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Genetic relationships of the western Mediterranean painted frogs based on allozymes and mitochondrial markers: evolutionary and taxonomic inferences (Amphibia, Anura, Discoglossidae)

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Allozymes and sequencing of mitochondrial cytochrome *b* (cyt *b*) and 12S genes were used to reconstruct the genetic structure and phylogenetic relationships of all *Discoglossus* taxa described so far (except the probably extinct *D. nigriventer*). This is the first time that a comprehensive study on the *Discoglossus* painted frogs has used nuclear and mitochondrial markers, evidencing a discordant pattern between the two datasets. Comparison of these discrepancies suggests a role of stochastic sorting of ancestral polymorphisms, possibly associated with male-biased dispersal and present or past secondary contact. The genetic relationships between taxa with intermediate levels of divergence were well defined by allozyme data, but showed short internodes and low bootstrap values for mitochondrial data, suggesting a rapid radiation of their lineages during the Messinian Lago Mare phase. The results provide information about the taxonomic status of *D. galganoi* and *D. jeanneae*, considered as subspecies, and indicate *D. pictus* as nonmonophyletic, confirming *D. scovazzi* as a distinct species. *Biological Journal of the Linnean Society*, 2006, **87**, 515–536.

ADDITIONAL KEYWORDS: ancestral polymorphism sorting – genetic structure – molecular phylogeny – mtDNA sequencing – palaeogeography.

INTRODUCTION

Biogeographical patterns of terrestrial fauna in the western Mediterranean region are still under discussion because of the complexity of the palaeogeographical processes influencing possible dispersal and vicariance events leading to speciation. Common patterns of dispersal and isolation have been proposed for different animal taxa, taking into account main geological events, such as the Corsican–Sardinian microplate disjunction from the mainland (27–30 Mya; Alvarez, 1972; Cocuzza, 1975), the Messinian salinity crisis with the complete isolation of the Mediterranean basin from the Atlantic (5.59–5.33 Mya, Hsü *et al*., 1977; Krijgsman *et al*., 1999), and the climatic oscillations from the early Pliocene to the Pleistocene. The study of circum-Mediterranean taxa has, however, proposed a role for some less well-known events, because some taxa from the Moroccan Rif–Atlas region showed higher affinities with Iberian than they did with Algerian and Tunisian Tell–Atlas taxa (Harris *et al*., 2002). Molecular studies have shown a common pattern of geographical subdivision between many Maghreb amphibians and reptiles just around the Moulouya River basin, separating East and West Maghreb, and suggested it as a relevant barrier with perhaps the same importance as the Gibraltar Straits (Lanza *et al*., 1986; Mateo, López-Jurado & Guillaume, 1996; Álvarez *et al*., 2000; Harris *et al*., 2002).

In this perspective the study of the archaeobatrachian *Discoglossus* painted frogs (Anura, Archeobatrachia, Discoglossidae) appears relevant because this genus has a relict range located in the western district

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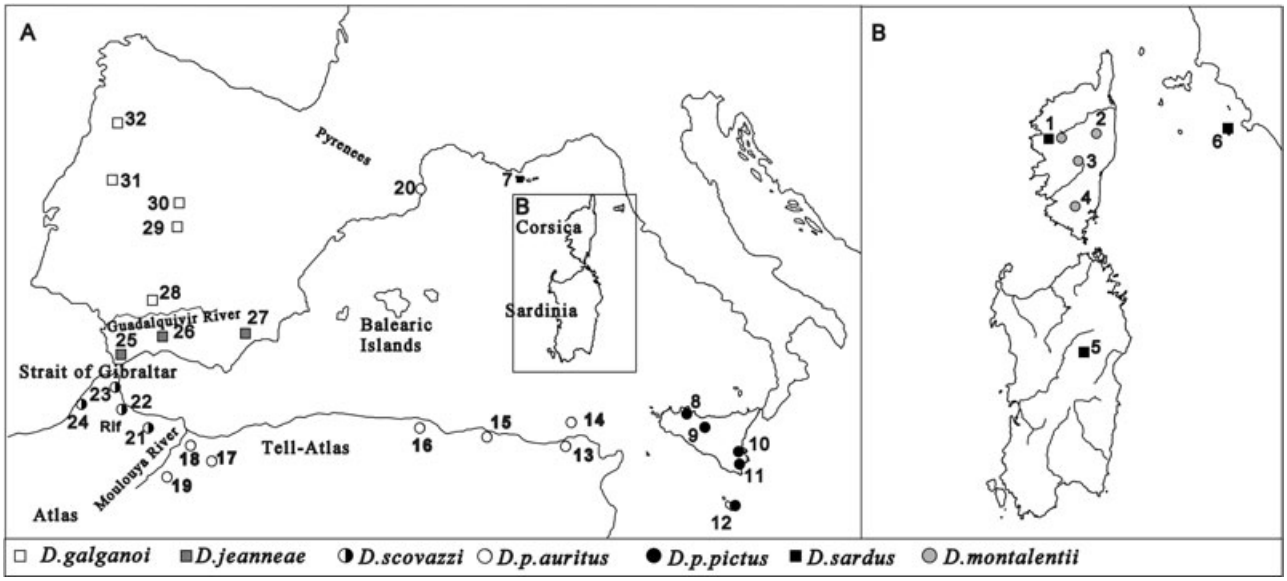


