A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys* (Reptilia, Testudines, Emydidae)

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Geographic variation in the mtDNA haplotypes (cytochrome *b* gene) of 127 European pond turtles from Italy was investigated. Thirty-eight of the Italian samples were also studied by nuclear fingerprinting (ISSR PCR) and compared with samples from other parts of the range representing all nine currently known mtDNA lineages of *Emys orbicularis*. Our genetic findings were compared against morphological data sets (measurements, colour pattern) for 109 adult turtles from southern Italy. Italy is displaying on a small geographical scale the most complicated variation known over the entire distributional area of *Emys* (North Africa over Europe and Asia Minor to the Caspian and Aral Seas). The Tyrrhenic coast of the Apennine Peninsula, the Mt. Pollino area and Basilicata are inhabited by *Emys orbicularis galloitalica*, a subspecies harbouring a distinct mtDNA lineage. The same lineage is also found in Sardinia. Along the Adriatic coast of Italy and on the Salentine Peninsula (Apulia, southern Italy), another morphologically distinctive subspecies (*Emys orbicularis hellenica*) occurs, which also bears a different mtDNA lineage. A higher diversity of mtDNA haplotypes in the south of the Apennine Peninsula suggests that the glacial refugia of *E. o. galloitalica* and *E. o. hellenica* were located here. A further refuge of *E. o. hellenica* probably existed in the southern Balkans. The west coasts of the Balkans and Corfu have probably been colonized from Italy and not from the geographically closer southern Balkanic refuge. In Sicily, a third mtDNA lineage is distributed, which is sister to all other known lineages of *Emys*. Morphologically, Sicilian pond turtles resemble *E. o. galloitalica*. However, nuclear fingerprinting revealed a clear distinctiveness of the Sicilian taxon, whereas no significant divergence was detected between representatives of the other eight mtDNA lineages of *Emys*. Furthermore, nuclear fingerprinting provided no evidence for current or past gene flow between the Sicilian taxon and the mainland subspecies of *E. orbicularis*. Therefore, Sicilian pond turtles are described here as a species new to science. Some populations in Calabria and on the Salentine Peninsula comprise individuals of different mtDNA lineages. We interpret this as a natural contact. However, we cannot exclude that these syntopic occurrences are the result of human activity. For example, in other parts of Italy, the natural distribution pattern of *Emys* is obscured by allochthonous turtles. This could also be true for southern Italy. The discovery of the complex taxonomic differentiation in southern Italy requires reconsidering conservation strategies.

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Introduction

The endangered European pond turtle, *Emys orbicularis* (L., 1758), is the sole Palearctic representative of Emydidae, a taxon otherwise distributed exclusively in the New World. It is a small to medium-sized freshwater terrapin, with shell lengths of 10–23 cm, and leaves the water only for aerial basking, seasonal overland movements, hibernation, or aestivation. *E. orbicularis* inhabits a vast distributional area from western North Africa over most of southern, central, and eastern Europe to Asia Minor and the Caspian and Aral Seas in the east. Its range extends approximately 6000 km in a west-east direction from the Moroccan Atlantic coast to the Aral Sea and about 2000 km in a north-south direction from the region of Moscow to the Turkish-Syrian border near Antakya (Fritz 2003). For decades, the European pond turtle was thought to be a textbook example of a wide ranging, monotypic species (Boulenger 1889; Wermuth & Mertens 1961; Arnold & Burton 1978). However, recent years have seen a rapid increase in the recognition of its geographical differentiation. Morphological and genetic (mtDNA) investigations demonstrated that *E. orbicularis* is amongst the most taxonomically structured reptile species in the western Palearctic. Currently, 13 subspecies are distinguished (Fritz 1998, 2001, 2003). Their morphological differentiation is, as far as is currently investigated, largely paralleled by a deep genetic differentiation into distinct mtDNA lineages (Lenk *et al*. 1999; Fritz 2001, 2003).

Southern Italy is one of the least studied parts of the range in terms of the systematics and phylogeography of the species. Preliminary investigations of morphology (Fritz 1995) and mtDNA haplotypes (Lenk *et al*. 1999) demonstrated considerable variation and differentiation that far exceeded other parts of the range. It is highly probable that southern Italy represents a refuge area for several ancient pond turtle lineages (Fritz 1996, 2003; Lenk *et al*. 1999). However, all previous studies were based only on very limited sample sizes. Therefore, the exact differentiation pattern (i.e. the number and the distribution of the taxa occurring in southern Italy) remains unclear until today.

Here we present the first detailed investigation covering the entire southern Italian range of *E. orbicularis*. Our goals are (i) to reveal the diversity of mtDNA lineages in southern Italy, (ii) to describe the geographical distribution of the mtDNA lineages, (iii) to find out whether nuclear genomic and morphological data match the differentiation of the mitochondrial genome, and (iv) to investigate whether gene flow exists between populations harbouring different mtDNA lineages. Finally, the implications of our findings on taxonomy, zoogeography, and biodiversity conservation are discussed and a taxon from Sicily that is highly distinct genetically is described as a species new to science.

Materials and methods

Sampling

We obtained one tissue and 57 blood samples from southern Italian turtles and blood samples of 69 turtles from other Italian regions as specified in Table 1. Most turtles were captured between 1993 and 2003, either personally or by local nature conservation authorities and released after study; a few individuals are long-term captives in local breeding projects. Blood was obtained by coccygeal vein puncture. A turtle that was found dead in the wild was alcohol-preserved and thigh muscle was extracted. The specimen is now in the herpetological collection of the Museum of Zoology, Dresden (MTD D 45294, adult male, Rauccio, Apulia).

Samples were preserved either in an EDTA (ethylene diamine tetra acetate) buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) or in ethanol (Wink 1998) and stored at –20 °C until processing. The remaining samples are permanently kept at –70 °C in the blood and tissue collection of the Museum of Zoology, Dresden. The same is true for the remaining comparative samples of *Emys orbicularis* from other parts of the species' range and of the outgroups *Actinemys marmorata* and *Emydoidea blandingii* (see succeeding discussion). The outgroups, many samples from more northern parts of Italy and other portions of the range, and a few samples from Sicily and Calabria represent previously published mtDNA sequence data (Lenk *et al*. 1999; Fritz *et al*. 2004, in press).

In addition, basic morphological data and voucher photographs (dorsal and ventral aspects; also details of head and extremities in part) were taken of nine males and seven females from Sicily (L), eight males and 11 females from the Neto River (E), 15 males and 23 females from the Mt. Pollino area plus Basilicata (F), and 13 males and 23 females from the Salentine Peninsula (G) (letters refer to Table 1). Several subadults and juveniles from these localities were also measured and photographed. Genetic and morphological data sets of turtles from the Neto River and Mt. Pollino were derived from distinct samples that did not necessarily represent the same individuals. Morphological data of Sicilian pond turtles were partially obtained from specimens in the Museo Zoologico 'La Specola', Florence, and the Muséum National d'Histoire naturelle, Paris, as specified below in the Systematics section. Photographs and data are filed in the Museum of Zoology, Dresden.

Molecular techniques

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, 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

Table 1 Geographic origin of Italian pond turtle samples and their mtDNA haplotypes.

					Haplotype										
	Locality	Region	Native	*n*	IIa	IIIa	IIIc	IVa	IVd	IVh	IVi	IVj	Va	Vb	Vc
–	Milano and Lacchiarella	Lombardia	–	4	–	–	–	4*	–	–	–	–	–	–	–
–	Pavia	Lombardia	–	1	–	–	–	1*	–	–	–	–	–	–	–
A	Chioggia	Veneto	+	1	–	–	–	1	–	–	–	–	–	–	–
A	Mestre	Veneto	+	1	–	–	–	1	–	–	–	–	–	–	–
A	Caorle	Veneto	+	1	–	–	–	1	–	–	–	–	–	–	–
A	Bibione	Veneto	+	6	–	–	–	6	–	–	–	–	–	–	–
B	Camp Darby	Toscana	+	13	–	–	–	–	–	–	–	–	13	–	–
B	San Piero a Grado	Toscana	+/–	2	1*	–	–	–	–	–	–	–	1	–	–
B	Calambrone	Toscana	+	1	–	–	–	–	–	–	–	–	1	–	–
B	Magliano in Toscana	Toscana	+	1	–	–	–	–	–	–	–	–	1	–	–
C	Tenuta di Castelporziano	Lazio	+/–	20	–	–	–	15*	–	–	–	–	5	–	–
D	Succivo	Campania	+	1	–	–	–	–	–	–	–	–	1	–	–
E	Neto River	Calabria	+?	5	–	1*?	–	–	–	1	–	–	1	2	–
F	Lametia and Sierra da Caviole, Mt. Pollino	Calabria	+	5	–	–	–	–	–	–	–	–	5	–	–
F	Canale Duglia, Mt. Pollino	Basilicata	+	1	–	–	–	–	–	–	–	–	–	–	1
F	Metaponto	Basilicata	+	2	–	–	–	–	–	–	–	–	2	–	–
G	Li Foggi	Puglia	+	3	–	–	–	–	–	1	2	–	–	–	–
G	Ugento	Puglia	+	2	–	–	–	–	–	–	–	2	–	–	–
G	Cesine	Puglia	+	4	–	–	–	1	3	–	–	–	–	–	–
G	Torre Rinalda	Puglia	+?	2	–	–	–	1	–	–	–	–	–	1*?	–
G	Rauccio	Puglia	+	2	–	–	–	–	2	–	–	–	–	–	–
G	Giancola	Puglia	+	3	–	–	–	1	2	–	–	–	–	–	–
G	Torre Guaceto	Puglia	+	6	–	–	–	2	4	–	–	–	–	–	–
G	Pantanagianni	Puglia	+	6	–	–	–	6	–	–	–	–	–	–	–
G	Morelli — Torre Canne	Puglia	+?	1	–	–	–	–	–	–	–	–	1*?	–	–
H	Lago di Lesina, Mt. Gargano	Puglia	+	1	–	–	–	1	–	–	–	–	–	–	–
I	Chieti	Abruzzo	+	3	–	–	–	3	–	–	–	–	–	–	–
–	Montesilvano	Abruzzo	–	1	–	–	–	–	–	–	–	–	1*	–	–
–	Terrano	Abruzzo	–	1	–	–	–	1*	–	–	–	–	–	–	–
K	Olmedo	Sardegna	+	1	–	–	–	–	–	–	–	–	1	–	–
K	Alghero	Sardegna	+	4	–	–	–	–	–	–	–	–	4	–	–
K	Olbia	Sardegna	+	2	–	–	–	–	–	–	–	–	2	–	–
K	Budoni	Sardegna	+	6	–	–	–	–	–	–	–	–	6	–	–
L	Agrigento	Sicilia	+	2	–	–	2	–	–	–	–	–	–	–	–
L	Mistretta	Sicilia	+/–	11	–	10	–	–	–	–	–	–	1*	–	–
L	Nicosia	Sicilia	+	1	–	–	1	–	–	–	–	–	–	–	–
	Total			127	1	11	3	45	11	2	2	2	46	3	1

Letters preceding localities refer only to combined locality groups for native samples in Fig. 4 as follows: A = Veneto, B = Toscana, C = Lazio, D = Campania, E = Neto River, F = Mt. Pollino plus Basilicata, G = Salentine Peninsula, H = northern Puglia, I = Abruzzo, K = Sardegna, L = Sicilia. + = yes; – = no; *haplotypes considered to be allochthonous.

isopropanol, and centrifuged, washed, dried and resuspended in TE buffer.

mtDNA. Our target sequence of mitochondrial cytochrome *b* gene (cyt *b*) is phylogenetically highly informative in emydid turtles (Lenk *et al.* 1999; Feldman & Parham 2002; Stephens & Wiens 2003). It was amplified by PCR (polymerase chain reaction) as outlined in Lenk *et al.* (1999). Sequence data of 1031–1106 bp were obtained directly from the sequencer for all studied samples (Table 1). No deletions, insertions or inversions were detected. Furthermore, only a single product was encountered, even when using different PCR primers. Because no internal stop codons were found and because nucleotide frequencies corresponded to those known for mtDNA, we conclude that we have amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

PCR was performed in 50 µL volume containing 1 unit 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 of 5 min at 94 °C, 31 cycles were performed with annealing of 52 s at 60 °C, primer extension of 80 s at 72 °C, and denaturing of 45 s at 94 °C. PCR products were sequenced directly using the dideoxy chain termination method with the cycle sequencing kit (Amersham Life Science,

RPN 2438/RPN 2538) in combination with internal CY-5 labelled primers (1.5 pmol). For cycle sequencing, a two-stage program containing an initial denaturing step at 94 °C for 4 min and 25 cycles at 60 °C (40 s) and 94° (30 s) was used. The primers employed were as given in Lenk *et al.* (1999). Labelled fragments were analysed on an automated DNA sequencer (Amersham Pharmacia Biotech, ALF-Express II).

