888317		22039
Testudo marginata	Greece: Peloponnes: Dídimi	+	AJ888318		22047
Testudo marginata	Greece: Peloponnes: 2 km E Kapanitza	+	AJ888319		22020
Testudo marginata	Greece: Peloponnes: Loutro Valley near Fília	+	AJ888320		25663
Testudo marginata	Greece: Peloponnes: Loutro Valley near Fília	+	AJ888321		25664
Testudo marginata	Greece: Peloponnes: Mykene	+	AJ888322		22045
Testudo marginata	Greece: Peloponnes: Mykene	+	AJ888323		22312
Testudo marginata	Greece: Peloponnes: Mykene	+	AJ888324		25695
Testudo marginata	Greece: Peloponnes: Kórinthos	+	AJ888325		25653
Testudo marginata	Greece: Peloponnes: Kórinthos	+	AJ888326		25655
Testudo marginata	Greece: Peloponnes: Kórinthos	+	AJ888327		25767
Testudo marginata	Greece: Peloponnes: Poros Town 37°30′37N, 23°28′14E	+	AJ888328		22038
Testudo marginata	Greece: Peloponnes: Poros Town 37°30′37N, 23°28′14E	+	AJ888329		25693
Testudo marginata	Greece: Peloponnes: Poros Town 37°30′37N, 23°28′14E	+	AJ888330		25694
Testudo marginata	Italy: Sardinia: near Olbia			+	25696
Testudo marginata	Italy: Sardinia: near Olbia			+	25697
Testudo marginata	Italy: Sardinia: near Olbia			+	25698
Testudo marginata	Italy: Sardinia: near Olbia			+	25699
Testudo marginata	Italy: Sardinia: Porto Rotondo	+	AJ888331	+	22307
Testudo marginata	Italy: Sardinia: Pittulongo	+	AJ888332		25322
Testudo marginata/weissingeri?	Greece: Peloponnes: Kalámata	+	AJ888333	+	25773
Testudo weissingeri	Greece: Peloponnes: Áyios Nikólaos	+	AJ888334	+	22059
Testudo weissingeri	Greece: Peloponnes: Áyios Nikólaos	+	AJ888335	+	22310
Testudo weissingeri	Greece: Peloponnes: Ithilo	+	AJ888336	+	25659
Testudo weissingeri	Greece: Peloponnes: Ithilo	+	AJ888337	+	25665
Testudo weissingeri	Greece: Peloponnes: Kardamíli	+	AJ888338	+	25764
Testudo weissingeri	Greece: Peloponnes: Neo Ithilo	+	AJ888339	+	25666
Testudo weissingeri	Greece: Peloponnes: Stoupa	+	AJ888340	+	25662
Testudo graeca anamurensis	South Turkey: Anamurium	+	AJ888347	+	22032
Testudo graeca anamurensis	South Turkey: Gazipaşa near Alanya	+	AJ888348	+	22033
Testudo graeca graeca s. l.	Libya	+	AJ888341		22070
Testudo graeca graeca s. l.	Mallorca: N Calvia	+	AJ888342		22072
Testudo graeca graeca s. l.	Italy: Sardinia: Sinis Peninsula	+	AJ888343		25318
Testudo graeca ibera s. l.	Bulgaria: Albena 43°22′527N, 28°04′972E	+	AJ888349	+	22055
Testudo graeca ibera s. l.	Bulgaria: Albena 43°22′366N, 28°05′139E	+	AJ888350	+	22057
Testudo graeca ibera s. l.	Greece: Kos Island (Asia Minor)	+	AJ888351		25324
Testudo graeca ibera s. l.	Greece: Kos Island (Asia Minor)	+	AJ888352		Tg817
Testudo graeca ibera s. l.	East Turkey: Van Gölü	+	AJ888353		22061
Testudo graeca ibera s. l.	East Turkey: 10 km N Van	+	AJ888354		22044
Testudo graeca ibera s. l.	Central Turkey: outskirts of Seydişehir	+	AJ888355		22023
Testudo graeca ibera s. l.	South Turkey: Sahayi Sitige 36°50′02N, 31°09′01E, S Serik and Aspendos	+	AJ888356		22024
Testudo graeca ssp.	Israel: Tiberias	+	AJ888344		25716
Testudo graeca ssp.	Israel: Tiberias	+	AJ888345		25717
Testudo graeca ssp.	Jordan: Jarash	+	AJ888346		25718
Testudo hermanni boettgeri	Greece: SW Corfu near Great Salt Lake 39°47′25N, 19°55′16E			+	22027

(*continued on next page*)

Table 1(*continued*)