Figure 1. Collecting localities of *Discoglossus* samples. Population numbers according to those reported in Table 1.

of the Mediterranean Basin. An ancient, wider distribution is witnessed by fossil records of Germany (Oligocene–Miocene), central Italy, the Balearic Islands and Crete (Plio-Pleistocene) (Sanchiz, 1977; Kotsakis, 1981, 1982a, b; Alcover, Sanders & Sanchiz, 1984). Today, *Discoglossus*survives in the Iberian Peninsula, Maghreb and the islands of Sicily, Malta, Sardinia, Corsica, the Tuscan Archipelago, Argentario (a fossil island, now a promontory) and the Hyères Islands (Fig. 1). The eastern Mediterranean taxon *D. nigriventer*, from Syria, Palestine and Israel, has not been detected since 1955 and is supposed to be extinct (Werner, 1988).

The different degrees of genetic divergence found between taxa led various authors to propose differing taxonomic arrangements. Consequently, conflicting biogegraphic scenarios have been proposed regarding the origin of the extant *Discoglossus* taxa. Taxonomy has changed according to the use of morphological, biochemical or molecular approaches. Morphological characters identified *D. pictus* (Otth, 1837) from Sicily, Malta and Gozo, *D. sardus* (Tschudi, 1837) from Sardinia, Corsica, the Tuscan Archipelago and the Hyères Islands, *D. scovazzi* (Camerano, 1878) from Morocco, and *D. auritus* (Heron-Royer, 1888a, b) from Algeria and Tunisia. Allozyme studies identified *D. montalentii* (Lanza *et al*., 1984) from Corsica, and *D. galganoi* (Capula *et al*., 1985) and *D. jeanneae* (Busack, 1986) from the Iberian Peninsula. Controversy surrounds the Iberian and Maghrebian plus Sicilian taxa. According to Busack (1986) and Garcia-Paris & Jockusch (1999), *D. jeanneae* is a distinct species, while Lanza *et al*. (1986) considered it a subspecies of *D. galganoi.* The Sicilian and Maghrebian *D. pictus*, *D. scovazzi* and *D. auritus* have been recognized as distinct species or subspecies of *D. pictus s.l.* by different authors. Nascetti *et al*. (1986) included only two taxa within *D. pictus s.l. D.p. pictus* (comprising *auritus*) and *D.p. scovazzi*, with a likely subspecific rank. Gasc *et al*. (1997) reported three subspecies in their Atlas: *D. p. pictus*, *D. p. auritus* and *D. p. scovazzi*. More recently, Garcia-Paris & Jockusch (1999) and Fromhage, Vences & Veith (2004) questioned the monophyly of *D. pictus s.l.* using mitochondrial markers, recognizing the Moroccan *D. scovazzi* and *D. pictus* from Malta and Tunisia as distinct species. The up-to-date *Discoglossus* taxonomy is reported in Figure 1.