Alternatively, a cycle sequencing reaction (final volume 10 μL) was carried out after the initial PCR. Reaction buffer consisted of 2 μL reaction mix with BigDye terminators (according to the BigDye Terminator Protocol; ABI Applied Biosystems), 4 μL primers (10 pmol), and 4 μL PCR product. The cycle sequencing was carried out in 25 cycles at 96 °C for 10 s, 52 °C for 5 s and 60 °C for 4 min. Sequencing products were purified by precipitation in 1 volume reaction mix, 1/10 3 M Na acetate (pH 4.6), and 2.5 volumes ethanol. After centrifugation for 15 min at 13 000 rpm, DNA pellets were washed in 70% ethanol and taken up in 20 μL distilled water. The purified DNA was diluted 1 : 5 in water and applied to a 16-column automatic capillary sequencer (ABI 3100) using 50 cm capillaries and POP6 as a polymer.

We define haplotypes and haplotype clades according to individual mtDNA sequences (Lenk *et al.* 1999). For each sequence, variable sites were checked individually to guard against sequencer output errors. Nomenclature of mtDNA haplotypes and lineages follows Lenk *et al.* (1999) and Fritz *et al.* (2004, in press). Newly identified haplotypes belonging to one of the previously known major clades bear the Roman numbers of the lineages in Lenk *et al.* (1999) and Fritz *et al.* (2004, in press) and are specified by the addition of consecutive letters.

Data were analysed using maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML) with PAUP* version 4.0b10 (Swofford 2002). Genetic distances (uncorrected p-distances) were calculated from an aligned data set of 1031 bp. Unweighted MP analyses were performed and ML trees were reconstructed using 'tree-bisection-reconnection' (TBR) branch swapping and the heuristic search option. The most appropriate model of evolution with respect to estimates of nucleotide substitutions, invariant sites and gamma parameters was tested using MODELTEST version 3.5 (Posada & Crandall 1998) from an initial NJ tree. A molecular clock was not enforced. A GTR + G model was selected using the hierarchical likelihood ratio test implemented in MODELTEST, where rates were allowed to vary between sites according to a gamma distribution (discrete approximation; shape parameter alpha = 0.5; number of rate categories = 4; representation of average rate for each category = mean). For comparative purposes, we included all previously identified haplotypes from other parts of the range of *E. orbicularis* into the analyses (Lenk *et al.* 1999; Fritz *et al.* 2004, in press). For tree rooting, we used the two most closely related outgroup species (Lenk *et al.* 1999; Feldman & Parham 2002; Stephens & Wiens 2003), the North American western pond turtle (*Actinemys marmorata*) and Blanding's turtle (*Emydoidea blandingii*).

Finally, we calculated a minimum spanning network for *Emys* haplotypes using ARLEQUIN version 2.000 (Schneider *et al.* 2000). In the network, haplotypes are connected in the most parsimonious way such that the overall number of putative mutations leading from one haplotype to another is minimized. In contrast to dichotomous phylogenetic trees, networks allow for persistent ancestral nodes and reticulations. Thus, a network is able to demonstrate alternative evolutionary pathways at the same time, with the occurrence of reticulations visualizing ambiguous or uncertain domains. In haplotypic data, loops can also indicate the occurrence of reverse or parallel mutations. Moreover, the position of a haplotype in a network implies some information about its age because older haplotypes are thought to have a greater likelihood of being located internally in a network (Posada & Crandall 2001).

EMBL (European Molecular Biology Laboratory) accession numbers for sequences used in this paper are: AJ131407–131426, AJ131430, AJ131432, and AY652865–AY652890.

Nuclear fingerprinting. To obtain a measure for the variation within the nuclear genome and to determine whether gene flow exists between populations harbouring different mtDNA lineages, we conducted nuclear fingerprinting with inter-simple sequence repeats (ISSR) for 38 Italian specimens. To compare the variation with other parts of the range, we also included one or two samples representing each *E. orbicularis* mtDNA lineage not occurring in Italy (Table 2). ISSR PCR is a simple and cheap method of mapping the nuclear genome and discovering rearrangements. It generates nuclear fingerprints that are usually diagnostic for species-level taxa (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994; Wink *et al.* 1998, 2001; Wolfe & Liston 1998; Wolfe *et al.* 1998; Nagy *et al.* 2003). ISSR employs only a single PCR primer that binds to di- or trinucleotide repeat motifs (microsatellites), which are abundant in eukaryotic genomes (Tautz & Renz 1984; Condit & Hubbell 1991). Because sequences of microsatellites are conserved over a wide range of organisms, ISSR PCR can use universal primers. A 5′ or 3′ anchoring sequence of one to three nucleotides (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994) avoids strand-slippage artefacts and results in unique, reproducible banding profiles. 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). It is thought that SSR regions are scattered evenly throughout the genome (Tautz & Renz 1984; Condit & Hubbell 1991), resulting in a large number of polymorphic bands. In this study, the anchored ISSR primer L18 (CTCGGGAAGGGA) was used.

For ISSR amplification, 15 ng of total DNA and 3 pmol of primer were selected as the best reaction conditions and were

Table 2 Geographic origin of pond turtle samples used for ISSR PCR.

Number	mtDNA haplotype	Locality
1	IIIa	Italy: Sicilia: Mistretta
2	IIIa	Italy: Sicilia: Mistretta
3	IIIa	Italy: Sicilia: Mistretta
4	IIIa	Italy: Sicilia: Mistretta
5	IIIa	Italy: Sicilia: Mistretta
6	IIIa	Italy: Sicilia: Mistretta
7	IVa	Italy: Abruzzo: Chieti
8	IVa	Italy: Abruzzo: Chieti
9	IVa	Italy: Abruzzo: Chieti
10	IVa	Italy: Puglia: Giancola
11	IVa	Italy: Puglia: Pantanagianni
12	IVa	Italy: Puglia: Pantanagianni
13	IVa	Italy: Puglia: Pantanagianni
14	IVa	Italy: Puglia: Pantanagianni
15	IVa	Italy: Puglia: Cesine
16	IVa	Italy: Puglia: Torre Rinalda
17	IVa	Italy: Puglia: Torre Guaceto
18	IVa	Italy: Puglia: Lago di Lesina, Mt. Gargano
19	IVd	Italy: Puglia: Rauccio
20	IVd	Italy: Puglia: Torre Guaceto
21	IVd	Italy: Puglia: Giancola
22	IVd	Italy: Puglia: Giancola
23	IVd	Italy: Puglia: Torre Guaceto
24	IVd	Italy: Puglia: Cesine
25	IVd	Italy: Puglia: Cesine
26	IVd	Italy: Puglia: Cesine
27	IVh	Italy: Calabria: Neto River
28	IVj	Italy: Puglia: Ugento
29	IVj	Italy: Puglia: Ugento
30	Va	Italy: Calabria: Mt. Pollino
31	Va	Italy: Calabria: Mt. Pollino
32	Va	Italy: Campania: Succivo
33*	Va	Italy: Abruzzo: Montesilvano
34	Va	Italy: Puglia: Morelli — Torre Canne
35	Va	Italy: Basilicata: Metaponto
36	Va	Italy: Calabria: Neto River
37	Vb	Italy: Puglia: Torre Rinalda
38	Vb	Italy: Calabria: Neto River
39	Ia	Poland: Chelm
40	Ia	Poland: Chelm
41	IIa	Hungary: Velencei-tó
42	IIa	Spain: Gerona Province
43	VIa	Spain: Ebro Delta
44	VIa	Spain: Ebro Delta
45	VIIa	Azerbaijan: Baku
46	VIIa	Azerbaijan: Baku
47	VIIIa	Turkey: Anamurum
48	VIIIa	Turkey: Anamurum
49	IXa	Unknown

The single allochthonous specimen is marked with an asterisk.

used for all further amplifications in a total volume of 12.5 µL comprising 1.5 mM $MgCl_2$, 0.1 mM of dGTP, dCTP, and dTTP, 0.075 mM dATP, 1 µCi [α-33P]-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 units *Taq* polymerase (Amersham Pharmacia Biotech). After an initial denaturation period (120 s at 94 °C), 33 cycles of 60 s at 94 °C, of 120 s at 55 °C, and of 120 s at 72 °C were performed on a thermocycler (Biometra, Goettingen, Germany); the reaction was then held at 72 °C for 4 min and stored thereafter 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 analysed by eye. Thirty-two unambiguously identifiable bands were transferred into a binary presence/absence matrix (Appendix) scoring each particular fragment. A UPGMA tree was obtained from the binary matrix using PAUP* and its robustness was tested by bootstrapping (1000 replicates) under the 50% consensus criterion. Clustering of samples representing different mtDNA haplotypes indicates a high percentage of shared bands in the UPGMA tree, suggesting past or current gene flow. If the tree splits in clearly differentiated branches that parallel mtDNA haplotypes, no or limited gene flow is thought to have occurred.

Morphology

The following straight-line maximum measurements as described in Fritz (1995) were taken with a caliper to the nearest 0.5 mm: carapacial length, carapacial width, shell height, and plastral length. For statistical purposes, only fully adult specimens were considered.

Statistics were performed with the computer software SPSS version 7.5 and included the standard procedures of a two-tailed *t*-test and discriminant analyses combined with a Levene test for determining significant differences of variance. Individuals suspected to be allochthonous were excluded from descriptive statistics or treated as ungrouped cases in discriminant analyses. Because a clear sexual dimorphism in shell ratios of *Emys* is known to exist (Fritz 2001, 2003), data of both sexes were processed separately and compared between and within the geographical groupings of Sicily, Neto River, Mt. Pollino plus Basilicata, and the Salentine Peninsula.

Based on the voucher photographs, the colour and pattern of investigated southern Italian turtles were compared individually according to the criteria of Fritz (1995) for western Mediterranean *E. orbicularis* subspecies.

Results

Sequence variation

When the two North American species, *Actinemys marmorata* and *Emydoidea blandingii* were included in the analyses, 73 of 1031 aligned sites were variable but parsimony-uninformative; 70 sites were parsimony-informative. When only *Emys* sequences were analysed, 71 of 1031 characters were variable, of which 41 were parsimony-informative.