Taxon	Locality	cyt *b*	EMBL Accession No.	ISSR	MTD T/HD
Testudo hermanni boettgeri	Greece: Epirus: Parga	+	AJ888357	+	25712
Testudo hermanni boettgeri	Greece: Epirus: Parga	+	AJ888358	+	25713
Testudo hermanni boettgeri	Greece: Epirus: near Great Parga Lake 39°20′48N, 20°26′24E			+	22025
Testudo hermanni boettgeri	Greece: Epirus: near Great Parga Lake 39°20′48N, 20°26′24E			+	22026
Testudo hermanni boettgeri	Greece: Central Makedonia: Stavros			+	25711
Testudo hermanni boettgeri	Greece: Thessalia: Meteora			+	25765
Testudo hermanni boettgeri	Greece: Thessalia: Mt. Olymp			+	22048
Testudo hermanni boettgeri	Greece: Thessalia: Mt. Olymp	+	AJ888359		22050
Testudo hermanni boettgeri	Greece: Thessalia: Platamonas	+	AJ888360		25710
Testudo hermanni boettgeri	Greece: Thessalia: Platamonas			+	25769
Testudo hermanni hermanni	Italy: Sardinia: Porto Palmas			+	25319
Testudo hermanni hermanni	Italy: Sardinia: Porto Palmas	+	AJ888361	+	25320
Testudo hermanni hermanni	Italy: Toscana: Roccatederighi	+	AJ888362	+	22066
Testudo hermanni hermanni	Italy: Toscana: Roccatederighi	+	AJ888363	+	25327
Testudo hermanni hermanni	South Italy	+	AJ888364		22060
Testudo horsfieldii kazachstanica	Kazakhstan	+	AJ888365		22058
Testudo horsfieldii rustamovi	Iran: Golestan Province: Gorgan Town	+	AJ888366	+	26458
Testudo horsfieldii rustamovi	Iran: Golestan Province: Miankaleh Peninsula			+	26459
Testudo horsfieldii rustamovi	Iran: Golestan Province: Maraveh Tappeh Town			+	26460
Testudo horsfieldii rustamovi	Iran: Semnan Province: 60 km S Damghan Town			+	26463
Testudo kleinmanni	Unknown	+	AJ888370	+	25325
Testudo kleinmanni	Unknown	+	AJ888371	+	25326
Testudo kleinmanni	Unknown			+	25571
Indotestudo forstenii	Indonesia: Sulawesi: Manado	+	AJ888372		22030
Malacochersus tornieri	Unknown	+	AJ888373		25568
Malacochersus tornieri	Unknown	+	AJ888374		25569

Note. MTD T/HD—Museum of Zoology Dresden Tissue Collection Number.

2.4. Phylogenetic analysis

Data were analyzed using maximum parsimony (MP) and neighbor joining (NJ) with PAUP* 4.0b10 (Swofford, 2002). For tree rooting, we used sequences of *Indotestudo forstenii* and *Malacochersus tornieri* (Table 1), representatives of tortoise genera that are thought to be closely related to *Testudo* (Crumly, 1985; Gaffney and Meylan, 1988; van der Kuyl et al., 2002) and an earlier published sequence of the European pond turtle (*Emys orbicularis orbicularis* haplotype Ia; Lenk et al., 1999, EMBL Accession No. AJ131417). Unweighted MP analyses were executed using "tree-bisection–reconnection" (TBR) branch swapping and the heuristic search option. For the ingroup species, 713 of 1124 aligned sites were constant, 122 characters were variable but parsimony-uninformative, and 289 variable characters were parsimony-informative. We computed a strict consensus tree of 1000 equally parsimonious MP trees. Due to the enormous computational time, bootstrap values (Felsenstein, 1985) were calculated under MP only for a pruned data set, consisting of the outgroups, one *Testudo horsfieldii kazachstanica* and *T. h. rustamovi* sequence, and two representatives of all other ingroup taxa. Genetic distances (uncorrected *p* distances) were calculated from a data set of 1000 bp. EMBL accession numbers of newly studied samples are in Table 1.

2.5. Nuclear genomic fingerprinting

To get a measure of variation of the nuclear genome and to find out whether gene flow exists between *Testudo marginata* and *T. weissingeri*, we conducted nuclear genomic fingerprinting with inter-simple sequence repeats (ISSR) for nine *Testudo marginata*, seven *T. weissingeri* and an individual from Kalámata. To compare the variation with other species, we included 24 samples of other *Testudo* species, representing the same major clades as used for cyt *b* sequencing (Table 1).

ISSR PCR is a simple and cheap method for mapping the nuclear genome and for discovering rearrangements. It generates nuclear fingerprints that are usually diagnostic for species-level taxa (Gupta et al., 1994; Nagy et al., 2003; Wink et al., 1998, 2001; Wolfe and Liston, 1998; Wolfe et al., 1998; Zietkiewicz et al., 1994). ISSR employs a single PCR primer, binding to di- or trinucleotide repeat motifs (microsatellites), which are abundant in eukaryotic genomes (Condit and Hubbell, 1991; Tautz and Renz, 1984). Since sequences of microsatellites are conserved over a wide range of organisms, ISSR

PCR can use universal primers. The amplified regions correspond to the nucleotide sequence between two simple sequence repeat (SSR) priming sites orientated on opposite DNA strands (Wolfe et al., 1998). SSR regions appear to be scattered evenly throughout the genome (Condit and Hubbell, 1991; Tautz and Renz, 1984), resulting in a large number of polymorphic bands. In this study, the primers $(GACA)_4$ and $(GAA)_5$ were used.

