Phylogeographic Inferences from the mtDNA Variation of the Three-Toed Skink, *Chalcides chalcides* (Reptilia: Scincidae)

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ABSTRACT   Genetic diversity was analyzed in *Chalcides chalcides* populations from peninsular Italy, Sardinia, Sicily and Tunisia by sequencing 400 bp at the 5′ end of the mitochondrial gene encoding cytochrome *b* (cyt *b*) and by restriction fragment length polymorphism (RFLP) analysis of two mitochondrial DNA segments (ND-1/2 and ND-3/4). The results of the phylogenetic analysis highlighted the presence of three main clades corresponding with three of the four main geographical areas (Tunisia, Sicily and the Italian peninsula), while Sardinia proved to be closely related to Tunisian haplotypes suggesting a colonization of this island from North Africa by human agency in historical times. On the contrary, the splitting times estimated on the basis of cyt *b* sequence data seem to indicate a more ancient colonization of Sicily and the Italian Peninsula, as a consequence of tectonic and climatic events that affected the Mediterranean Basin during the Pleistocene. Finally, the analysis of the genetic variability of *C. chalcides* populations showed a remarkable genetic homogeneity in Italian populations when compared to the Tunisian ones. This condition could be explained by a rapid post-glacial expansion from refugial populations that implied serial bottlenecking with progressive loss of haplotypes, resulting in a low genetic diversity in the populations inhabiting the more recently colonized areas. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:297–307, 2007.*   © 2007 Wiley-Liss, Inc.

The geographical distribution and phylogenetic relationships of animal mitochondrial DNA (mtDNA) variants reflect both contemporary gene flow and biogeographical events in the past history of species (Avise, '94). In this respect, paleoclimatic and paleogeographic events are regarded as important factors in fostering allopatric isolation and subsequently the genetic divergence among populations, through the fragmentation of the distributional range of species (see La Greca, '90; Avise, '94; Hewitt, '96).

During the last 6 million years, North Africa and the Mediterranean area went through a series of tectonic and climatic phenomena that promoted intraspecific genetic divergence, that may ulti-mately lead to speciation (Hewitt, '96, 2000). In particular, intense tectonic phenomena that led to the closing of the Strait of Gibraltar in upper Miocene about 6 Million years ago (Ma), connected Africa and Europe. This possibility of faunal exchange was interrupted again by the separation of the European and African plates in the Early Pliocene (approximately 5 Ma) (Rosenbaum et al.,

2002). This caused the allopatric isolation of the populations that had colonized Europe from Africa and vice versa. Regional flora and fauna were also influenced by the climatic changes during ice ages starting in the Tertiary (see Hewitt, '96). The effect of climatic oscillations was particularly strong in shaping intraspecific genetic structure and distributional patterns in temperate regions: species partly became extinct, dispersed or survived in refugia in the South, and subsequently recolonized the northern regions again (Hewitt, '96, 2000). Avise et al. ('98) and Avise and Walker ('98) used mtDNA of extant species to estimate the temporal spans of and the Pleistocene effects upon speciation events. They found considerable impact of Pleistocene on phylogeography of many vertebrate species. In this context, due to the limited dispersal capacities and temperature dependence, terrestrial and freshwater reptiles represent sensitive indicators of paleoclimatic and paleogeographic events and the study of their genomic markers substantially contributes to the understanding of biogeographical processes (Lenk et al., '99).

The genus *Chalcides* occurs in the Macaronesian and Mediterranean areas and from central and eastern Africa to Pakistan. This group comprises 28 lizard species, all viviparous, differing in the degree of limb reduction and body elongation (Caputo et al., '95; Greer et al., '98). The three-toed skink (*Chalcides chalcides*) is a short-limbed snake-like species distributed in peninsular (south of Po river) and insular Italy and North Africa (Tunisia and Libya). It belongs to the *C. chalcides* complex, comprising five taxa with allopatric or parapatric distribution in the western Mediterranean (Caputo, '93, 2006). Their very specialized ecology confines these lizards to mesophilous polyphytic grasslands, where they live as a ''grass-swimmer''. This remarkable ecological specialization explains the extremely scattered distribution of the three-toed skink, for which only 1–10% of the Mediterranean basin represents a potentially suitable habitat (Orsini and Cheylan, '81). The distribution of Mediterranean grasslands roughly corresponds with that of broad leaf deciduous forests (Hewitt, '93), and seemingly underwent the same range contractions/expansions as those experienced by these woods during the Pleistocene climate oscillations (see Cattani, '92; Ravazzi and Aceti, 2004; Ravazzi et al., 2004). The aim of the present work was to evaluate the extent of genetic variability in populations of *C. chalcides* across most of its range in order to:

(i) infer the phylogenetic relationships among the mtDNA lineages of the sampled locations to depict a possible phylogeographic scenario; (ii) study the role played by the climatic oscillations that affected the Northern Hemisphere during the late Pliocene and Pleistocene in shaping the genetic structure of the three-toed skink. This was done by means of the restriction fragment length polymorphism (RFLP) analysis of two fragments of the mtDNA comprising NADH dehydrogenase subunits 1 and 2 (ND-1/2) and NADH dehydrogenase subunits 3, 4L and 4 (ND-3/4), and by sequencing a portion of the mitochondrial gene encoding cytochrome *b* (cyt *b*).