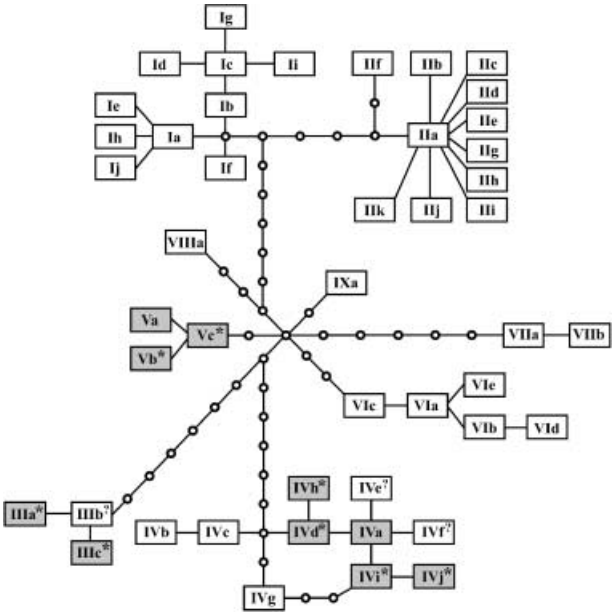

Fig. 1 Minimum spanning network of all 46 mtDNA haplotypes of *Emys* currently known. Branch lengths correspond to the inferred number of nucleotide changes along each branch. Boxes and open circles symbolize identified and hypothesized haplotypes, respectively. Each line between boxes or circles indicates one substitution. Boxes of haplotypes occurring in southern Italy are grey; haplotypes endemic to southern Italy are asterisked. Haplotypes bearing question marks were identified from individuals of unknown geographical origin (e.g. haplotype IIIb, which was found in an allochthonous turtle from Germany).

We detected a striking diversity of 10 distinct haplotypes in southern Italy, representing three of the nine hitherto identified major basal branches of mtDNA haplotypes in *E. orbicularis* (Lenk *et al*. 1999; Fritz *et al*. 2004, in press). The southern Italian haplotypes correspond to lineages III, IV, and V in the terminology of Lenk *et al*. (1999). Of the 10 southern Italian haplotypes, eight are restricted to this area (IIIa, IIIc, IVd, IVh, IVi, IVj, Vb, Vc) and seven were identified for the first time in this study. Haplotype IIIb, which was identified earlier (Lenk *et al*. 1999) from an allochthonous pond turtle caught in Germany, is thought to originate in southern Italy as well (Fritz *et al*. 2004). The individual haplotypes of each lineage differ in only a few mutation steps from one another. However, the lineages are highly distinct (Fig. 1) and separated by mean sequence divergences ranging from 0.48% to 1.84% (Table 3).

Phylogeny of mtDNA haplotypes

Maximum parsimony (MP), neighbour joining (NJ), and maximum likelihood (ML) analyses for all known *Emys* mtDNA haplotypes consistently produced the same general tree topology, reflecting nine major phylogeographical lineages (Fig. 2). For all tree-building methods, lineage III was found to be sister to all other lineages. For the remaining lineages, lineages I and II formed a well-supported clade that is the sister group of the other lineages. This general branching pattern III + ((I + II) + (other lineages)) was supported by high bootstrap values under an MP analysis; however, the sister group relation of I + II and the lineages IV, V, VI, VII, VIII, and IX was only moderately supported in an NJ analysis. The monophyly and branching pattern of the lineages IV, V, VI, VII, VIII, and IX was supported only weakly by the bootstrap in either analyses, resulting in polytomies for the lineages (IV + VII), V, VI, and IX in a strict consensus of all the equally most parsimonious trees (Fig. 3).

When only *Emys* haplotypes were analysed using MP, a single unrooted most parsimonious tree of 82 steps was obtained (not shown), which again displayed the nine lineages. The minimum spanning network is similar to the unrooted MP tree and differs significantly only with regard to lineage IV where the only loop occurs (Fig. 1). Moreover, in the unrooted MP tree, haplotype IIIa is ancestral to IIIb, and IIIb to IIIc. In both analyses, the lineages found in Italy (III, IV, V) are clearly distinct, both from one another and from all other mtDNA lineages occurring in other parts of the species' range. Lineage IV, which also occurs along the Adriatic and

Table 3 Genetic distances (uncorrected p-distances) between *Emys* mtDNA lineages based on a dataset of 1031 bp of cyt *b*. Mean distances between haplotypes are given below, and ranges (in italics) above the diagonal. Within-lineage mean distances are given in bold on the grey diagonal. All estimates are expressed as percentages.

	I	II	III	IV	V	VI	VII	VIII	IX
I	**0.24**	*0.53–0.95*	*1.23–1.68*	*1.46–2.00*	*1.07–1.49*	*1.16–1.79*	*1.26–1.69*	*1.07–1.38*	*1.12–1.37*
II	0.76	**0.22**	*1.46–1.84*	*1.61–2.05*	*1.26–1.55*	*1.36–1.84*	*1.46–1.75*	*1.26–1.44*	*1.13–1.32*
III	1.49	1.66	**0.14**	*1.01–1.71*	*0.90–1.17*	*1.01–1.46*	*1.35–1.55*	*1.07–1.17*	*0.79–1.12*
IV	1.67	1.84	1.40	**0.28**	*0.97–1.13*	*1.07–1.61*	*1.16–1.51*	*1.17–1.41*	*1.02–1.23*
V	1.25	1.44	1.05	1.13	**0.13**	*0.49–0.88*	*0.78–0.98*	*0.58–0.68*	*0.41–0.52*
VI	1.44	1.62	1.24	1.32	0.71	**0.18**	*0.87–1.26*	*0.68–0.97*	*0.51–0.82*
VII	1.43	1.62	1.44	1.31	0.89	1.08	**0.10**	*0.97–1.07*	*0.83–0.93*
VIII	1.18	1.37	1.12	1.26	0.65	0.84	1.02	—	—
IX	1.23	1.23	0.98	1.11	0.48	0.68	0.88	0.62	—

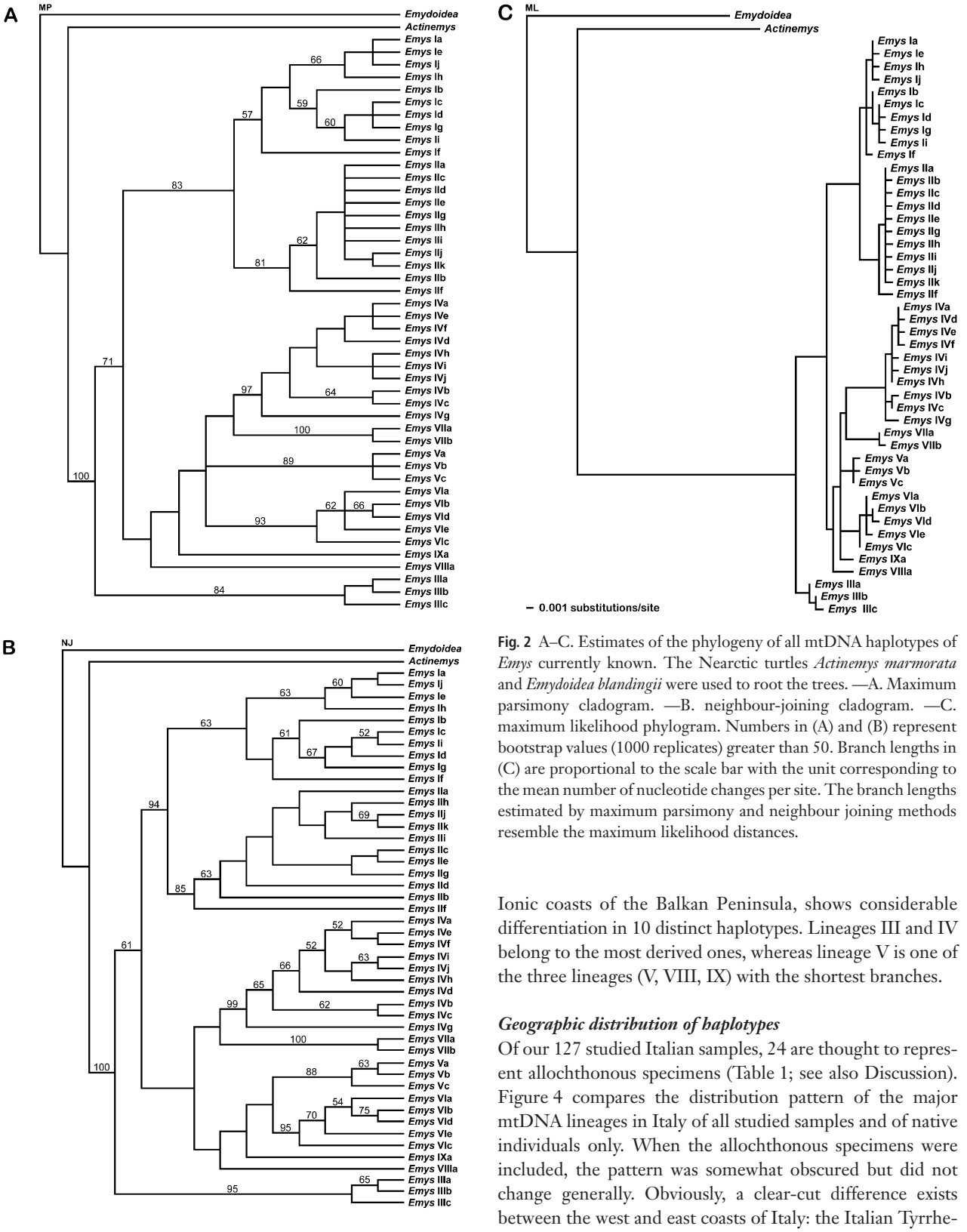

Fig. 2 A–C. Estimates of the phylogeny of all mtDNA haplotypes of *Emys* currently known. The Nearctic turtles *Actinemys marmorata* and *Emydoidea blandingii* were used to root the trees. —A. Maximum parsimony cladogram. —B. neighbour-joining cladogram. —C. maximum likelihood phylogram. Numbers in (A) and (B) represent bootstrap values (1000 replicates) greater than 50. Branch lengths in (C) are proportional to the scale bar with the unit corresponding to the mean number of nucleotide changes per site. The branch lengths estimated by maximum parsimony and neighbour joining methods resemble the maximum likelihood distances.

Ionic coasts of the Balkan Peninsula, shows considerable differentiation in 10 distinct haplotypes. Lineages III and IV belong to the most derived ones, whereas lineage V is one of the three lineages (V, VIII, IX) with the shortest branches.