For ISSR amplification 15 ng of total DNA and 3 pmol primer were selected as best reaction conditions and used for all further amplifications in a total volume of 12.5 μl: 1.5 mM $MgCl_2$, 0.1 mM of dGTP, dCTP, and dTTP, 0.075 mM dATP, 1 μCi [α-^{33}P]dATP, 1.25 μl of 10× amplification buffer (100 mM Tris–HCl, pH 8.5, 500 mM KCl, 5% Triton X-100) and 0.4 U *Taq* polymerase (Amersham-Pharmacia Biotech). After an initial denaturation (120 s at 94 °C), 33 cycles of 60 s at 94 °C, 120 s at 55 °C, and 120 s at 72 °C were performed on a Biometra thermocycler, followed by 4 min at 72 °C and storage at 4 °C. After electrophoresis on 0.2 mm denaturing polyacrylamide gels (size 45 × 30 cm) at 65 W for 3 h, the gel was exposed to Kodak Hyperfilm for several hours. ISSR fingerprinting was repeated several times to ensure reproducibility of the pattern.

Fragment patterns were analyzed manually. Eighty-four unambiguously identifiable bands were transferred into a presence/absence matrix scoring each particular fragment. Any ISSR PCR product is a DNA sequence between two microsatellites. The position of these microsatellites may differ between distinct taxa, resulting in species-specific banding patterns. We interpret the occurrence of a certain fragment as representing a derived character and used for tree rooting a virtual outgroup, assuming absence of a fragment as the plesiomorphic character state. Based on this assumption and the presence/absence matrix, a NJ tree as implemented in PAUP* 4.0b10 (Swofford, 2002) was calculated and its robustness was tested under the 50% consensus criterion by bootstrapping (1000 replicates).

3. Results

3.1. mtDNA phylogeny

Both tree-building methods (NJ, MP) yielded the same topology, which contains clades corresponding to the five traditionally recognized species within *Testudo* (*T. graeca*, *T. hermanni*, *T. horsfieldii*, *T. kleinmanni*, and *T. marginata*). While we detected some variation among our samples of the *Testudo graeca* complex and the subspecies of *T. hermanni* and *T. horsfieldii*, our data do not distinguish *T. marginata* and *T. weissingeri*. The phylogenetic analyses do not resolve clades corresponding to *T. marginata* or *T. weissingeri*, and thus do not support monophyly of either species (Figs. 2–4). *Testudo marginata* from different parts of Greece (Epirus, Thessalia, Peloponnese) and Sardinia and *T. weissingeri* show no geographical differentiation of haplotypes, in contrast to the subspecies of *T. graeca*, *T. hermanni*, or *T. horsfieldii*. Uncorrected mean p distances within the *T. graeca* complex, *T. hermanni*, and the two investigated subspecies of *T. horsfieldii* range from 1.2 to 2.6%. Between these and the other *Testudo* species, mean sequence divergence varies between 6.8 and 12.6%. However, comparing *T. marginata* and *T. weissingeri* we found only a low average sequence divergence of 0.2%, matching the variation found within each of these species and significantly lower than the within-taxon sequence divergence of *T. graeca*, *T. hermanni*, and *T. horsfieldii* (Table 2).

Our data corroborate the finding of van der Kuyl et al. (2002) that *T. kleinmanni* is the sister taxon to the cluster containing *T. marginata* and *T. weissingeri* (bootstrap support 87% under NJ, however only 45% under MP). The sister taxon of this group is *T. graeca*. This clade is supported by high bootstrap values of 99% under NJ and 88% under MP. *Testudo hermanni* is the sister taxon to the cluster ((*T. marginata*/*weissingeri* + *T. kleinmanni*) + *T. graeca*), and *T. horsfieldii* is the sister taxon to a clade comprising all other *Testudo* species. However, the sister group relationship of *T. hermanni* and ((*T. marginata*/*weissingeri* + *T. kleinmanni*) + *T. graeca*) is only moderately or weakly supported by the bootstrap (73% NJ; 48% MP), while the sister group relationship of *T. horsfieldii* and all other *Testudo* species has high bootstrap support under NJ (84%) and weak bootstrap support under MP (52%). The two traditionally recognized subspecies of *T. hermanni* are reflected by our phylogeny in that the samples from the West Mediterranean form a monophyletic group corresponding to *T. h. hermanni*, supported by a bootstrap value of 100% (NJ, MP). The bootstrap support for the monophyly of the eastern subspecies *T. h. boettgeri* is lower (61% NJ, 92% MP).