MATERIALS AND METHODS

A total of 168 specimens of *C. chalcides* were caught in 11 of the 13 sampling locations reported in Figure 1 and Table 1. Tail tips were removed and preserved in 99% ethanol until DNA extraction. The two individuals from Sardinia were ethanol preserved specimens. Total genomic DNA was extracted from the preserved tissues by Proteinase K/SDS dissolution and purified with an extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and an additional extraction with chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation with a 1/10 volume of sodium acetate (3 M, pH 5.2) (Kocher et al., '89; Sambrook et al., '89).

For the study of genetic variability of *C. chalcides* populations, RFLP analysis was performed on 168 *C. chalcides* specimens. Additionally, four specimens of *C. striatus* and four of *C. boulengeri* (subgenus *Sphenops*, *sensu* Caputo, 2004) were included as outgroups in the phylogenetic analysis. Two segments of mtDNA were PCR-amplified: a segment of approximately 2.7 kb comprising NADH dehydrogenase subunits 1 and 2 (ND-1/2) was amplified using the primers L3827 and H6313 (Sorenson et al., '99); a segment of about 2.4 kb comprising NADH dehydrogenase subunits 3, 4L and 4 (ND-3/4) was amplified with the primers ND-3/4 F and ND-3/4 R designed by Nielsen et al. ('98). Both ND-1/2 and ND-3/4 were amplified with the same PCR protocol comprising an initial denaturation step at 95°C for 5 min followed by 35 cycles of 94°C/45 sec denaturation, 52°C/1 min annealing and 72°C/1.5 min extension, then a final extension at 72°C for 10 min. This analysis was not performed on the two specimens from Sardinia because it was not possible to successfully amplify these segments.
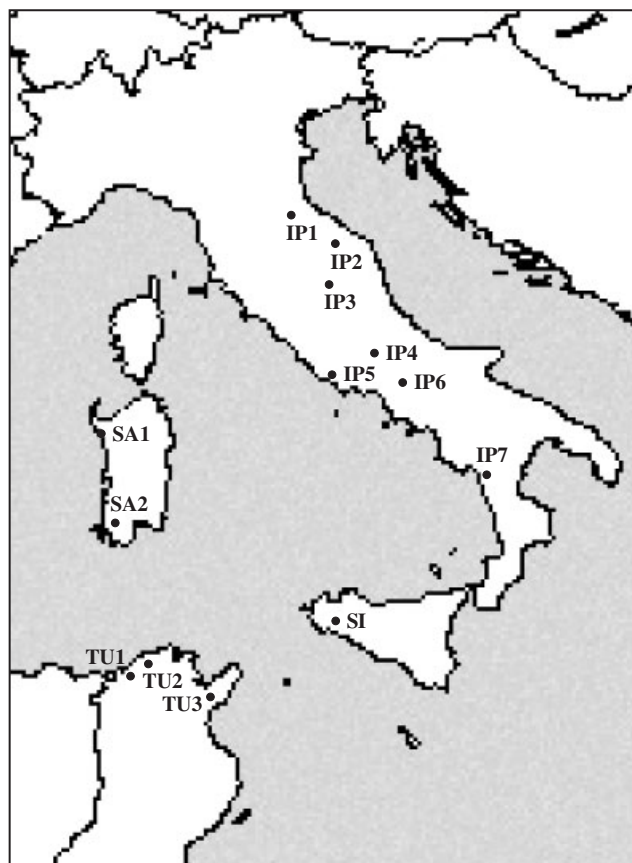
Fig. 1. Geographical distribution of the sampling localities of the three-toed skink (*Chalcides chalcides*). For locality codes see Table 1.

TABLE 1. *Sampling localities, number of specimens collected (n) and locality code*

Locality	n	Locality code
Urbino	18	IP1
Ancona	15	IP2
Macerata	15	IP3
Isernia	18	IP4
Latina	20	IP5
Caserta	8	IP6
Potenza	7	IP7
Agrigento	10	SI
Alghero	1	SA1
Cagliari	1	SA2
Tabarka	20	TU1
Ouchtata	20	TU2
Korba	17	TU3

IP: Italian peninsula; SI: Sicily; SA: Sardinia; TU: Tunisia.

The amplified fragments were then digested with restriction endonucleases. Nine endonucleases were used for ND-1/2 (*Alu*I, *Apa*I, *Ava*II, *Dra*I, *Hinf*I, *Hpa*II, *Rsa*I, *Sca*I and *Taq*I) and

seven for ND-3/4 (*Ava*II, *Dra*I, *Hinf*I, *Hpa*II, *Rsa*I, *Sca*I and *Taq*I). Restriction digestions were carried out in 20 μl volumes using 3–5 μl of PCR product, 10 U of the enzymes and buffers according to the manufacturer's instruction (Fermentas International, Inc., Burlington, ON). Restriction fragments were electrophoresed on 2% agarose gels (Bio-Rad Laboratories, Hercules, CA) using ethidium bromide staining, alongside 100 bp ladder and visualized on an ultraviolet transilluminator. Fragments shorter than 100 bp were not generally detectable and some hypothetical fragments were assumed in order to explain all the mutational steps (see Jaarola and Tegelstrom, '96).