Geographic distribution of haplotypes

Of our 127 studied Italian samples, 24 are thought to represent allochthonous specimens (Table 1; see also Discussion). Figure 4 compares the distribution pattern of the major mtDNA lineages in Italy of all studied samples and of native individuals only. When the allochthonous specimens were included, the pattern was somewhat obscured but did not change generally. Obviously, a clear-cut difference exists between the west and east coasts of Italy: the Italian Tyrrhenic coast and Sardinia are inhabited by lineage V turtles,

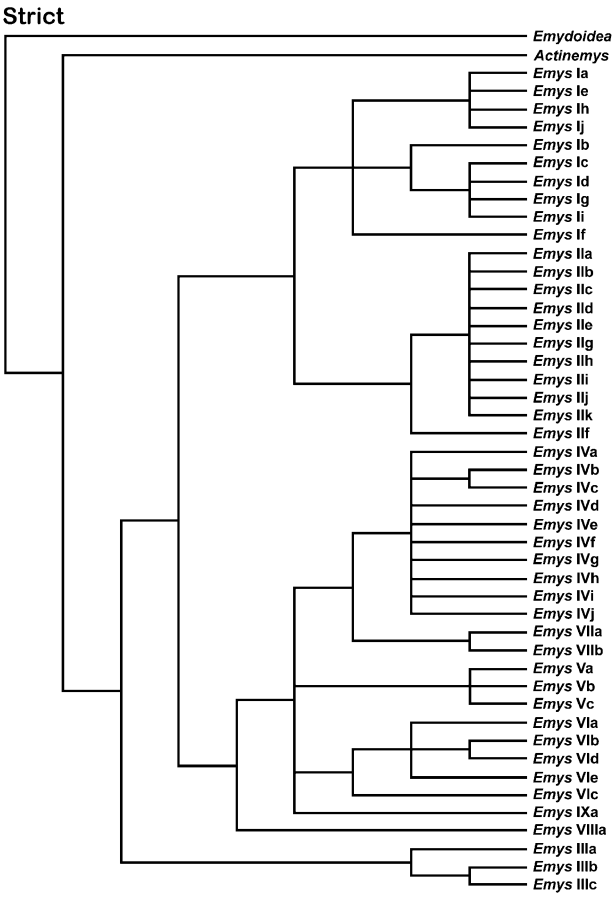

Fig. 3 Strict consensus tree of 14 maximum parsimony trees for all mtDNA haplotypes of *Emys* currently known.

whereas lineage IV turtles occur along the Adriatic coast. For the first time, lineage IV turtles were also recorded from the southern Italian Salentine Peninsula (Apulia; the 'heel' of Italy), thereby extending the range of lineage IV turtles in Italy considerably to the south. Before, the southernmost record was Chieti (Abruzzo Region; Lenk *et al*. 1999), approximately 300 km to the north. We confirmed the finding of Lenk *et al*. (1999) that an endemic lineage (III) occurs on Sicily and in Calabria. However, our data demonstrate for the first time that continental southern Italy is a contact zone. In Calabria, all three lineages (i.e. III, IV, and V) were detected, and lineage V just enters the Salentine Peninsula. On a finer scale, it is remarkable that the highest diversity of individual haplotypes occurs on the Salentine Peninsula and in Calabria. Here, the endemic haplotypes IVd, IVh, IVi, IVj, Vb, and Vc were found (Fig. 5), whereas all more northerly localities harboured only haplotypes IVa and Va.

Nuclear fingerprinting

The UPGMA tree clearly separated the six Sicilian pond turtles studied bearing an mtDNA haplotype of lineage III from all other specimens. This difference between lineage III and all other studied samples is robust as assessed by bootstrapping (Fig. 6). By contrast, the fragment patterns of all other samples were very similar to one another, resulting in a largely unresolved region after bootstrapping. The only two Salentine samples with lineage V haplotypes (Va, Vb; nos. 34 and 37 in Table 2, Fig. 6) are also found in this multifurcation.

Within the polytomy, only two samples of haplotype Ia turtles and two samples of haplotype VIIIa turtles cluster together with high bootstrap support. Moderate support was indicated for a group of two haplotype Va turtles from the Mt. Pollino area and a haplotype IVh individual from the Calabrian Neto River. This is the sole mainland locality where we caught turtles with haplotypes of lineages III, IV, and V syntopically. However, the three Neto samples studied by ISSR PCR (haplotypes IVh, Va, Vb; nos. 27, 36, 38 in Table 2, Fig. 6) do not cluster with the Sicilian pond turtles and do not possess the three private bands that occurred only in Sicilian lineage III samples (bands *s*, *t*, *ab* in Appendix). By

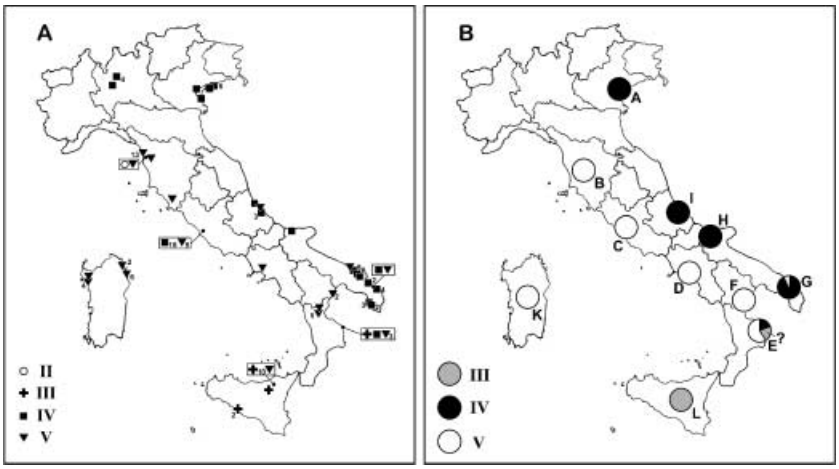

Fig. 4 A, B. Distribution of phylogeographical lineages II, III, IV, and V of European pond turtles in Italy. —A. All samples studied. Numbers by symbols indicate frequencies of individuals; symbols in boxes mark polymorphic populations. —B. Relative frequencies of lineages when only native individuals are considered (the occurrence of lineage III in Calabria is enigmatic and discussed in the text). See Table 1 for an explanation of the letters in (B), detailed localities, and numbers of studied samples.

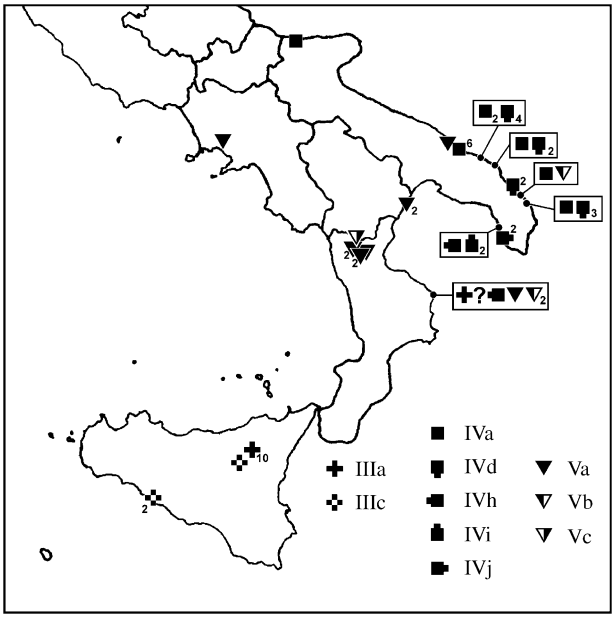

Fig. 5 Distribution of *Emys* mtDNA haplotypes in southern Italy (native specimens plus the enigmatic record of haplotype IIIa in the Neto River). Near Mistretta, Sicily, an allochthonous individual bearing haplotype Va was caught in addition to 10 turtles with the expected haplotype IIIa. Only haplotypes IVa (Adriatic coast) or Va (Tyrrhenic coast) occur in more northerly Italian localities.

contrast, the six Sicilian samples studied do not possess some common bands (*v*, *y*, *ac* in Appendix) present in lineages IV and V, despite the fact that an allochthonous haplotype Va turtle was caught in the same population (Table 1). Unfortunately, the Neto IIIa sample could not be studied by ISSR fingerprinting because it was used up for mtDNA sequencing during a previous study (Lenk *et al*. 1999).

Morphology of southern Italian pond turtles

Colouration and pattern

Males in all studied groups have an immaculate or nearly immaculate contrasting white iris. The iris of females is yellowish, often with a dark horizontal bar running through the pupil and sometimes with additional black elements. This sexual dichromatism is characteristic for many Mediterranean subspecies of *Emys orbicularis*, whereas males have an intensely reddish iris colour in other parts of the range (Fritz 2001, 2003). Locality groups as defined in Table 1 and Fig. 4(B) follow successively in a west-east direction.

Sicily (locality group L)

All pond turtles have a dark to black carapacial primary colour with a radiating pattern of yellow dots or lines; some aged individuals have an indistinctly mottled yellow pattern. The plastra of six males (67% of the nine Sicilian males studied) and five females (71% of the seven Sicilian females studied) are immaculate yellow. Three males (33%) and two females (29%) have well-defined, small black blotches at the distal seams of the central plastral scutes ('distal blotches'). The shell colouration of Sicilian pond turtles resembles *E. o. galloitalica* in that a similar blotched plastral pattern may occur in both (Fig. 7). However, when such a dark plastral pattern is present in Sicilian turtles, the dark elements are, with one exception (see succeeding discussion), smaller than in many *E. o. galloitalica*. Moreover, no Sicilian turtles with a light brownish primary colour of the carapace, as is characteristic for many *E. o. galloitalica*, were recorded. The extremities of most Sicilian males and females are irregularly speckled with yellow dots; these dots are arranged on the forelegs of three males and of one female into two confluent yellow lines. The crown of the head of most turtles from Sicily is dark, with indistinct yellow spots or vermiculations in the occipital region. A young male in the collection of the Museo Zoologico 'La Specola' (MZUF 19877, Sciacca) has a vermiform pattern across the entire top of the head, whereas a young live female from Mistretta possesses round yellow dots. This suggests that the crown of the head darkens anteriorly with increasing age. The throat is either immaculate yellow or speckled with black. With the exception of the iris colouration, a clear sexual dimorphism is not evident, although males tend to more irregularly spotted soft parts and a somewhat darker overall colouration than do females. One female from Mistretta, the only individual with haplotype Va, differs from other Sicilian turtles in that the forelegs are uniform ivory black with a single, contrasting yellow line (as is frequently found in *E. o. galloitalica*). Its plastral pattern also consists of larger black blotches than in the other individuals.

Neto River (locality group E)

Carapacial colouration and pattern could not be investigated because the shells of all turtles were heavily encrusted with algae, leading to a loss of the pattern as a result of a scarred shell surface. Six males (75% of $n = 8$) and seven females (64% of $n = 11$) have an immaculate yellow plastron; the remaining two males (25%) and four females (36%) bear well-defined, occasionally large dark distal blotches. The colouration of the soft parts in males is more irregular than in females. Females frequently have a prominent yellow line on the surface of the otherwise black foreleg; males have isolated yellow spots only. The throat colouration of some turtles is immaculate bright yellow; in others, the throat is intensely speckled with dark colour. A clear sexual dimorphism in the head colouration is not evident. However, one male (shown in colour in Fritz *et al*. 1993 and Fritz 1998) has a reticulate brownish head pattern, like the males of *E. o. bellenica*. With the exception of this male, all other turtles from the Neto River resemble *E. o. galloitalica* in the colour pattern characters described in Fritz (1995, 2001, 2003).