It is beyond the scope of the present paper to investigate the systematics of the *T. graeca* complex. However, we note that the studied samples cluster into three distinct groups that may correspond to distinct evolutionary lineages. One cluster contains individuals from central and eastern Asia Minor and the Middle East; the second corresponds to samples from western Asia Minor (Kos Island, Greece) and Bulgaria; and the third, which is the sister taxon to a clade containing the other two, represents tortoises from the West Mediterranean. The sequence divergence between the clusters from (central and eastern Asia Minor + Middle East) and (western Asia Minor + Bulgaria) corresponds approximately to the divergence observed between the two subspecies of *T. hermanni*, whereas the differentiation of the West Mediterranean samples is more pronounced (Figs. 2–4).

3.2. Nuclear fingerprinting

The branching pattern of the NJ tree agrees perfectly with the traditional *Testudo* taxa (Fig. 5). All *Testudo*

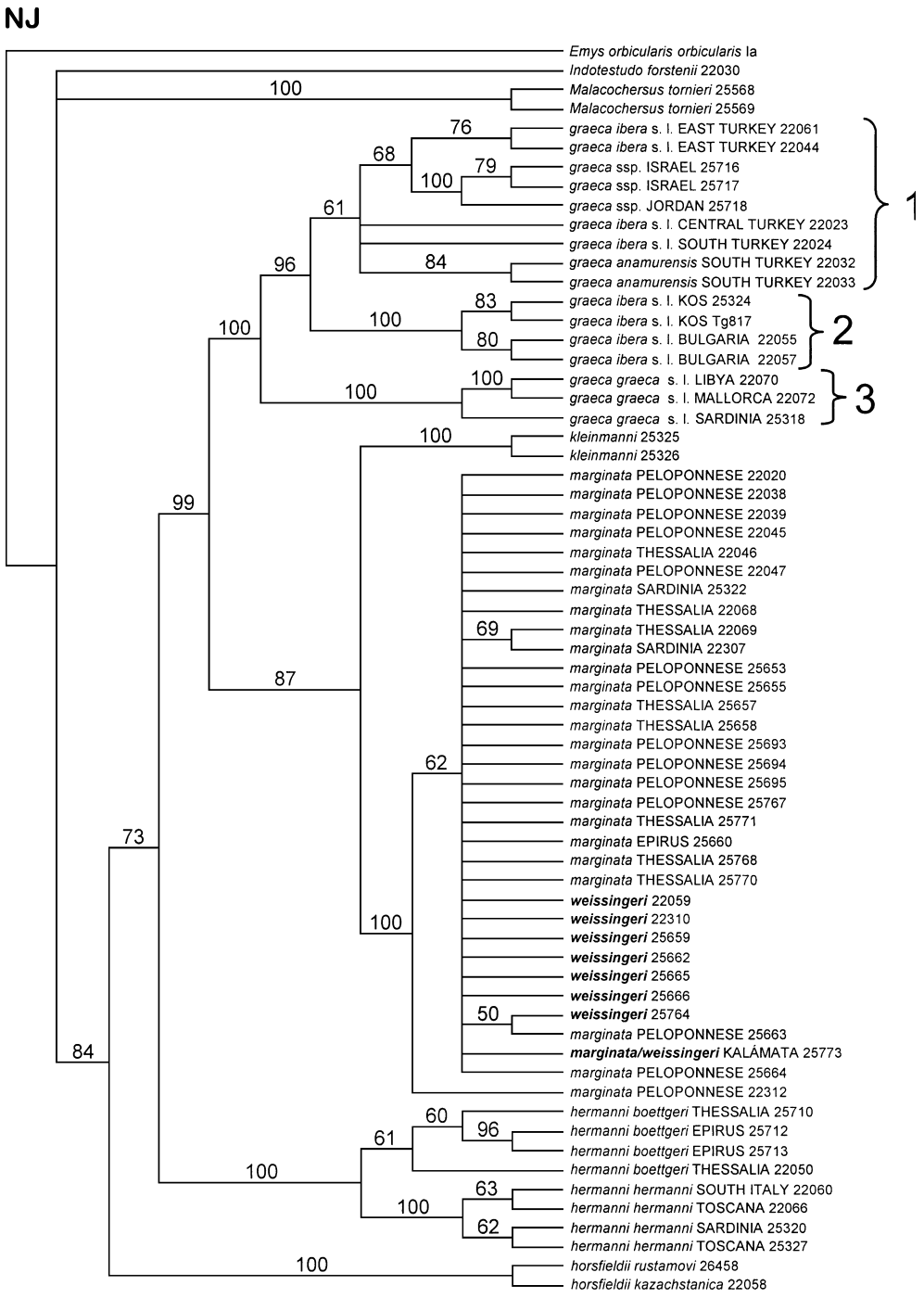

Fig. 2. NJ cladogram of mtDNA haplotypes from *Testudo*. Numbers following taxon names or localities refer to Table 1. Numbers along the branches represent bootstrap values (1000 replicates) equal to or greater than 50. Note the lacking differentiation of *T. marginata* and *T. weissingeri*. Three distinct clades occur within the *T. graeca* complex: (1) central and eastern Asia Minor and the Middle East, (2) western Asia Minor and Bulgaria, and (3) West Mediterranean.