Concerning the palaeogeographical scenarios, quite different hypotheses have been proposed based on nuclear (allozymes; Lanza *et al*., 1986) or mitochondrial (partial 12S and 16S genes; Fromhage *et al*., 2004) data. According to Lanza *et al*. (1986), *D. montalentii* is considered a palaeoendemism, inhabiting the Sardinian–Corsican plate since the salinity crisis of the Messinian epoch (around 5 Mya) or even since the time of its disjunction from the mainland (27–30 Mya). The other western species were all regarded as being of recent origin and Plio-Pleistocenic glaciations were suggested as the palaeogeographical events promoting divergence. Fromhage *et al*. (2004) proposed a quite different scenario: *D. montalentii* originated after the separation of Corsica from Sardinia (20–15 Mya), and its sister taxon colonized the mainland from Sardinia via a land bridge (11.5–6 Mya). Two different vicariance events

were considered to explain the other splits: Moroccan *D. scovazzi* separated from Iberian *D. galganoi* during the fragmentation of the Betic region (12–6 Mya), and the divergence of *D. pictus* from *D. sardus* was because of the separation of the Calabro-Peloritan massif from Sardinia (8.6–7.6 Mya).

Evolutionary and phylogenetic studies on *Discoglossus* have been based so far on different markers (allozymes or mtDNA sequences), leading to conflicting scenarios and confirming the necessity of the simultaneous use of several independent markers to depict the evolutionary history of species (Monsen & Blouin, 2003; Rokas *et al*., 2003; Ballard & Whitlock, 2004).

In this study, 33 populations of *Discoglossus*were analysed genetically, 31 by means of allozymes and 25 by mitochondrial markers (partial sequences of cytochrome *b* (cyt *b*) and 12S genes). This allowed us to compare the degree of genetic differentiation within and among the *Discoglossus* taxa, and to test specifically the monophyly of the polytypic taxon *D. pictus* from Maghreb and Sicily, using a likelihood-based test and parametric bootstrapping approach (Ruedi, Auberson & Savolainen, 1998; Emerson, Ibrahim & Hewitt, 2001; Rees *et al*., 2001). A biogeographical scenario was inferred, reconciling the contrasting pattern that emerged between mitochondrial and nuclear markers.

MATERIAL AND METHODS

POPULATIONS SAMPLED

The collecting sites of the *Discoglossus* population samples studied are listed in Table 1 and shown in

Table 1. Collecting sites of *Discoglossus* samples, listed with their identification code and the number of specimens studied using different markers: cytochrome b (N_{cytb}), 12S (N_{12s}) and allozymes (N_{all})

Taxon	Site code	Collection locality	N_{cytb}	N_{12s}	N_{all}
D. montalentii	m1	Evisa (Corsica, France)	1	1	5
	2	Stazzona (Corsica, France)	1	2	17
	3	Vizzavona (Corsica, France)	2	2	12
	4	Ospedale (Corsica, France)	–	–	8
D. sardus	s1	Evisa (Corsica, France)	1	2	13
	5	Nuoro (Sardinia,Italy)	2	2	33
	6	Giglio Isl. (Tuscan Archipelago, Italy)	–	2	8
	7	Port Cros (Hyères Isl., France)	–	2	13
D. pictus pictus	8	Palermo (Sicily, Italy)	3	2	14
	9	Piana degli albanesi (Sicily, Italy)	1	1	7
	10	Ciane (Sicily, Italy)	–	–	12
	11	Noto (Sicily, Italy)	2	–	6
	12	Malta	1	1	6
D. p. auritus	13	Tabarca (Tunisia)	3	3	8
	14	Galite Island (Tunisia)	1	2	4
	15	El Milia (Algeria)	1	2	–
	16	Akfadou (Algeria)	2	2	–
	17	Sebdou (Algeria)	1	2	17
	18	Taforalt (Morocco)	2	3	13
	19	Debdou (Morocco)	2	2	7
	20	Banyuls sur Mer (France)	1	2	17
D. scovazzi	21	MatMata (Morocco)	–	–	5
	22	Oued Laou (Morocco)	1	3	9
	23	Tetouan (Morocco)	–	–	9
	24	Kenitra (Morocco)	3	3	5
D. jeanneae	25	Facinas (Spain)	–	–	15
	26	Ronda (Spain)	3	3	16
	27	Pico Veleta (Sierra Nevada, Spain)	–	–	13
	28	Between Lora del Rio and Puebla de Los Infantes (Sierra Morena, Spain)	7	7	7
D. galganoi	29	Talavera de la Reina (Spain)	–	2	5
	30	Arenas de San Pedro (Sierra de Gredos, Spain)	8	2	17
	31	Beira Baixa (Serra da Guardunha, Portugal)	–	–	5
	32	S. João de Campo (Serra do Geres, Portugal)	–	–	7

Figure 1. A total of 337 *Discoglossus* specimens, collected from 1984 to 2000, were analysed genetically.