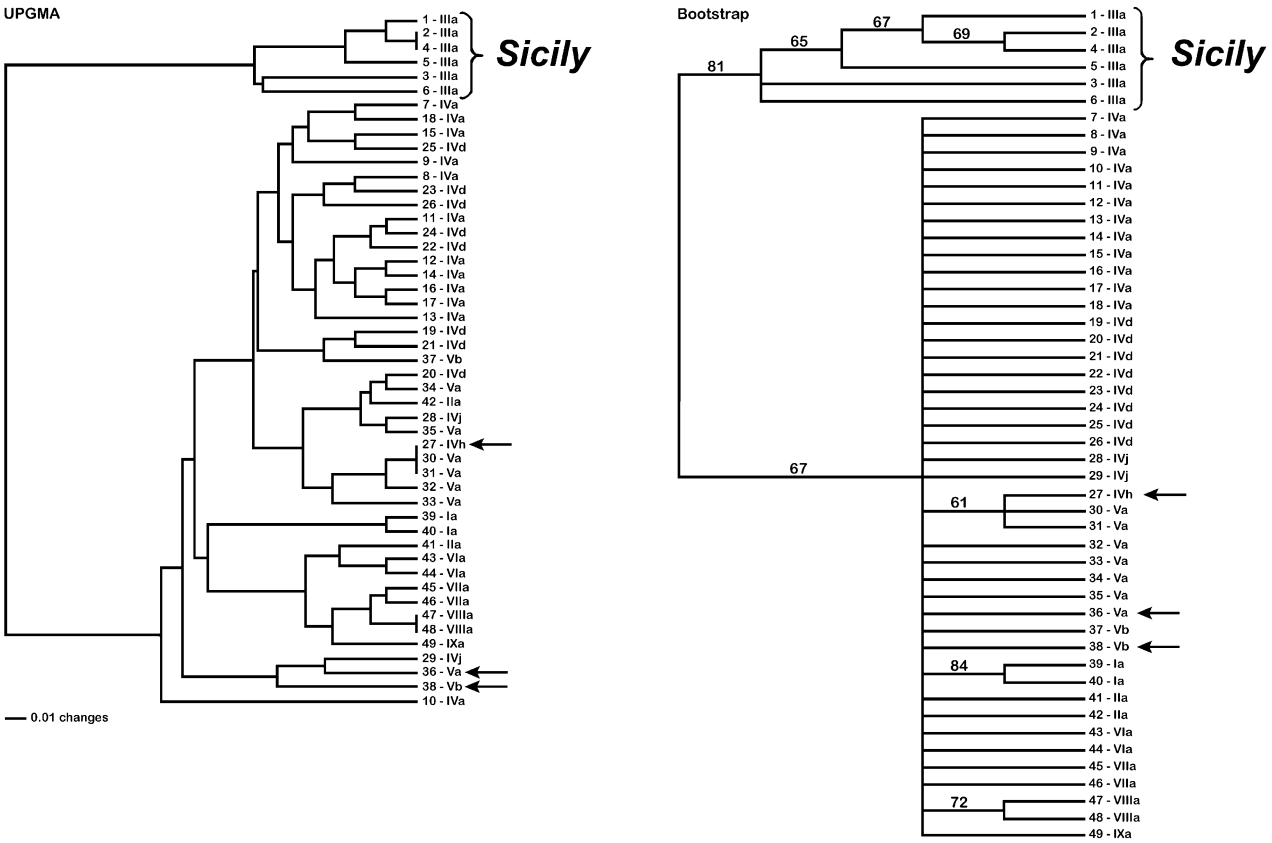

Fig. 6 UPGMA trees of ISSR fingerprints of 38 Italian *Emys* samples based on 32 scored fragments. For comparative purposes, samples of all currently known mtDNA lineages of *Emys* are included. Individual numbers refer to Table 2 and are followed by the mtDNA haplotypes of the respective samples. Arrows indicate samples from the Calabrian Neto River, the only known syntopic occurrence of haplotypes of lineages III, IV and V. Right: 50% bootstrap consensus tree; the gap between the Sicilian and other samples is consistent.

Mt. Pollino and Basilicata (locality group F)

The carapace colouration is polymorphic as is characteristic of *E. o. galloitalica* (Fritz 1995, 2001, 2003), with a high percentage of turtles (60% of n = 15 males and 39% of n = 23 females) having a medium to light brownish or yellow-brownish primary colour. The primary colour of the carapace of the other individuals is dark to black, with a somewhat less contrasting yellow radiating pattern than in Sicilian or Salentine pond turtles. Like in the two preceding groups, the plastra are either immaculate yellow (six males = 40%; nine females = 39%) or with a prominent pattern of contrasting black blotches at the distal seams of the scutes (nine males = 60%; 14 females = 61%; Fig. 7). These blotches are often of considerable size, making up to 50% of the plastral surface in four turtles. Individuals with a light carapace colouration tend to have smaller or no plastral blotches, whereas turtles with a dark carapace have, on average, a more intensely patterned plastron. The colouration of the soft parts is similar to that in the Neto group turtles, although males with a reticulate brownish head pattern were not recorded. In conclusion, turtles from Mt. Pollino and Basilicata match the colouration characteristics of *E. o. galloitalica* as defined in Fritz (1995, 2001, 2003).

Salentine Peninsula (locality group G)

European pond turtles from the Salentine Peninsula are, with two exceptions, indistinguishable from *E. o. hellenica*. Two females from the localities Morelli — Torre Canne and Torre Rinalda have a plastral pattern consisting of well-defined dark blotches positioned at the distal seams of the plastral scutes, as is often found in *E. o. galloitalica*. Their forelegs also bear only a single conspicuous yellow line characteristic of *E. o. galloitalica* as well. Moreover, these two specimens are the sole Salentine turtles bearing mtDNA haplotypes of lineage V (Table 1: Va, Vb) instead of lineage IV. This agrees with colouration and pattern because lineage V corresponds to *E. o. galloitalica* and lineage IV to *E. o. hellenica*. A description for the other Salentine turtles is as follows: the carapace has a dark to black primary colour with a conspicuous radiating pattern of yellow dots or lines. Turtles with a light primary

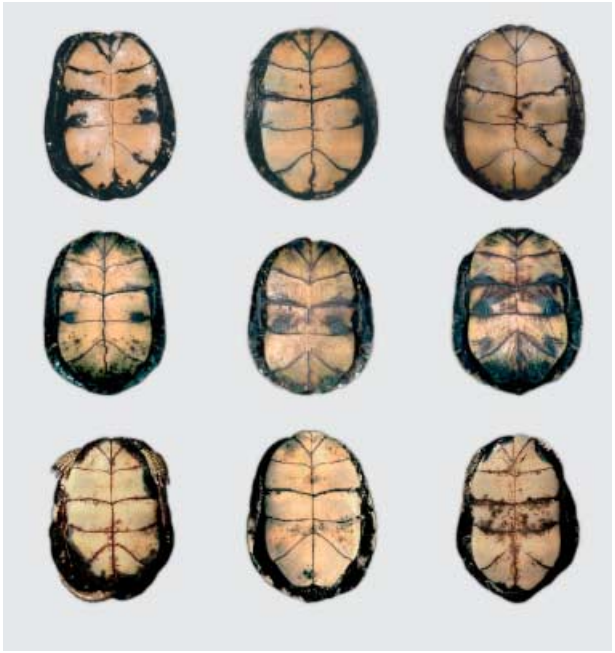

Fig. 7 Plastral pattern of southern Italian pond turtles. Rows from top to bottom are turtles from Sicily (Mistretta), Mt. Pollino (Lametia), and the Salentine Peninsula (Pantanagianni; on the right is an aged specimen with sooty dark pattern). The left turtle in the upper- and lowermost rows are males, all others are females. Note the blotched pattern in the Sicilian and Mt. Pollino turtles. The dark areas along the plastral edges of the Salentine male are a layer of green algae. Individuals from Mt. Pollino can alternatively have entirely yellow plastra.

colour of the carapace are extremely rare in *E. o. hellenica* (Fritz 1995, 2001, 2003), and such colouration was not found in any Salentine individual. With the exception of aged adults displaying an indistinct sooty dark plastral mottling (mainly males), the plastra of most individuals are entirely yellow (Fig. 7). There is a sharp sexual dimorphism in that males have a reticulate brownish head and neck colouration whereas females have dark to black heads with round yellow dots. The throat of all turtles is bright yellow. The extremities and the tail of males are intensely patterned with irregular yellow and brown speckles; sometimes the extremities and the tail are almost uniformly yellow. The forelegs of females bear two well-defined, conspicuous yellow lines. An indistinct V-shaped yellow figure occurs on the tail of females. As in many other subspecies, the carapacial pattern of males consists of yellow spots whereas females have a radiating pattern consisting of more elongated yellow elements like lines or streaks.

Morphometry

As for most Mediterranean populations, the southern Italian pond turtles are small-sized (Table 4), far removed from the

Table 4 Basic morphological data for pond turtles from Sicily, Neto River, Mt. Pollino plus Basilicata, and the Salentine Peninsula.

	Sicily		Neto River		Mt. Pollino plus Basilicata		Salentine Peninsula	
	Males $n = 9$	Females $n = 6$	Males $n = 8$	Females $n = 11$	Males $n = 15$	Females $n = 23$	Males $n = 13$	Females $n = 21$
CL	125.0 ± 5.5	133.0 ± 3.0	118.0 ± 2.5	123.0 ± 1.0	119.0 ± 2.0	131.0 ± 2.0	115.5 ± 2.0	121.0 ± 3.5
	(96.0–141.0, *SD* 16.7)	(127.0–145.0, *SD* 7.8)	(108.0–128.0, *SD* 7.3)*	(120.0–127.5, *SD* 2.5)	(109.0–135.5, *SD* 8.0)***	(115.0–149.0, *SD* 8.6)	(105.0–128.0, *SD* 7.2)	(97.0–149.0, *SD* 15.4)
CW	100.5 ± 4.0	104.5 ± 1.5	89.0 ± 2.0	93.0 ± 1.0	93.0 ± 2.5	100.0 ± 1.5	94.0 ± 3.5	104.0 ± 4.5
	(75.0–116.0, *SD* 12.7)	(98.0–109.0, *SD* 3.7)	(84.0–98.5, *SD* 5.6)	(86.5–97.5, *SD* 3.8)	(85.0–119.5, *SD* 8.8)***	(85.0–111.0, *SD* 6.6)	(72.0–110.0, *SD* 12.6)	(79.0–142.0, *SD* 21.0)
SH	47.0 ± 1.5	54.5 ± 2.1	42.5 ± 1.5	52.0 ± 0.5	43.0 ± 1.0	54.0 ± 1.0	40.0 ± 1.0	49.0 ± 2.0
	(41.0–52.0, *SD* 3.7)*	(47.0–62.0, *SD* 5.2)	(38.0–52.5, *SD* 4.2)***	(49.0–55.5, *SD* 2.1)	(39.5–53.0, *SD* 4.2)***	(49.0–61.0, *SD* 3.3)	(37.0–45.0, *SD* 3.4)*	(35.0–67.0, *SD* 8.1)
PL	111.0 ± 3.5	122.0 ± 3.5	103.5 ± 3.0	115.5 ± 1.0	105.0 ± 2.0	123.5 ± 1.5	90.0 ± 2.6	100.0 ± 3.0
	(91.0–123.0, *SD* 10.4)**	(113.0–133.0, *SD* 8.1)	(92.0–116.0, *SD* 8.1)***	(112.0–120.5, *SD* 2.9)	(97.0–119.5, *SD* 8.0)***	(112.0–140.0, *SD* 7.3)	(85.0–108.0, *SD* 9.4)***	(77.0–139.0, *SD* 14.5)

Values are means ± standard errors (to the nearest 0.5 mm); ranges and standard deviations (*SD*) are given in brackets. All values are in mm. CL = carapacial length, CW = carapacial width, SH = shell height, PL = plastral length.
Asterisks indicate significant differences between the sexes of one locality group (two-tailed *t*-test): * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 5 Significant differences between basic morphological data of southern Italian pond turtles (two-tailed *t*-test).