species, except *T. marginata* and *T. weissingeri*, appear as monophyletic units, supported by high bootstrap values (100%). The monophyly of the clade containing *T. kleinmanni* and *T. marginata/weissingeri* is confirmed by high bootstrap support (96%). Moreover, the ISSR banding patterns allow discrimination between the two subspecies of *T. hermanni* and between *T. graeca ibera* from Bulgaria and *T. g. anamurensis*. Within *T. h. hermanni*, even discrimination between Sardinian and Tuscan samples is permitted. In contrast, no differences between *T. marginata* and *T. weissingeri* are detected. The investigated samples of both species cluster together, and no geographically correlated pattern is evident.

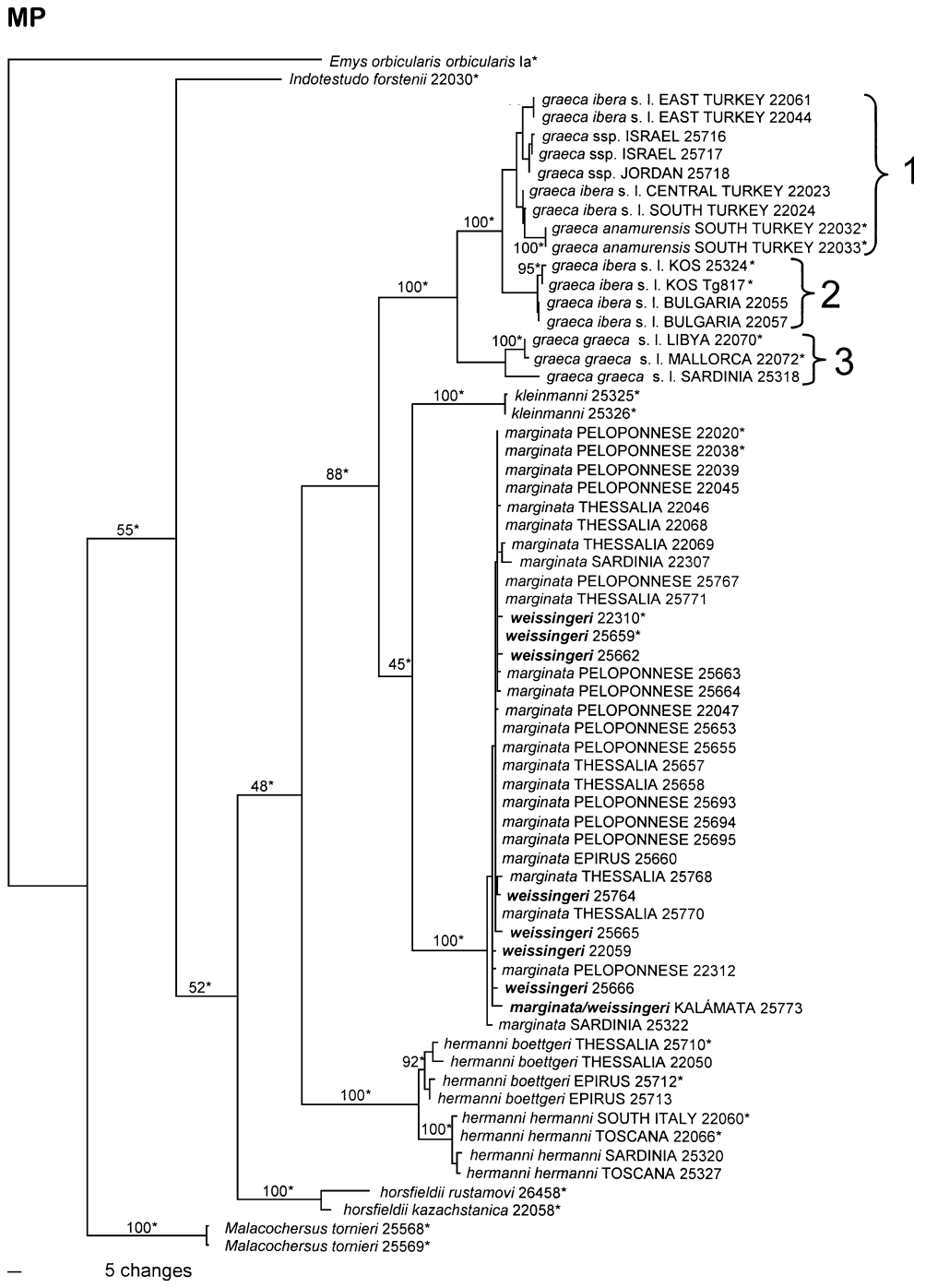

Fig. 3. MP phylogram of mtDNA haplotypes from *Testudo* with bootstrap values for a pruned data set including only the asterisked specimens. Branch lengths are proportional to the scale bar with the unit corresponding to the mean number of nucleotide changes per site. For further explanations see Fig. 2.