Restriction patterns generated by each enzyme were identified with capital letters which were then combined to define composite mtDNA haplotype patterns (Table 2). These patterns were subsequently used to infer the presence or absence of restriction sites in the different haplotypes. Restriction site data were analyzed with the computer package REAP 4.0 (McElroy et al., '91), program DA, to calculate haplotype (h) and nucleotide diversity (π) of the sampled populations.

The population genetic structure was determined by estimating the molecular variance and calculating *F*-statistics and their analogs (Φ-statistics) by AMOVA analysis (Excoffier et al., '92) (10,000 replications), using ARLEQUIN 2.000 (Schneider et al., 2000). The analysis is performed at three hierarchical levels: among groups, among populations within groups and within populations. We defined three groups according to the geographical distribution of the analyzed specimens (Italian peninsula, Sicily and Tunisia), and 11 populations, corresponding to as many sampling locations. AMOVA analysis was performed in two ways: the one based on the pairwise differences between haplotypes and that based on haplotype frequencies.

The statistical significance of haplotype frequency differentiation among populations was tested by the Monte Carlo simulation with 10,000 replications, as described by Roff and Bentzen ('89), using the program MONTE of the REAP package.

The distribution of pair-wise genetic differences between individual haplotypes within a population (mismatch distribution) was analyzed using the sudden expansion model of Rogers and Harpending ('92) as implemented in ARLEQUIN.

In order to increase the number of informative characters for the analysis of the phylogenetic relationships between haplotypes and to estimate the splitting times between the main genetic

TABLE 2. *Genetic composition of the populations studied*

Composite haplotype	Cyt b haplotype	Populations														Outgroups	
		Italy										Tunisia				
		IP1	IP2	IP3	IP4	IP5	IP6	IP7	SI	SA1	SA2	TU1	TU2	TU3	CS	CB
H1 AAAAAAAAAAAAAAAA	C1	18	15	15	18	20										
H2 AABAAAAAAAAAAAAA	C1						8									
H3 DABAAAABABABBAAA	C2							7								
H4 ABAAAAECCAAADAAA	C3								8							
H5 ABBAAAECCAAADAAA	C4								2							
— —	C5									1	1					
H6 CABADDDBCABDDACA	C6											11				
H7 CABACDCDCABDDACA	C7											2				
H8 CABACCCDCABDDACA	C7											1				
H9 CABACDDBCABDDACA	C8											3				
H10 CABACDCBCBBDDDDA	C7											2	8			
H11 CABADDDDCABDDACA	C6											1				
H12 CABACDCDCBBDDDDA	C7												7			
H13 CABAFDCDCBBDDDDA	C7												3			
H14 CABAFDCBCBBDDDDA	C7												2			
H15 CACACCCBCBBDDCCA	C9													16		
H16 CABACCCBCBBDDACA	C10													1		
H17 BABBBBBBBAACCBBB	C11														4	
H18 ECDCEEFBDABEEEBC	C12															4
n		18	15	15	18	20	8	7	10	1	1	20	20	17	4	4

PCR-RFLP composite haplotypes, their frequency in the sampled populations, the corresponding cyt *b* haplotypes and sample size (*n*). The last two haplotypes (H17, H18) and the last two columns refer to the species used as outgroups in the phylogenetic analysis.

lineages, a sequence analysis was performed on individuals representing the haplotypes detected with the RFLP analysis. A 450 bp segment of the mtDNA including the 5′ end of the cyt *b* gene was PCR-amplified using the universal primers L14724 (Palumbi, '96) and H15149 (Kocher et al., '89). The PCR profile comprised an initial denaturation step at $95°C$ for 2 min followed by 35 cycles of 1 min $95°C$/1 min denaturation, $52°C$/45 sec annealing and $72°C$/1 min extension, then a step of final elongation at $72°C$ for 7 min. Sequencing of the PCR products was performed with above primers on ABI PRISM 3730XL (Applied Biosystems, Foster City, CA) automatic sequencer. Sequences were aligned in Clustal W (Thompson et al., '94) with the default parameters. MEGA version 2.1 (Kumar et al., 2001) was used to determine number and type of nucleotide substitutions in pair-wise comparisons of sequences and the genetic distance between haplotypes, calculated using Kimura 2-parameters (K2P). This portion of the cyt *b* gene was successfully amplified also in the two specimens from Sardinia.