	Sicily	Neto River	Mt. Pollino plus Basilicata	Salentine Peninsula
Sicily	**SH*, PL****	*CW*, SH**	*SH**	*SH***, PL****
Neto River	CL*, CW***	**CL*, SH***, PL*****	—	*PL****
Mt. Pollino plus Basilicata	—	CL***, CW**, PL***	**CL***, CW***, SH***, PL*****	*PL****
Salentine Peninsula	PL**	CW*, PL***	CL*, SH*, PL***	**SH*, PL*****

Data for males (in italics) are above, and those for females (not italicized) are below the diagonal. On the grey diagonal, measurements with significant differences between the sexes of the respective locality group are given in bold. Samples are the same as those in Table 4. For abbreviations and significance levels see Table 4.

Table 6 Parameters of the discriminant analyses involving the complete character set.

	Function	Eigenvalue	% Variance	Cumulative Percentage	Canonical Correlation
Males	1	1.645	86.5	86.5	0.789
	2	0.179	9.4	95.9	0.390
	3	0.077	4.1	100.0	0.268
Females	1	1.478	90.6	90.6	0.772
	2	0.149	9.1	99.7	0.360
	3	0.005	0.3	100.0	0.068
	Test of Function	Wilk's Lambda	Chi^2	d.f.	*P*
Males	1–3	0.298	48.472	12	< 0.001
	2–3	0.787	9.567	6	0.144
	3	0.928	2.982	2	0.225
Females	1–3	0.350	58.847	12	< 0.001
	2–3	0.866	8.026	6	0.236
	3	0.995	0.258	2	0.879

maximum shell lengths reported from the Rhône Valley in southern France or from the north of the range (185–230 mm; Fritz 2003). Within the four southern Italian locality groups exist statistically significant sexually dimorphic differences (two-tailed *t*-test). In Sicilian and Salentine turtles, significant differences were found with respect to shell height and plastral length in that females have a relatively higher shell and a longer plastron than males of the same size. There are no significant differences between the sexes in carapacial length and width, despite females being slightly larger. Moravec (2003) found the same pattern of sexual dimorphism in a population of *E. o. hellenica* from Pag Island (Croatia). Likewise, Neto River males and females differ significantly in shell height and plastral length. However, the carapace of Neto River females is also significantly longer than in males. Pond turtles from Mt. Pollino and Basilicata differ significantly in all characters studied: males have smaller, flatter and narrower carapaces and shorter plastra than females (Table 4).

If males or females were compared between the four groupings, only males from Mt. Pollino plus Basilicata and Neto River and females from Sicily and Mt. Pollino plus Basilicata did not differ significantly in any character (Table 5).

For both sexes, parameters for the discriminant analyses (DAs) involving the complete character set are given in Table 6 and standardized discriminant coefficients are presented in Table 7. DA without cross-validation based on all four characters recorded (carapacial length and width, shell height, and plastral length) classified most of the males from Mt. Pollino plus Basilicata (86.7%) and from the Salentine Peninsula (76.9%) correctly, reflecting their general distinctiveness. However, only approximately half of the Sicilian males and 25% of the Neto River males were properly grouped. Similar results were obtained in cross-validated and stepwise DA, although stepwise DA classified none of the Neto River turtles correctly (Table 8). Stepwise DA with cross-validation yielded somewhat poorer results for the other groups (not shown). In stepwise DA, the combination of plastral and carapacial lengths were selected as being optimal for discrimination. In summary, DAs were unable to group many male pond turtles from Sicily and from the Neto

Table 7 Standardized canonical discriminant coefficients.

	Males Function			**Females Function**		
	1	2	3	1	2	3
CL	–1.168	–0.256	1.772	–1.142	–0.063	–2.673
CW	0.152	0.785	–1.794	0.398	1.579	1.845
SH	–0.010	0.767	1.042	–0.135	–1.404	–0.720
PL	1.613	–0.431	–0.947	1.726	0.821	1.639

CL = carapacial length, CW = carapacial width, SH = shell height, PL = plastral length.

Table 8 Classification of southern Italian pond turtles by discriminant analyses.

Group		Predicted grouping			
Males	*n*	L = Sicily	E = Neto River	F = Mt. Pollino plus Basilicata	G = Salentine Peninsula
L	9	5 (55.6%)	0 (0.0%)	4 (44.4%)	0 (0.0%)
E	8	1 (12.5%)	2 (25.0%)	4 (50.0%)	1 (12.5%)
F	15	2 (13.3%)	0 (0.0%)	13 (86.7%)	0 (0.0%)
G	13	0 (0.0%)	1 (7.7%)	2 (15.4%)	10 (76.9%)
Cross-validated					
L	9	5 (55.6%)	0 (0.0%)	4 (44.4%)	0 (0.0%)
E	8	1 (12.5%)	1 (12.5%)	5 (62.5%)	1 (12.5%)
F	15	4 (26.7%)	0 (0.0%)	11 (73.3%)	0 (0.0%)
G	13	0 (0.0%)	1 (7.7%)	2 (15.4%)	10 (76.9%)
Stepwise					
L	9	5 (55.6%)	0 (0.0%)	4 (44.4%)	0 (0.0%)
E	8	1 (12.5%)	0 (0.0%)	7 (87.5%)	0 (0.0%)
F	15	2 (13.3%)	0 (0.0%)	13 (86.7%)	0 (0.0%)
G	13	0 (0.0%)	0 (0.0%)	3 (23.1%)	10 (76.9%)
Females					
L	6	0 (0.0%)	1 (16.7%)	4 (66.7%)	1 (16.7%)
E	11	0 (0.0%)	5 (45.5%)	6 (54.5%)	0 (0.0%)
F	23	0 (0.0%)	2 (8.7%)	21 (91.3%)	0 (0.0%)
G	21	0 (0.0%)	2 (9.5%)	3 (14.3%)	16 (76.2%)
Cross-validated					
L	6	0 (0.0%)	1 (16.7%)	4 (66.7%)	1 (16.7%)
E	11	0 (0.0%)	5 (45.5%)	6 (54.5%)	0 (0.0%)
F	23	0 (0.0%)	2 (8.7%)	21 (91.3%)	0 (0.0%)
G	21	0 (0.0%)	2 (9.5%)	3 (14.3%)	16 (76.2%)
Stepwise					
L	6	0 (0.0%)	0 (0.0%)	5 (83.3%)	1 (16.7%)
E	11	0 (0.0%)	0 (0.0%)	11 (100.0%)	0 (0.0%)
F	23	0 (0.0%)	1 (4.3%)	22 (95.7%)	0 (0.0%)
G	21	0 (0.0%)	2 (9.5%)	3 (14.3%)	16 (76.2%)

River correctly whereas a high percentage of the other two groupings were classified appropriately. In all discriminant analyses, Sicilian males were grouped either into Sicily or into Mt. Pollino plus Basilicata. A considerable percentage of the Neto River turtles was predicted to belong to the Mt. Pollino plus Basilicata group (Table 8, top; Fig. 8), matching the finding that the colour pattern in both groups is very similar and fits the characteristics of *E. o. galloitalica*.

In females, DA with and without cross-validation yielded the same results for the complete set of four characters. In stepwise DA, the same combination of characters as for males was selected; stepwise DA with and without cross-validation produced identical results. Sicilian females were consistently misidentified in all DAs and placed most frequently into the Mt. Pollino plus Basilicata group. In DA of the complete character set, about half of the females from Neto River were grouped properly, the other half were placed in the Mt. Pollino plus Basilicata group. Most females from Mt. Pollino plus Basilicata (91.3%) and from the Salentine Peninsula (76.2%) were classified correctly. Stepwise DA produced results considerably different from the other DAs with respect to the Neto River females, which were completely misidentified as belonging to the Mt. Pollino plus Basilicata group (Table 8, bottom). When the females from Sicily (n = 1) and the Salentine Peninsula (n = 2) with unusual mtDNA haplotypes for these areas (i.e. lineage V) were included into the analyses as ungrouped cases, they were consistently put into the Salentine Peninsula assemblage in all DAs (Fig. 8). In conclusion, the distinctiveness of females of the Mt. Pollino plus Basilicata group vs. the Salentine group was confirmed by DAs. The DAs also indicate a similarity between Neto River females and individuals from Mt. Pollino plus Basilicata. Unfortunately, the only Neto River turtle with haplotype IIIa, which was

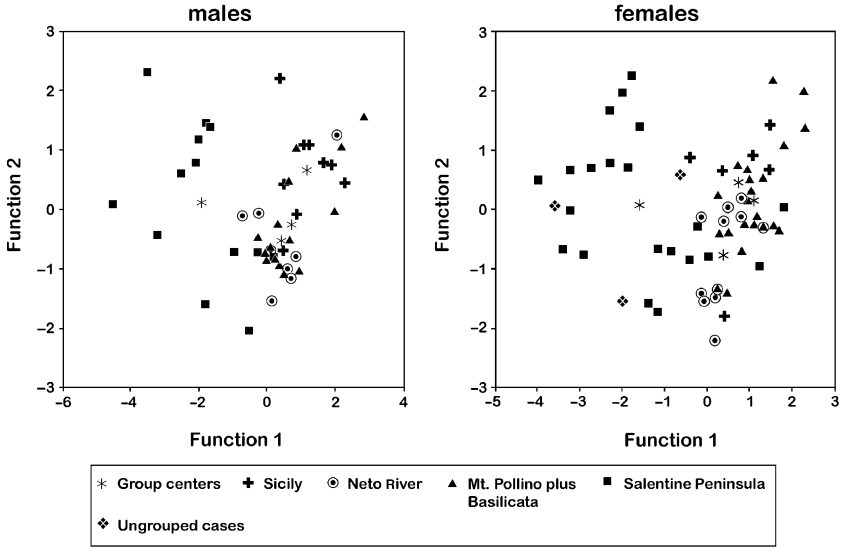

Fig. 8 Scatter diagrams for the canonical discriminant function for pond turtles from Sicily (males: n = 9, females: n = 6), Neto River (males: n = 8, females: n = 11), Mt. Pollino plus Basilicata (males: n = 15, females: n = 23), the Salentine Peninsula (males: n = 13, females: n = 21); and ungrouped cases (females, n = 3).

found during a previous study (Lenk *et al*. 1999), could not be included in the analysis because the necessary morphometric measurements were not recorded.

Systematics

Until recently, there were so few pond turtles known from Sicily and continental southern Italy that Fritz (1995, 1996, 2001, 2003) refrained from making any definite taxonomic allocations on the basis of morphology for these regions. In this study, we provide evidence that specimens from the Mt. Pollino area and Basilicata exhibit colour and pattern characteristics of *E. o. galloitalica*. Their mitochondrial haplotypes also fit this subspecies, which is known to harbour lineage V haplotypes (Fritz 2001, 2003). Nearly all turtles from the Salentine Peninsula are morphologically distinct and match *E. o. hellenica* in colouration, pattern and mtDNA haplotypes (lineage IV). Based on these findings, European pond turtles from the Mt. Pollino area and Basilicata should be assigned to *E. o. galloitalica* and those from the Salentine Peninsula to *E. o. hellenica*. Both subspecies were previously known from the Tyrrhenic (*galloitalica*) or Adriatic coast (*hellenica*) of the Apennine Peninsula, but from localities distinctly more northwards (*E. o. galloitalica*: Gulf of Sant'Eufemia; *E. o. hellenica*: Chieti, Abruzzo Region; Fritz 2003). Outside of Italy, *E. o. galloitalica* has been recorded from the northern Mediterranean coast of Spain and the Mediterranean coast of France and *E. o. hellenica* from the Adriatic and Ionic coasts of the Balkan Peninsula and the Peloponnese (Fritz 2003).