4. Discussion

Our mtDNA and nuclear fingerprinting data do not support the distinctiveness of *Testudo weissingeri*. While we found all traditionally recognized *Testudo* species and some subspecies or geographic populations clearly distinct in both data sets, neither the mtDNA data nor nuclear fingerprinting revealed any significant differences between *T. marginata* and *T. weissingeri*. Our mtDNA findings are in line with the results of van der Kuyl et al. (2002). These authors sequenced the slowly evolving 12S rRNA gene of two *T. marginata* and two *T. weissingeri* and found no differences. We used the faster-evolving cytochrome *b* gene and greater sample sizes for *T. marginata* ($n = 25$) and *T. weissingeri* ($n = 7$), and found no distinction between the species. However, it is

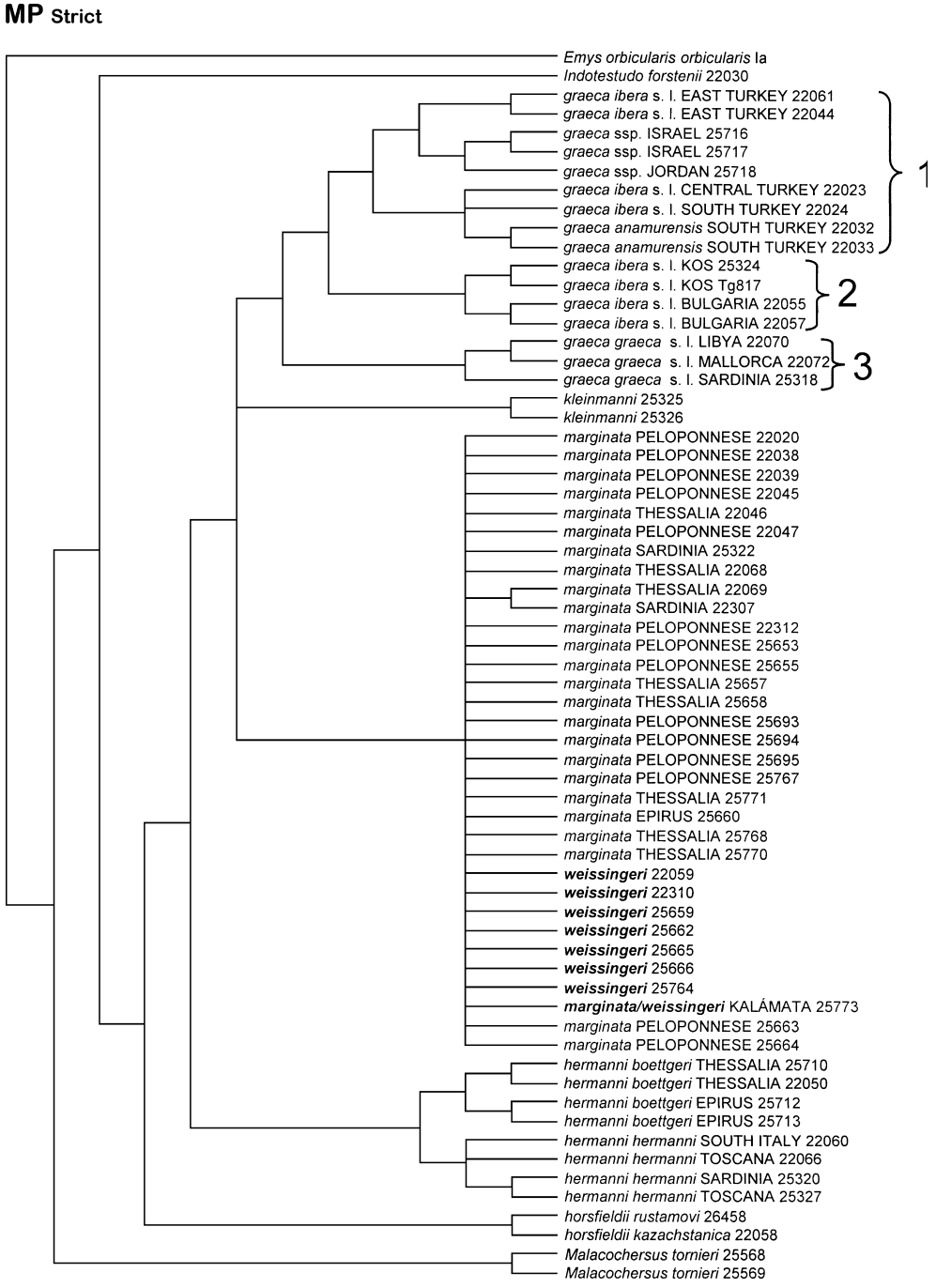

Fig. 4. Strict consensus of 1000 equally most parsimonious trees of mtDNA haplotypes from *Testudo* (768 steps); CI = 0.643, HI = 0.357, RI = 0.905, RC = 0.58. For further explanations see Fig. 2.