Sequence and RFLP data were then combined in a single data set for the phylogenetic analysis. For this purpose, the variable sites of the sequences were transformed into binary format by excluding the sites that presented more than two character states (see Koskinen et al., 2000) The resulting binary matrix was analyzed with the computer package PHYLIP (Felsenstein, '95). It was replicated 1,000 times with the program SEQBOOT (PHYLIP) and then analyzed by Wagner parsimony method using the program MIX (PHYLIP). The trees obtained were combined with CONSENSE (PHYLIP). The splitting times between main lineages of *C. chalcides* and between *C. chalcides* and *C. striatus* were estimated on the basis of cyt *b* sequence data. The net nucleotide divergence between groups was calculated from K2P pair-wise distances using MEGA. This method allows the calculation of the genetic divergence between clades corrected for the within-clade haplotype variation and can be used to calculate the splitting times of groups (Nei, '87). These latter were estimated applying an

evolutionary rate of 2.1% sequence divergence per million years, as estimated by Carranza and Arnold (2003) for the genus *Chalcides*. The relative rate test was performed to verify whether there was a statistically significant difference in cyt *b* substitution rates among the lineages. This analysis was carried out as implemented in RRTREE version 1.1 (Robinson-Rechavi and Huchon, 2000), taking an NJ tree as a reference topology and using *C. boulengeri* as an outgroup. Relative rate tests were performed on the proportions of synonymous (K_s) and nonsynonymous (K_a) substitutions.

RESULTS

PCR-RFLP analysis of the ND-1/2 and ND-3/4 regions of 168 *C. chalcides* individuals from 11 different sampling locations revealed 16 composite haplotypes (Table 2). In *C. chalcides*, the 16 endonucleases recognized an average of about 48 restriction sites per composite haplotype, corresponding to an average of about 205 bp.

The length of the 18 *C. chalcides* cyt *b* sequences analyzed was 400 bp. The analysis of these sequences (16 from individuals representing the RFLP composite haplotypes and 2 from the Sardinian specimens) revealed 10 haplotypes (Tables 2 and 3). No indels were detected, 96 substitutions occurred with 15 amino acid changes when outgroups were included (Table 3), and 31 substitutions with 8 amino acid changes when excluded. The average base composition (outgroups included) was A: 27.0; C: 28.0; G: 16.3; T: 28.6. This strong bias against guanine is typical of mitochondrial but not nuclear genes (see Desjardins and Morais, '90). The transitions/transversions ratio (ti:tv) was 3.8 with the outgroups and 5.2 without the outgroups. The sequence analysis of the two *C. chalcides* individuals from Sardinia revealed a close affinity to Tunisian haplotypes. In particular, the two specimens had the same sequence haplotype (C5) that differed for only one substitution from haplotype C10 (Tunisia) (Table 3). Distances between haplotypes, calculated following the K2P method, ranged from 0.3% to 5.8% without the outgroups and from 0.3% to 22.2% including the outgroups.

The phylogenetic relationships among *C. chalcides* haplotypes were inferred using combined data set of RFLP and sequence data. It provided a total of 81 parsimony informative characters: 47 from RFLP and 34 from sequence data. The Wagner parsimony analysis suggests the monophyly of *C. chalcides* with 100% bootstrap support.

Within this group the parsimony analysis revealed three main clades. Clade I contains the haplotypes from Tunisian populations; clade II the Sicilian haplotypes, and clade III the haplotypes from the Italian peninsula. All clades are statistically well supported (96%, 99% and 94%, respectively) even though the relationships among them are unresolved (bootstrap values <50%) (Fig. 2).

The relative rate test indicated no significant heterogeneity (both K_a and K_s nonsignificant) in cyt *b* substitution rates among lineages. The splitting times between the three clades, estimated from net nucleotide divergence values, revealed that the genetic divergence between them is referable to Early Pleistocene. The separation between the Italian peninsula and Tunisia and between Sicily and Tunisia is almost contemporary, dating back to about 1.8 Ma; the divergence between Italian peninsula and Sicily dates back to 1.1 Ma. Finally, the divergence between the *C. chalcides* and *C. striatus* lineages seems to have occurred about 4.5 Ma in the Early Pliocene.

The RFLP composite haplotypes observed in the sample showed a clear geographical distribution congruent with the three main clades revealed by the Wagner parsimony analysis. Haplotypes 1–5 occurred in the Italian peninsula (H1, H2, H3) and Sicily (H4, H5) while the haplotypes H6–H16 were represented in the Tunisian populations. The genetic diversity was sensibly different between Italy and Tunisia. The seven populations of the Italian peninsula were monomorphic with five of them sharing the same haplotype (H1: IP1, IP2, IP3, IP4, IP5), while the population from Sicily presented two haplotypes (H4 and H5) with one of them represented in only two individuals. On the contrary, the Tunisian population showed a higher diversity with 11 haplotypes found in three local samples. The lower genetic diversity of Italian populations emerged also by the values of haplotype and nucleotide diversity of the single populations which were different from zero only in the Sicilian population ($h = 0.3368 \pm 0.1098$, $\pi = 0.0010$) (Table 4). Pooling the samples according to their geographical origin (Italian peninsula: $h = 0.2652 \pm 0.0389$, $\pi = 0.0022$; Sicily: $h = 0.3368 \pm 0.1098$, $\pi = 0.0010$; Tunisia: $h = 0.8365 \pm 0.0171$, $\pi = 0.0112$) further corroborated the above findings.