Allozymes

Standard horizontal starch gel electrophoresis was performed on leg muscle tissue. The electrophoretic techniques used were those reported in Capula *et al.* (1985) and in Lanza *et al.* (1984, 1986). The 26 putative loci studied were: glycerol-3-phosphate dehydrogenase (*G3pdh;* EC 1.1.1.8), lactate dehydrogenase (*Ldh-1, Ldh-2;* EC 1.1.1.27), malate dehydrogenase (NAD^+) (*Mdh-1, Mdh-2*; EC 1.1.1.37), malate dehydrogenase (NADP+) (*Mdhp-1, Mdhp-2*; EC 1.1.1.40), isocitrate dehydrogenase (*Icdh-1, Icdh-2;* EC 1.1.1.42), phosphogluconate dehydrogenase (*6Pgdh*; EC 1.1.1.44), superoxide dismutase (*Sod-1, Sod-2*; EC 1.15.1.1), nucleoside phosphorylase (*Np*; EC 2.4.2.1), aspartate aminotransferase (*Aat-1, Aat-2*; EC 2.6.1.1), alanine aminotransferase (*Alat*; EC 2.6.1.2), hexokinase (*Hk*; EC 2.7.1.1), creatine kinase (*Ck*; EC 2.7.3.2), acid phosphatase (*Acph*; EC 3.1.3.2), adenosine deaminase (*Ada*; EC 3.5.4.4), carbonate dehydratase (*Ca;* EC 4.2.1.1), mannose-6-phosphate isomerase (*Mpi-1, Mpi-2*; EC 5.3.1.8), glucose-6-phosphate isomerase (*Gpi*; EC 5.3.1.9), and phosphoglucomutase (*Pgm-1, Pgm-2*: EC 5.4.2.2). In addition, an achromatic region (*Acr*) from an unknown enzyme and four unidentified nonenzymatic proteins (*Pt*) were analysed.

Statistical analysis of allozyme data

Allele frequencies and deviations from the Hardy–Weinberg equilibrium were calculated using BIOSYS–1 and BIOSYS–2 computer programs (Swofford & Selander, 1981, 1989). The genetic divergence between populations was estimated using Nei's (1972) genetic distances (D_{Nei}) as implemented in BIOSYS–1. Nei's distance modified by Hillis (1984; D^*_{Nei}) was calculated with POPDIST vers. 1.1.1 (Guldbrandtsen, Tomiuk & Loeschcke, 2000) in order to estimate times of divergence in accordance with Beerli, Hotz & Uzzell (1996).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from leg muscle using a slightly modified version of the Doyle & Doyle (1987) CTAB method (2% C-TAB, 1.4 M NaCl, 0.2% 2-beta-mercaptoethanol, 100 mM Tris-HCl pH 8.0, 20 mM EDTA) after overnight incubation in proteinase k.

A 384-bp fragment of cyt *b* was studied in order to compare our data with those of Garcia-Paris & Jockusch (1999). This fragment was amplified using PCR primers MVZ15-L (5′GAACTAATGGCCCACACW WTACGNAA3′, Moritz, Schneider & Wake, 1992) and H15149-H (5′AAACTGCAGCCCCTCAGAAT GATATTTGTCCTCA3′, Kocher *et al.*, 1989). The small ribosomal 12S gene was amplified using primers 12SZ–L (5′AAAGGTTTGGTCCTAGCCTT3′) and 12SF–H (5′CTTGGCTCGTAGTTCCCTGGCG3′) (Goebel, Donneley & Atz, 1999) for the first part of the region (about 400 bp) and 12sA–L (5′AAACTGG GATTAGATACCCCACTAT3′, Palumbi *et al.*, 1991) plus tRNAval–H (5′GGTGTAAGCGARAGGCTTTKG TTAAG3′, Goebel *et al.*, 1999) for the second part (about 400 bp). The complete secondary structure of the ribosomal 12S gene was compared for all the taxa to obtain a reliable alignment. Only the variable first 400-bp region was used for the phylogenetic analysis.