The record of two lineage V turtles patterned like *E. o. galloitalica* on the Salentine Peninsula, but associated by discriminant analyses with Salentine lineage IV turtles fitting the morphological characteristics of *E. o. hellenica* could indicate gene flow between both subspecies. In the Calabrian Neto River, both subspecies interbreed as well. Most turtles studied there are morphologically similar to *E. o. galloitalica*, although one male resembles *E. o. hellenica* in colouration and pattern. An influence of *hellenica* is also apparent by the syntopic occurrence of mtDNA haplotypes of lineages IV and V in this population. Furthermore, nuclear genomic fingerprinting shows that a Neto River turtle with haplotype IVh clusters with two haplotype Va specimens (*E. o. galloitalica*) from southern Italy (Fig. 6).

In an earlier study (Lenk *et al*. 1999), an individual with an mtDNA haplotype (IIIa) of the Sicilian lineage III was also reported for the Neto River. However, our nuclear fingerprint analysis did not reveal a genetic influence of lineage III on the Neto River turtles studied. This finding could (1) argue for a syntopic occurrence of noninterbreeding taxa that differ in their nuclear and mitochondrial genomes and thus fit the criteria of distinct biological species (Mayr 1942, 1963, 2000); (2) indicate that the haplotype IIIa turtle was introduced in the Neto area by humans; or (3) indicate a recently immigrated member of this population that is not yet genetically contributing to the other studied individuals. In any case, the record of haplotype IIIa in the Neto River remains enigmatic.

In the UPGMA tree of nuclear fingerprints (Fig. 6), only the Sicilian samples are clearly distinct under the 50% consensus criterion; representatives of the other two Italian mtDNA lineages (IV, V) were placed in the same multifurcation with samples of lineages from other parts of the range of *E. orbicularis*. This indicates a high percentage of shared bands, suggesting past (e.g. during Pleistocene interglacial or interstadial phases because some lineages are now distributed fully allopatrically) or current gene flow between lineages I, II, IV, V, VI, VII, VIII, and IX. By contrast, the remote position of lineage III speaks for a distinctly longer and more effective reproductive isolation. This is remarkable because a repeated faunal interchange between Sicily and Calabria took place during glacial low sea level stands (Palombo 1986, 2001; Bonfiglio *et al*. 1997). Moreover, as outlined previously, a haplotype IIIa turtle was caught in the Neto River area, where individuals with haplotypes of lineages IV and V were also recorded, but no evidence for interbreeding was detected in nuclear fingerprinting.

The obvious distinctiveness of the nuclear fingerprints of lineage III turtles compared to representatives of all other known mtDNA lineages of *Emys* argues for a long unique evolutionary history and a high degree of differentiation. Furthermore, the data from the only known locality where a lineage III turtle has been caught syntopically with lineage IV and V individuals (Neto River, Calabria) provide no evidence for intergradation or hybridization. The same is true for the fingerprints of six Sicilian haplotype IIIa samples, which originate from a locality where an allochthonous haplotype Va turtle was found. Because of the lack of evidence for intergradation or hybridization and the distinctiveness of both their nuclear fingerprints and their mitochondrial genome, we regard the Sicilian and Calabrian lineage III turtles as representatives of a second *Emys* species that is the sister taxon of *E. orbicularis*. There is no scientific name available for Sicilian or Calabrian pond turtles (Fritz 2001, 2003). Consequently, we describe them below as a species new to science.

Genus *Emys* Duméril, 1806

Emys trinacris n. sp.

Suggested vernacular name: Sicilian pond turtle (English), Testuggine palustre siciliana (Italian).

Holotype. Museo Zoologico 'La Specola', Florence (MZUF 11136, adult male, Lago Gian Fenaro, below the pass of Pizzo Laminaria approximately 1400 m above sea level, Monte Nebrodi, Sicily, leg. E. Kramer & S. Dereani, 9 May 1968).

Paratypes. Muséum National d'Histoire naturelle, Paris (MNHN 409, 1989: 3805–3806, two adult males, one juvenile, Sicily, don. G. Bibron, *c*. 1830), Museo Zoologico 'La Specola', Florence (MZUF 1648, adult male, Monalo River, Castelbuono, Madonie, Sicily, don. F. M. Palumbo, August 1881; MZUF 7736–7737, hatchling and adult male, Stagno grande near Faro, Messina, Sicily, don. G. Cevanne, May 1877 and R. Gollette, 23 November 1878; MZUF 19877, adult male, surroundings of Sciacca, Agrigento, Sicily, leg. D. Manni, 18 August 1974). All types except MZUF 1648 (mounted) are alcoholic specimens. The type series comprises all known museum specimens of *E. trinacris*.

Etymology. The species name *trinacris* is the Latin adjective derived from the ancient Greek word for Sicily, Trinacria (Τρινακρία) = triangular island, which is still used for Sicily in Italy. The Greek origin of the species name matches the generic name *Emys*, which has its roots in ancient Greek as well (ἐμύζ = turtle).

Diagnosis. A small *Emys* species (known maximum straight-line carapacial length: 145 mm) with a dark carapace and a mainly or entirely yellow plastron. Small dark blotches at the distal seams of the plastral scutes are rare but may occur; most frequently on the pectoral-abdominal and on the abdominal-femoral seams. *Emys trinacris* differs from the highly polytypic and morphologically variable *E. orbicularis* by its distinct mitochondrial and nuclear genome, indicating reproductive isolation. Morphologically, *E. trinacris* differs from the northern *E. orbicularis* subspecies by its distinctly smaller size and lighter colouration of the shell and soft parts; it differs from the *orbicularis* subspecies group of *E. orbicularis*, *E. o. luteofusca*, and *E. o. iberica* in that the iris of males is white instead of reddish. Among the small-sized southern subspecies of *E. orbicularis*, *E. trinacris* most closely resembles *E. o. galloitalica* in gross morphology; however, *E. trinacris* has a more ovoid, and not elongated, shell outline in dorsal view on average. A light brownish or yellowish-brown primary colour of the carapace, which is frequent in *E. o. galloitalica*, is not known to occur in *E. trinacris*.

Description of the holotype. Carapacial length 132 mm, maximum carapacial width 103 mm. Maximum shell height 49.5 mm. Plastral length 119 mm. All values are straight-line measurements to the nearest 0.5 mm. Primary carapacial colouration is dark, patterned with radiating wide yellow spots and streaks. Hyo-hypoplastral hinge present, plastron entirely yellow, with slightly brownish staining due to environmental conditions (Fig. 9).

Fig. 9 Dorsal and ventral aspect of the holotype of *Emys trinacris* (MZUF 11136, adult male, Lago Gian Fenaro, Monte Nebrodi, Sicily).

Discussion

Morphological crypsis of Emys trinacris

In contrast to the southern Italian populations of *E. o. galloitalica* and *E. o. hellenica*, all applied discriminant analyses failed to separate the genetically highly distinctive *E. trinacris* on the basis of morphometry. Although we cannot exclude that this situation will change with additional characters or a larger sample size, this finding is paralleled by gross morphology. *E. trinacris* is characterized by the lack of striking morphological traits present in some *E. orbicularis* subspecies more than by own distinct characters. Colour and pattern are powerful tools for discriminating some subspecies of *E. orbicularis* (Fritz 2001, 2003), but none of the characters of *E. trinacris* are unique. For example, the conspicuously blotched dark plastral pattern also occurs frequently in *E. o. galloitalica*, and a similar pattern is found in several other emydine turtle species (e.g. *Clemmys guttata*, *Emydoidea blandingii*, *Glyptemys insculpta*, and some individuals of *Actinemys marmorata*; Carr 1952; Ernst *et al*. 1994), of which *C. guttata* and *G. insculpta* are not closely related to *Emys* (Lenk *et al*. 1999; Feldman & Parham 2002; Stephens & Wiens 2003). This suggests that the blotched plastral pattern is a plesiomorphic character state in *E. trinacris*, which could be true for other morphological traits of this species as well. Moreover, turtles are generally known for their particularly homoplastic morphology (Feldman & Parham 2002; Claude *et al*. 2003). As such, the weak morphological differentiation of *E. trinacris* could explain why this taxon was not recognized earlier. With the advent of modern molecular genetic techniques, several cryptic chelonian species-level taxa have been identified (Guicking *et al*. 2002; Feldman & Parham 2004) and we expect that *E. trinacris* will not remain the most recent example for long.

The question of autochthony

Italy is a densely populated country, and introductions and relocations of turtles and tortoises have probably occurred for centuries. A well-known example is an established

population of the Greek marginated tortoise (*Testudo marginata*) in Sardinia, which was introduced long ago, possibly in ancient or prehistoric times (Bringsøe *et al*. 2001). In recent years, the introduction of the North American red-eared slider *Trachemys scripta elegans* became a problem for nature conservation in Italy (Ferri & di Cerbo 2000; Bringsøe 2001), cogently illustrating the current human impact. In addition to the introduction of foreign species, the release of native turtles in other places or regions, whether for intentional introduction or simply as a result of being picked up out of curiosity and transported for a distance, is likely to occur for a long time, obscuring the natural distribution pattern more and more.

The pond turtles treated as allochthonous in this study were captured either in habitats close to urban areas or in regions where native populations are rare or known to be extinct; they were morphologically different than expected or bore mtDNA haplotypes that did not agree with the general picture. The latter is, for example, the case for a turtle of unknown morphology caught in Tuscany that bears a haplotype (IIa) not otherwise encountered in Italy. The nearest documented natural occurrences of haplotype IIa are in the Camargue (France) and the Danube catchment basin (Lenk *et al*. 1999; Fritz *et al*. in press), 400–500 km away. Another example is the population in the Tenuta di Castelporziano Reserve in the neighbourhood of Rome. Here, the majority of turtles studied bear haplotype IVa. This haplotype is otherwise found only along the Adriatic coast, on the Salentine Peninsula, and on the Balkans (Lenk *et al*. 1999; this study). Only five out of 20 analysed individuals from Castelporziano bore the expected haplotype of Va. Morphologically, all Castelporziano turtles resemble *E. o. galloitalica* (Fritz *et al*. 1995), a subspecies normally characterized by haplotypes of lineage V (Fritz 2001, 2003). It is very likely that the native Castelporziano population of *E. o. galloitalica* was contaminated with introduced haplotype IVa turtles (*E. o. hellenica*) sometime in the past.

An individual from Sicily warrants special mentioning. It was caught together with 11 other turtles near Mistretta, Sicily (Demone Valley; 1000 m above sea level). Eleven of these 12 specimens were haplotyped and 10 bore the mtDNA haplotype IIIa typical for *E. trinacris*, whereas one had Va. This Va turtle exhibits, compared with the other Mistretta individuals, a distinct pattern on the soft parts and plastron. Nuclear genomic fingerprinting revealed no influence of *E. o. galloitalica*, the taxon normally bearing haplotypes of lineage V, in six haplotype IIIa turtles from Mistretta. Thus, we conclude that the haplotype Va individual was introduced.

We are confident that our identification of allochthonous specimens is reliable. However, we cannot exclude that we were not strict enough and have accepted some introduced individuals as being native. This could be true for the two specimens with haplotypes of lineage V recorded on the Salentine Peninsula (localities Torre Rinalda, Morelli — Torre Canne). In contrast to the majority of Salentine turtles, which are morphologically and genetically indistinguishable from *E. o. hellenica*, the two lineage V turtles are patterned like the subspecies *E. o. galloitalica*, suggesting an introduction as for the above-mentioned Sicilian turtle. However, we cannot entirely exclude that they immigrated naturally from the west, despite long-distance migrations not having been reported in *E. o. galloitalica* (Fritz 2003), or that they are the result of the hybridization of a lineage IV *hellenica* male with an introduced or naturally immigrated lineage V *galloitalica* female. Support for the latter hypothesis derives from the two questionable turtles being associated with the other Salentine turtles, and not with *E. o. galloitalica*, in discriminant analyses based on morphometric characters.