The genetic structure of populations was inferred by AMOVA analysis performed on 11 populations and three groups of populations. When this test was conducted with the pair-wise difference method, the results showed that the

TABLE 3. The 96 variable positions of cyt b sequences

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                                  1 1 2 2 3 3 3 3 4 4 4 5 5 5 5 5 6 6 6 6 7 7 9 9 9 9 1 1 1 1 1
                                  6 8 1 8 3 4 6 7 3 5 8 1 2 4 5 6 0 3 6 9 2 5 0 3 6 9 0 0 0 1 1
PCR-RFLP haplotype  Cyt b haplotype                                                  3 5 8 1 2
-------------------------------------------------------------------------------------------------
H1, 2               C1   A T C G C G C T A T T G C C G C A G T T A C G C T A A C T A A
H3                  C2   · · · · · · · · · · · · · · · · · · · · · · · · · · · · · · ·
H4                  C3   · · A · · A · · · · · · · · · · · · · · · · · · · · C · ·
H5                  C4   · · A · · A T · · · · · · · · · · · · · · · · · · · C · ·
—                   C5   · C · · · T · · · · · · · · · · G · · · · · A · · · T · · ·
H6, 11              C6   G C · · · T · · · · · · · · · · G · · · · · A · · · T · · ·
H7, 8, 10, 12, 13, 14  C7  · C · · · T · · · · · · · · · · G · · · · · A · · · T · · ·
H9                  C8   · C · · · T · · · C G · C G G · · · · · A · · · T · · ·
H15                 C9   · C · · · · · · · · · · · · · · G · · · · · A · · · T · · ·
H16                 C10  · C · · · T · · · · · · · · · · G · · · · · A · · · T · · ·
H17                 C11  · C · A T · · A · · · C · · · · · · · · · C · T · · · C T G · C · ·
H18                 C12  · C T A · A · · G C C A · A · · · A C C C · A T C C · · C T G
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                                  1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2
                                  1 1 2 2 2 2 3 4 5 5 6 6 6 6 7 9 9 0 0 0 1 1 1 2 3 3 4 4 4 4 5 6
PCR-RFLP haplotype  Cyt b haplotype  5 6 0 1 6 9 8 7 0 3 2 3 5 8 7 2 8 1 7 0 3 9 2 4 7 0 3 6 9 5 1
-------------------------------------------------------------------------------------------------
                         G T A G C G C A C T C A T C T C T A T A C G T T T T T G T G C
                         · · · · · · · · · · · · · · · · · · · · · · · · · · · · · A ·
H4                  C3   A · · · · · · · · · · · · · · · · · · · C · T · · · · C · · A ·
H5                  C4   A · · · · · · · · · · · · · · · · · · · C · T · · · · C · · A ·
—                   C5   A · · · · · T · · · · · · · · · · · · · · · · · · · C · · C A · A ·
H6, 11              C6   A · · · · · T · · · · · · · · · · · · · · · · · · · C · · C A · A ·
H7, 8, 10, 12, 13, 14  C7  A · · · · · T · · · · · · · · · · · · · · · · · · · C · · C A · A ·
H9                  C8   A · · · · · T · · · · · · · · · · · · · · · · · · · C · · C A · A ·
H15                 C9   A · · · · · T · · · · · · · · · · · · · · · · · · · C · · C A · A ·
H16                 C10  A · · · · · T · · · · · · · · · · · · · · · · · · · C · · C A · A ·
H17                 C11  · C · · T C · · T C T · C · · · C · C · · A C · C · C A A A ·
H18                 C12  · C G A T T T G · C · G C A C T C G G · A G A C C C C A A T
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                                  2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
                                  6 6 7 7 7 8 8 9 9 9 0 0 0 1 1 2 3 3 4 4 4 5 5 5 5 6 7 7 7 8 8 8 8 9
PCR-RFLP haplotype  Cyt b haplotype  4 9 0 3 9 5 8 1 2 4 0 3 6 2 5 4 0 9 0 5 6 1 2 4 7 9 2 5 8 1 4 5 7 0
-------------------------------------------------------------------------------------------------
                         T T T C C C A C T A C C A G C T C G G C C C T A C T T T C C C C G A
                         · · · · · · · · C · · · · · · · · · · · · · · · · · · · · · · · · ·
H4                  C3   · · · T · · · · · · · · · · · · · · · · · · · · · · · · · · · · T C
H5                  C4   · · · T · · · · C · · · · · · · · · · · · · · · · · · · · · · · T C
—                   C5   · C · · · · · · · · · · · · · · · · A · · · · · · · · · · · · · T A C
H6, 11              C6   · · · · T · · · · · · · · · A · · · · A · · · · · · · · · · · · A C
H7, 8, 10, 12, 13, 14  C7  · · · · · · · · · · · · · · · · · T · A · · · · · · · · · · · · A C
H9                  C8   · · · · T · · · · · · · · · A · · · · A · · · · · · · · · · · · A C
H15                 C9   · · · · · · · · · · · · · · · · · · · A · · · · · · · · · · · · A C
H16                 C10  · · · · · · · · · · · · · · · · · · · A · · · · · · · · · · · · T A C
H17                 C11  C · C · · A · · · G · · · · · · · T · A · · · C G T C · · T · A · · C
H18                 C12  C · C · A G G · · T A C · T C T A · T T T · · · · · C C A T T · · ·
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First two columns, correspondence between PCR-RFLP and cyt *b* haplotypes. Bold columns indicate the variable positions that were not utilized for the RFLP/sequence combined analysis as three character states were present. Position 269 was also excluded since the ND segments of the individuals bearing this cyt *b* sequence were not successfully PCR amplified, and therefore not included in the combined analysis