well known that recently split taxa may share mtDNA haplotypes, due to incomplete lineage sorting, introgression, or hybridization (Ballard and Whitlock, 2004; Funk and Omland, 2003). To get a measure of the variation in the nuclear genome, we conducted fingerprinting with ISSR. This technique proved to be more sensitive on the intraspecific level. ISSR fingerprints discriminated even geographically distinct populations of *T. hermanni hermanni* (but not of the eastern subspecies *T. h. boettgeri*). However, even ISSR did not reveal any differences between *T. marginata* and *T. weissingeri*, reflecting the lack of diagnostic inter-simple sequence repeat fragments in the nuclear genome of *T. weissingeri*.

Testudo weissingeri differs from *T. marginata* mainly by its smaller size and some color-pattern characteristics. The shell length in *T. weissingeri* is normally below 21.5 cm, while *T. marginata* usually exceeds 26.0 cm (Bour, 1996). The dwarfism of *T. weissingeri* and related allometric differences discriminate it morphologically by multivariate methods from large-sized *T. marginata*

Table 2
Uncorrected *p* distances (percentages) within and between *Testudo* species based on a dataset of 1000 bp

	Testudo graeca	*Testudo hermanni*	*Testudo horsfieldii*	*Testudo kleinmanni*	*Testudo marginata*	*Testudo weissingeri*
Testudo graeca	**2.585 (0.000–5.950)**	*11.325–13.623*	*9.931–12.964*	*8.123–9.990*	*7.349–10.122*	*7.457–10.091*
Testudo hermanni	12.562	**1.183 (0.000–2.752)**	*9.931–13.057*	*10.673–11.907*	*9.871–11.864*	*10.262–11.806*
Testudo horsfieldii	11.581	11.622	**2.461 (–)**	*9.990–11.333*	*10.072–11.788*	*10.328–11.774*
Testudo kleinmanni	9.139	11.454	10.662	**0.000 (–)**	*6.452–7.301*	*6.862–7.164*
Testudo marginata	8.748	11.042	10.942	6.843	**0.171 (0.000–1.010)**	*0.000–1.010*
Testudo weissingeri	8.922	11.272	11.068	6.976	0.240	**0.317 (0.101–0.505)**

Note. Mean distances between species are given below, ranges (in italics) above the diagonal. The within-taxon sequence divergence is given in bold on the diagonal (mean and range). The sequence of a Marginated Tortoise from the locality Kalámata, from where *Testudo marginata*, *T. weissingeri* and hybrids between both species have been reported, was not included.