The two turtles from the Neto River with haplotypes IIIa and IVh could also represent allochthonous individuals. However, this area is a strictly protected nature reserve that is not easily accessible. Moreover, the haplotype IVh turtle from Neto River clusters in nuclear fingerprinting with two haplotype Va specimens from southern Italy (Fig. 6), suggesting gene flow. One Neto River male also has soft parts patterned as is typical for *E. o. hellenica*. Thus, it is likely that *E. o. galloitalica* and *E. o. hellenica* interbreed in the Neto system. Furthermore, we cannot exclude that *E. trinacris* is also involved, although this is not corroborated by genetics.

Zoogeography

In this paper, we demonstrate for the first time the occurrence of *E. o. hellenica* in southern Apulia, a taxon that was recorded before now only from the Adriatic coast of the Italian regions Abruzzo and Veneto, the Adriatic and Ionic coasts of the Balkans, and from the Peloponnese (Fritz 2001, 2003). With our new records for southern Apulia, the range of this subspecies fits a circumadriatic distribution pattern known for several other animal species (La Greca 1995).

Like in the Balkans, where higher haplotypic diversity is found in the south of the range of *E. o. hellenica* (Lenk *et al*. 1999), we also encounter endemic mtDNA haplotypes of lineage IV in southern Italy. This observation agrees with the well-known phenomenon in many species that higher genetic diversities occur in the south of their ranges (e.g. Hayes & Harrison 1992; Merilä *et al*. 1997; Taberlet *et al*. 1998; Hewitt 1999, 2001; Cruzan & Templeton 2000), fitting Hewitt's (1996) long-distance dispersal model. Generally, this distributional pattern is thought to reflect a rapid postglacial range expansion, resulting in a decreasing diversity with increasing distance from the refugium that acts as radiation centre. Remarkably, the endemic southern Italian haplotypes (IVd, IVh, IVi, IVj) of *E. o. hellenica* differ from the southern Balkan haplotypes IVb and IVc that were reported by Lenk *et al*.

(1999) for the Peloponnese and Cephalonia. In the meantime, we identified a third endemic haplotype (IVg) from the Peloponnese (Kapsia). This could indicate the existence of two different glacial refugia of haplotype IV turtles: (a) in southern Italy, and (b) in the southern Balkans.

Haplotype IVa, which occurs alongside the endemic haplotypes IVd, IVh, IVi, and IVj in southern Italy but not on the southernmost Balkans, exclusively occupies the northern parts of the subspecies' range around the Adriatic and Ionic Seas: Apulia, Abruzzo, Veneto, Istria, Dalmatia, and Corfu (Lenk *et al*. 1999; this study). Because haplotype IVa is replaced on the southernmost Balkans by the endemics IVb, IVc, and IVg, we speculate that the west coast of the Balkans and Corfu might have been colonized from Italy and not from the geographically closer southern Balkanic refuge. During the late phases of the last glacial (18 000–14 000 years BP), the Adriatic basin was only about 1/7th of its present extent and the Po River was debouching at the edge of the Mid Adriatic Deep (Correggiari *et al*. 1996; Trincardi *et al*. 1996), approximately between Pescara and Šibenik. The maximum Holocene flooding of the Adriatic continental shelf was not reached before 6000 years BP (Langone *et al*. 1996). Thus, a postglacial colonization of the Balkan coast from the Italian Peninsula would have been much easier for a long time than it would be now.

A similar situation with respect to higher southern genetic diversity also emerges for *E. o. galloitalica*, which is characterized by mtDNA lineage V. This lineage is distributed from the northern Spanish and French Mediterranean coasts to the western Apennine Peninsula. In addition, it occurs on Corsica and Sardinia (Lenk *et al*. 1999; this study). In southern Italy, we identified for the first time new haplotypes for this lineage (Vb, Vc) occurring together with the previously known haplotype Va. Again, these new haplotypes were found exclusively in the very south of the range of lineage V turtles, and thus in the putative glacial refuge.

In an earlier paper (Lenk *et al*. 1999), the occurrence of the highly distinct mtDNA lineage III of *E. trinacris* in the Calabrian Neto River was recorded. This was not confirmed in our present study. Instead, we found evidence for the syntopic occurrence of two other lineages (IV, V) and for the interbreeding of *E. o. galloitalica* and *E. o. hellenica* there, but not for a genetic influence of *E. trinacris*. Additional research is needed to clarify this situation and whether and where *E. trinacris* is distributed in Calabria.

Compared with other parts of the range of *Emys*, Italy exhibits the most complicated variation for this genus that is currently known on a small geographical scale. This is especially true for the southern part of the peninsula and Sicily (Figs 4,5). There, three taxa with distinct mtDNA lineages occur, qualifying southern Italy as biodiversity hotspot for pond turtles. A somewhat similar situation is known only from the south-eastern Balkans and southern France plus adjacent north-eastern Spain in that two or three *E. orbicularis* subspecies bearing distinct mtDNA lineages meet in either location. In the latter cases, this situation is thought to be the result of Holocene range expansions of different taxa (Lenk *et al*. 1999; Mascort *et al*. 2000; Fritz 2001, 2003; Fritz *et al*. in press). By contrast, southern Italy seems to harbour several distinct taxa with old mtDNA lineages that have thrived there for a very long time. According to molecular clock calculations, the three southern Italian lineages already diverged in the Early to Middle Pliocene (Lenk *et al*. 1999). The rich fossil record in Italy reveals that pond turtles occurred on the Apennine Peninsula since approximately 1.8 million years ago at least; Delfino (2002) lists numerous Early Pleistocene sites scattered over almost the whole peninsula: Toscana: Montecarlo, S. Giovanni; Umbria: Pietrafitta; Abruzzo: Scoppito, L'Aquila; Lazio: Capena; Puglia: Cava Pirro/Dell'Erba, Apricena; Basilicata: Loreto di Venosa, Potenza (sites listed from north to south). The oldest known fossils from Sicily are somewhat younger and date back to the early Middle Pleistocene (Contrada Cozzo del Re, Comiso; Contrada Frategianni, Comiso; Grotta di Luparello; Delfino 2002). Thus, southern Italy was inhabited by pond turtles for a considerably longer time than the Würm glacial. This scenario suggests that not only were glacial refugia for the extant taxa (*E. o. galloitalica*, *E. o. hellenica*, *E. trinacris*) located in southern Italy, but that the ancestors of the recent taxa already occurred there prior to the onset of the great Pleistocene climatic oscillations that started in the Middle Pleistocene (700 000 years BP; Schwarzbach 1988; Hantke 1992). If this is correct, the taxonomic distinctiveness of *E. o. galloitalica* and *E. o. hellenica* could be underestimated on the subspecific level because they would have coexisted in close geographical proximity for several hundred thousands of years without losing their genetic integrity.

Implications for conservation

European pond turtles are seriously endangered in many parts of their range, mainly by former overexploitation and/or habitat destruction (Fritz 2003). The latter applies particularly to southern Italy, where 70–90% of suitable wetlands have been lost in the past century (Pozio & Frisenda 1973; Frisenda 1988; Fattizzo 2004). This general situation was responsible for the inclusion of *E. orbicularis* in the EU Directive (92/43/EEC) on the conservation of natural habitats and of wild fauna and flora (1992) and in Appendix II (Strictly Protected Fauna Species) of the Bern Convention on the conservation of European wildlife (1979). A shortcoming of the current legislative situation is that no distinction is made between *E. trinacris*, *E. orbicularis* and the various subspecies of *E. orbicularis*, each of which occupy ranges of very different sizes, and between the varying conservational situations and threats in different parts of the range of the genus *Emys*.

Sicily is well known for its endemic species (e.g. the wall-lizard *Podarcis wagleriana* or the Sicilian shrew *Crocidura sicula*). With the description of *Emys trinacris*, a further endemic Sicilian species becomes known to science. The discovery of this second European pond turtle species is a challenge for nature conservation. Because turtles and tortoises are extremely charismatic animals, *E. trinacris* has the potential of becoming a flagship species for wetland conservation in Sicily. Because of its restricted geographical distribution, we recommend strict national and international protection of *E. trinacris* and its habitats, as has been suggested earlier for some endemic *E. orbicularis* subspecies that occupy small ranges (Fritz 2000).

Pond turtle conservation in Italy often includes captive breeding projects of NGOs and (re)introduction measures (e.g. Gariboldi & Zuffi 1994; Ballasina 1995; Ferri *et al*. 1998; Jesu *et al*. 2000). Our findings on the taxonomic variation in Italy call for caution with reintroduction projects. Both the west and east coasts of Italy as well as Sicily are inhabited by distinct pond turtle taxa. Additionally, the Sardinian population, which has been present and isolated on that island since at least the Upper Pleistocene (Delfino 2002), has been described as a distinct subspecies as well (Fritz 1995). Because of the danger of outbreeding and loss of biodiversity, the introduction of any individual of unknown provenance to any population should be strictly avoided. The complicated pattern found in southern Italy calls for caution even when transferring individuals over small distances. If captive breeding programs are desired, they should focus only on local turtles that originate immediately in the area where a later re-introduction is intended.

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Appendix

Data matrix of nuclear DNA fragments obtained by ISSR.

No.	Haplotype	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	z	aa	ab	ac	ad	ae	af
1	IIIa	1	0	0	1	1	0	1	1	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0
2	IIIa	1	0	0	1	1	0	1	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0
3	IIIa	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0
4	IIIa	1	0	0	1	1	0	1	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0
5	IIIa	1	0	0	1	1	0	1	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	1	0	1	1	0
6	IIIa	1	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	0	0	1	0	1	1	0
7	IVa	1	1	1	1	1	0	1	0	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
8	IVa	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
9	IVa	1	1	0	1	1	0	1	0	0	1	1	0	0	0	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
10	IVa	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0
11	IVa	1	1	1	1	1	1	1	0	0	1	0	1	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
12	IVa	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
13	IVa	1	1	0	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0
14	IVa	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
15	IVa	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	0	1	1	0	0	1	1	1	1	1	0	0	0	1	1	1	0
16	IVa	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
17	IVa	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
18	IVa	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
19	IVd	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
20	IVd	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
21	IVd	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
22	IVd	1	1	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
23	IVd	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0
24	IVd	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
25	IVd	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
26	IVd	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
27	IVh	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
28	IVj	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
29	IVj	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	0	1	1	1	1	0	1	1	1	0
30	Va	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
31	Va	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
32	Va	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
33	Va	1	1	1	1	1	0	1	1	0	1	1	0	0	1	0	1	0	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
34	Va	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
35	Va	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
36	Va	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
37	Vb	1	1	1	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0
38	Vb	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	0	0	1	1	0	1	1	1	1	0	0	0	0	0
39	Ia	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1
40	Ia	1	0	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1
41	IIa	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0
42	IIa	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	1	1	0
43	VIa	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
44	VIa	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
45	VIIa	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
46	VIIa	1	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
47	VIIIa	1	1	1	1	0	1	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
48	VIIIa	1	1	1	1	0	1	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0
49	IXa	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0

Bands: a-af; bold numbers: individuals. Numbers correspond to Table 2. Differences of Sicilian pond turtles (haplotype IIIa) mentioned in the text are presented in grey; private bands of Sicilian samples are framed.