majority of the total molecular variance (88.87%, $P < 0.001$, $\Phi_{CT} = 0.88868$) was distributed among groups suggesting a pronounced geographical structure of the *C. chalcides* populations. Lower percentages of the total molecular variance were distributed among populations within groups (7.64%, $P < 0.001$, $\Phi_{'s} = 0.68671$) and within populations (3.49%, $P < 0.001$, $\Phi_{ST} = 0.96512$). The molecular variance among groups (39.62%, $P < 0.01$, $F_{CT} = 0.39619$) sharply decreased when
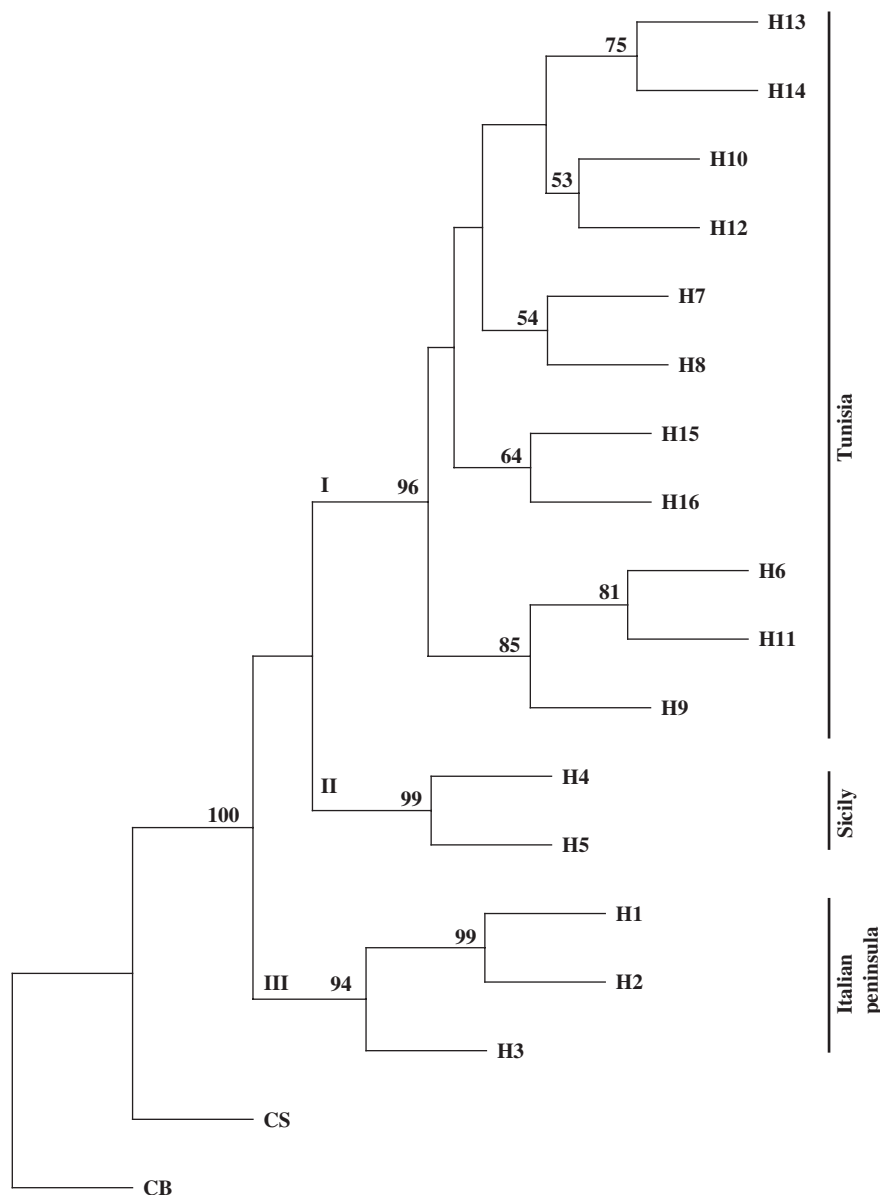
Fig. 2. Majority rule consensus tree based on the Wagner parsimony analysis of the RFLP/sequence combined data set showing the phylogenetic relationships among the 16 *Chalcides chalcides* haplotypes. *Chalcides boulengeri* (CB) and *C. striatus* (CS) were used as outgroups. Roman numerals indicate the three main clades, while the vertical bars highlight the geographic origin of the haplotypes. Bootstrap values higher than 50% are indicated at nodes.

the analysis was performed on the basis of haplotype frequencies, even though it remained the highest of the three variance components. This change can be attributed to the high haplotype diversity of the group formed by the Tunisian populations and to the high heterogeneity of these populations (only one of the 11 haplotypes shared between TU1 and TU2). Subsequently, the percentage of variation among populations within groups (38.48%, $P < 0.001$, $F_{sc} = 0.63721$) and within populations (21.91%, $P < 0.001$, $F_{ST} = 0.78094$) increased (Table 5).