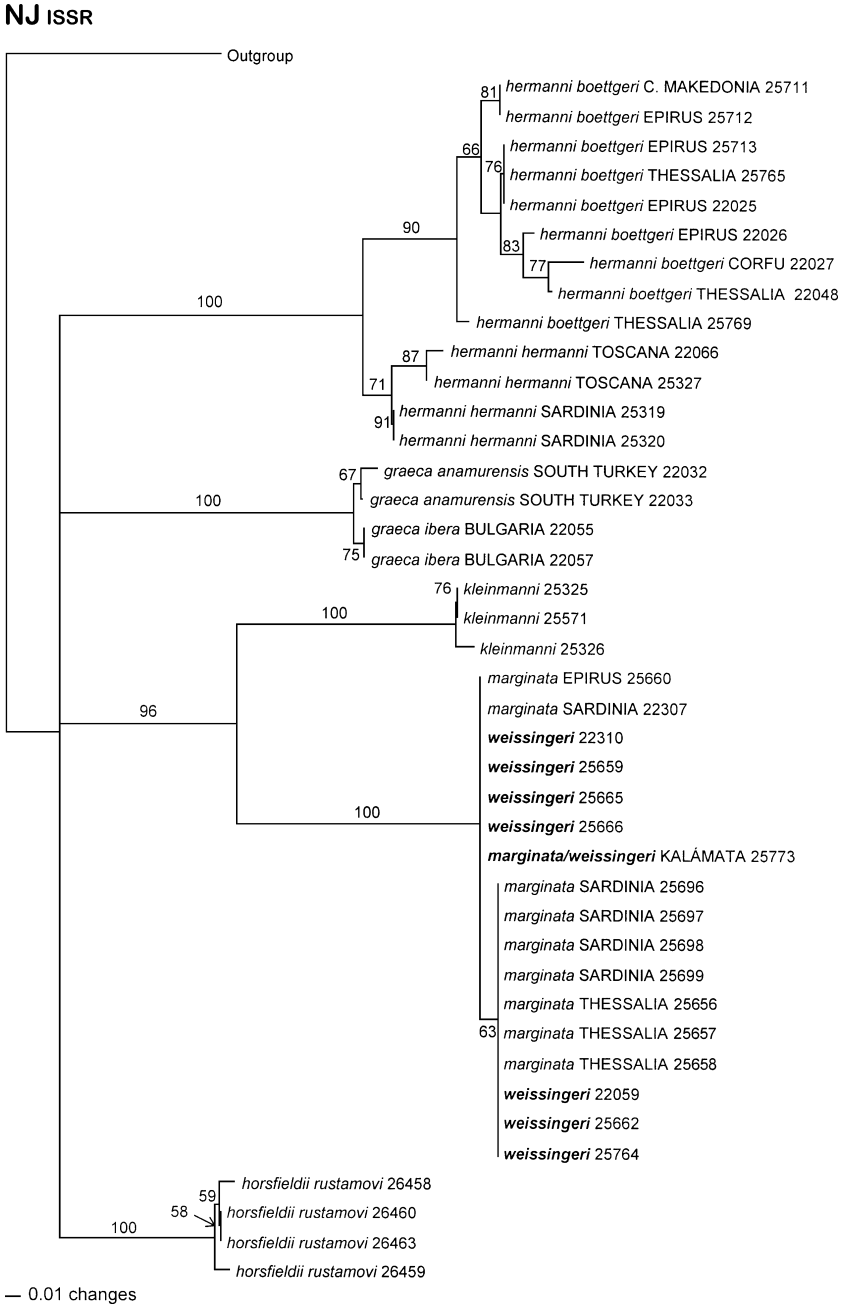

Fig. 5. NJ phylogram of ISSR fingerprints of *Testudo* based on 84 scored fragments (50% consensus tree). Numbers along the branches represent bootstrap values (1000 replicates) greater than 50. For further explanations see Fig. 2.

(Perälä, 2002b). However, small tortoises and individuals colored like *T. weissingeri* also occur throughout the range of *T. marginata* (Artner, 1996; U. Fritz unpubl. observation). Whether this represents individual of interpopulational variation is unclear, and we do not know whether such small individuals were included in the discriminant analyses of Perälä (2002b). Also, other characters originally thought to be diagnostic for *T. weissingeri*, like the presence of thigh tubercles, are common in *T. marginata* (Artner, 1996; Perälä, 2002d).

It is well known that many animal species show reduced body sizes in an environment with limited resources (e.g., *Emys orbicularis*: Fritz, 2003; *Homo sapiens*: Shea and Bailey, 1996). Another example of this phenomenon is the widespread island dwarfism (e.g., Jianu and Weishampel, 1999; Palombo, 2001; Sondaar, 1977; Vartanyan et al., 1993), currently attracting considerable public interest with the newly described dwarfed *Homo floresiensis* (Brown et al., 2004). Likewise, Bringsøe et al. (2001) suggested that the small size of *T. weissingeri* is a response to suboptimal habitat. *Testudo weissingeri* occupies the hottest, driest and poorest region within the range of *T. marginata* where it replaces the latter species. Taking the environmental pressure and the lack of differentiation in the mitochondrial and nuclear genome into account, we conclude that *Testudo weissingeri* Bour, 1996 is not a distinct evolutionary lineage and relegate it to the synonymy of *Testudo marginata* Schoepff, 1792.

The congruence of morphology-based traditional taxa, mtDNA phylogeny, and ISSR fingerprints indicates that nuclear fingerprinting with ISSR PCR is a powerful tool for species delineation in testudinids. However, compared with mtDNA data, the phylogenetic resolution of ISSR is generally poorer. In conclusion, ISSR is ideally suited for complementing mtDNA data when this data set alone is not decisive. On the other hand, our findings question the taxonomic value of minute morphological differences in color-pattern or morphometry as used in recent studies to erect new *Testudo* taxa. This is highlighted by the observation that the introduced Sardinian population of *T. marginata* differs in some morphological characters from Greek Marginated Tortoises. With the exception of its smaller average size, similar characters occur also in "*T. weissingeri*." In both, the hind shell margin is less flaring and less serrated, and the carapace is less distinctly concave when viewed from above than in normal-sized Greek *T. marginata*. These characters of Sardinian *T. marginata* and "*T. weissingeri*" can be interpreted as persisting juvenile traits (Bringsøe et al., 2001). It is unknown whether the Sardinian population of *T. marginata* was introduced in prehistoric or historic times. In any case, its morphological distinctiveness shows that tortoises are capable of modifying their morphology within a few hundred or thousand years, due to genetic bottlenecks or environmental pressure. This argues for caution when erecting further new *Testudo* species or subspecies without accompanying genetic studies. A future challenge will therefore be to expand the genetic investigations to test the genetic distinctiveness of the many recently described or resurrected *Testudo* taxa.

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