The extent of geographic heterogeneity in the frequency distribution of haplotypes among populations, tested with Monte Carlo simulation, showed significant heterogeneity in all the pairwise comparisons ($P < 0.001$), with the exception of the comparisons involving the five populations of central Italy (IP1, IP2, IP3, IP4, IP5), which were all nonsignificant.

TABLE 4. Haplotype (h ± standard deviation) and nucleotide (π) diversity and sample size (n) in the populations studied

Populations	h ± s.d.	π	n
IP1	0.0000 ± 0.0000	0.0000	18
IP2	0.0000 ± 0.0000	0.0000	15
IP3	0.0000 ± 0.0000	0.0000	15
IP4	0.0000 ± 0.0000	0.0000	18
IP5	0.0000 ± 0.0000	0.0000	20
IP6	0.0000 ± 0.0000	0.0000	8
IP7	0.0000 ± 0.0000	0.0000	7
SI	0.3368 ± 0.1098	0.0010	10
TU1	0.8165 ± 0.0393	0.0063	20
TU2	0.6985 ± 0.0489	0.0051	20
TU3	0.3680 ± 0.1002	0.0019	17

TABLE 5. AMOVA analysis performed on the 11 populations of Chalcides chalcides

Source of variation	Variance components	Percentage of variation	F-statistics
Among groups[1]	9.72685	88.87	0.88868*** (ϕ_{CT})
	0.18306	39.62	0.39619** (F_{CT})
Among populations within groups	0.83674	7.64	0.68671*** (ϕ_{SC})
	0.17778	38.48	0.63721*** (F_{SC})
Within populations	0.38173	3.49	0.96512*** (ϕ_{ST})
	0.10122	21.91	0.78094*** (F_{ST})

The test was carried out on the basis of both pair-wise differences between haplotypes (first line of each hierarchical level) and haplotype frequencies (second line of each hierarchical level).
[1]The populations were grouped as follows: Italian peninsula, Sicily and Tunisia.

The observed mismatch distribution in *C. chalcides* revealed different patterns between peninsular Italy and Tunisia. The results showed a distribution of differences for the Italian peninsula not statistically different from the one following a sudden expansion model ($P_{SSD} = 0.14$), therefore suggesting a recent sudden expansion or a bottleneck. On the contrary, the mismatch distribution of the Tunisian three-toed skinks is statistically different from that typical of a sudden expansion ($P_{SSD} = 0.02$), indicating the presence of a stable or slowly declining population in north Africa.

DISCUSSION

Maghreb can be regarded as a ''hot spot'' for the biodiversity of the *C. chalcides* complex. In fact, three of the five species of this complex live exclusively in this area of North Africa (*C.*

pseudostriatus, *C. minutus* and *C. mertensi*), one (*C. chalcides*) occurs in both Maghreb and insular and peninsular Italy, and only one (*C. striatus*) is exclusive to Europe. The distribution of these species seems to indicate that Maghreb was the area where this species complex probably differentiated owing to the geological and climatic events that have affected this part of North Africa during the last 6 million years (see Caputo, '93). Therefore, it is reasonable to suppose that the presence of two species in Europe is the result of colonization events that started from North Africa, and not the opposite.

The results of the present work indicate that the colonization of Europe from the *C. chalcides* complex occurred in two different periods (Early Pliocene and Early Pleistocene) and that geological events may have played a major role in this process. In particular, our estimate of the splitting time between *C. striatus* and *C. chalcides* lineages indicates that their separation took place about 4.5 Ma (Early Pliocene). The event that brought about the colonization of Iberian peninsula by the ancestor of *C. striatus* was the closing of the Strait of Gibraltar (about 6 Ma), that allowed extensive faunal exchange between North Africa and Europe (e.g., Busack, '86; Harris et al., 2004). Its subsequent re-opening, in the Early Pliocene (about 5 Ma) (Rosenbaum et al., 2002), led to the allopatric divergence of *C. striatus*, scenario also supported by allozyme data (Caputo, '93). Another example of Pliocene colonization of Europe by skinks comes from the Iberian endemism *Chalcides bedriagai* whose ancestor separated from a small-bodied Chalcides of Morocco with the re-opening of the Strait of Gibraltar about 5 Ma (Caputo et al., '99).

As for the separation between Tunisian and Sicilian lineages of *C. chalcides*, the estimate of splitting time is about 1.8 Ma. This suggests that the colonization of this island from the coasts of North Africa took place during Early Pleistocene, implying connections between Africa and Sicily in this period, as also proposed by anthropologists and paleontologists (see Vaufrey, '29; Kelling and Stanley, '72; Alimen, '75). Recently, the possible existence of such connections was re-considered by Bonfiglio and Piperno ('96) and Bonfiglio et al. (2002), hypothesizing a possible African provenance for both *Elephas falconeri* and human populations through the Sicilian–Tunisian isthmus, as a result of tectonic controlled changes of sea depth during Early Pleistocene. However, without a ''land bridge'' between Sicily and

Tunisia, the colonization of southern Italy may have occurred by transport on natural rafts of vegetation. In fact, this type of colonization seems to be quite common in reptiles like skinks of the genus *Mabuya* (Arnold, '76; Mausfeld et al., 2000; Carranza et al., 2001; Carranza and Arnold, 2003), chameleons (Raxworthy et al., 2002) and geckos (Carranza et al., 2000), even across long distances. This type of colonization might have been relatively easy for *C. chalcides*, considering the narrowness of the Sicily channel (about 60 km vs. the present 160 km) due to the decreases in the sea level during the Pleistocene glaciations (Antonioli et al., 2004).

According to our estimates, the colonization of the Italian peninsula has happened around 1.1 Ma, during Early Pleistocene. The dispersal to the mainland might have occurred during the temporary connections between Sicily and southern Italy. These connections were more frequent and extensive during the Late Middle Pleistocene and Late Pleistocene (see Bonfiglio et al., 2002), but evidence from the Quaternary faunal assemblages of Sicily suggests that connections between this island and the peninsula occurred also in the early part of this period (see Villa, 2001).

Another point worth discussing is the pronounced similarity between cyt *b* sequences from Sardinia and Tunisia. This corroborates the hypothesis of colonization of this island in historical times, by passive transportation, from North African populations, as already suggested by Caputo ('93) on the basis of the strong allozyme and morphological similarity between *C. chalcides* from Sardinia and North Africa. Considering the remarkable morphological and genetic homogeneity of Sardinian populations indicating founder effect, Caputo ('93) concluded that the colonization started from North Africa. The hypothesis of passive transportation is strengthened by evidence from other scincid lizards, such as the presence of *Chalcides ocellatus* in the ex royal garden of Portici (Naples, Italy) as explained by passive transportation of individuals from Sicily in the clods of soil of the trees used for the preparation of this garden in XVIII century (Monticelli, 1903; Caputo et al., '97). The passive transportation finds further support from data on mammals (Cosson et al.,'05; Vigne and Alcover, '85; Vogel et al., '86).

The results of the RFLP analysis performed on 168 specimens of *C. chalcides* underline the role played by the Quaternary climatic oscillations in shaping the genetic structure of this species. Our

data revealed a low level of genetic variability in the populations of Italian peninsula and in the one of Sicily as compared to Tunisia, as already observed on the basis of allozyme data (Caputo, '93). A possible explanation for the low variability of the Italian populations are the effects of glaciations that in Mediterranean Europe were more drastic than in North Africa (see Suc, '84, '89). Furthermore, the low vagility and the temperature dependence of these reptiles, coupled with strong ecological specialization, may have made them particularly vulnerable to periods of intense climatic deterioration. In particular, during the Würmian, with glaciers that occurred along the Apennines up to Pollino Mount (Calabria) (Cremaschi, 2004; Giraudi, 2004), the habitat suitable for these skinks was limited to southern Italy (see Cattani, '92; Ravazzi and Aceti, 2004; Ravazzi et al., 2004) that must have represented the glacial refuge of Italian populations. Interestingly, the existence of European refugia south of 40°N was proposed also for other reptile species, like *Emys orbicularis* (Lenk et al., '99).

The populations of the three-toed skink probably went through many events of contractions/expansions of range during the Quaternary climatic fluctuations, with the extinction of northern populations during the glacials and a northward expansion during the periods of climatic improvement. The large area of homozygosity observed in central Italy is likely to be related to the colonization process that took place with the climatic improvement, and that probably implied successive bottlenecks (see Hewitt, '96). In fact, a growing number of studies show that areas colonized after the glaciations are characterized by a low genetic diversity (Hewitt, '96), as demonstrated by a study performed on 41 North American fish species (Bernatchez and Wilson, '98). This large area of homozygosity could be explained by a leptokurtic mode of expansion. According to this model, the colonization would start from populations located at the northern limit of their refuge into large areas of suitable territory; this leading edge expansion would probably be by long-distance disperser that set up colonies and rapidly expanded to fill the area before the others arrived. This process would be repeated many times over a colonizing route, and these founding events would lead to the loss of alleles and to homozygosity (see Hewitt, '96, 2000). Ibrahim et al. ('96) showed, by computer simulations, that such large areas of homozygosity are indeed produced by leptokurtic dispersal

rather than dispersal achieved by normal or stepping-stone modes (see also Fraser et al., 2001). A recent sudden expansion for the three-toed skink populations of the Italian peninsula seems to be corroborated by the result of the mismatch analysis distribution that revealed demographic characteristics typical of expanding populations ($P_{SSD} = 0.14$). In Tunisia, where the effects of glaciations were probably less drastic than in Italy, the mismatch analysis showed the presence of a stable population ($P_{SSD} = 0.02$), seemingly not affected by past bottlenecks due to contractions/expansions of its range.

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