PCR conditions and the PCR thermal program were the same for both cyt *b* and 12S. PCR reactions were performed in a volume of 50 μL, containing 2.5 mM $MgCl_2$, 1× buffer, 0.2 μM each primer, 0.2 mM each dNTP, 0.5 U Promega Taq Polymerase and 2 μL target DNA (1 : 20 dilution of 20 ng/mL DNA extraction). The temperature profile, after 2 min denaturation at 96 °C, consisted of 40 cycles of: denaturation at 96 °C for 1 min, annealing at 50 °C for 45 s and extension at 72 °C for 1 min. This was followed by a final extension of 10 min at 72 °C. The purified PCR products were double-sequenced using the same six PCR primers on an ABI Prism automated sequencer (Perkin Elmer, Applied Biosystems) and the sequences were aligned using CLUSTALX (Thompson *et al.*, 1997).

All the haplotypes found were deposited in GenBank (Accession numbers AY347414–347438 for cyt *b* and AY347439–AY347473 for 12S).

Outgroup

Xenopus laevis was used as the outgroup for both cyt *b* and 12S (sequences are available for the complete mitochondrial genome from EMBL, Accession number NC001573). *Alytes muletensis* and *A. obstetricans* (Anura, Discoglossidae) were also included in the cyt *b* analyses (Accession numbers AY341728 and AF128916).

Phylogenetic analysis

The percentage of polymorphic, variable and informative sites and Tajima's *D*-test for neutrality (Tajima, 1989) were computed using DnaSP version 3.53 (Rozas & Rozas, 1999). An exhaustive search for all trees in PAUP* 4.0b10 (Swofford, 2003) was performed to calculate the skewness (g1) of the tree-length distribution, in order to discriminate the phylogenetic signal from noise. A subset of sequences (one haplotype per taxa) was analysed in order to com-

pare our results with tables of *P*-values published by Hillis & Huelsenbeck (1992).

The average nucleotide frequencies and the genetic distances between clusters and taxa, with standard error values, were calculated using MEGA vers. 2.1 (Kumar *et al*., 2001). Sequence divergence was estimated according to the Kimura 2-parameter model (Kimura, 1980), in order to compare our data with those of Garcia-Paris & Jockusch (1999). Saturation plots of transitions and transversions against genetic distances were obtained using DAMBE vers. 4.1.19 (Xia & Xie, 2001). Phylogenetic analyses were performed using maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML) methods as implemented in PAUP* 4.0b10 (Swofford, 2003). Bootstrap resampling was performed on 1000 replicates. Unweighted MP trees were obtained using the heuristic search option with TBR branch swapping using random addition of sequences. In the MP analysis attempts were made both treating gaps as new states (as suggested by Giribet & Wheeler, 1999) and excluding them (according to Swofford *et al*., 1996).

For NJ and ML likelihood analyses, different models of nucleotide substitution were used: uncorrected, Jukes–Cantor, Kimura 2-parameter and a model estimated with a likelihood ratio test implemented in the software program Modeltest (Posada & Crandall, 1998). Phylogenetic analyses were conducted separately for each gene and tested subsequently for congruence (Huelsenbeck, Hillis & Jones, 1996), running HomPart test as implemented in PAUP*.

Testing competing phylogenetic hypotheses and molecular clock

Competing phylogenetic hypotheses between mitochondrial and allozyme data and between different tree topologies were evaluated using two different methods. The first used the Kishino–Hasegawa nonparametric likelihood ratio test (KH test; Kishino & Hasegawa, 1989) and the modified Shimodaira–Hasegawa test (SH test; Shimodaira & Hasegawa, 1999) to estimate the standard error and confidence interval for the differences in log-likelihoods between two a priori chosen tree topologies, as implemented in PAUP* (Swofford, 2003). The second used parametric bootstrapping to test the monophyly of a specific clade, following the approach of Ruedi *et al*. (1998), with a method based on MP instead of ML, to reduce computation time. The parametric bootstrapping involved three main steps. The first step was to estimate scores for MP trees both with and without constraints enforced (in our case, constraint 1 = monophyly of *D. pictus*; constraint 2 = monophyly of *D. pictus* plus *D. sardus*). The second involved the simulation of 100 datasets of sequences using Seq-Gen vers. 1.2.5 (Rambaut & Grassly, 1997), applying an appropriate nucleotide substitution model selected with Modeltest and with a NJ constrained tree (enforcing constraint 1 or 2). The third was a comparison of the observed differences between unconstrained and constrained topologies with a distribution of differences generated by simulation to the significant level of 0.05 (Ruedi *et al*., 1998; Emerson *et al*., 2001; Rees *et al*., 2001).

The molecular clock hypothesis was evaluated using a likelihood ratio test implemented in PAUP* 4.0b10 (Swofford, 2003). The hypothesis was satisfied if DNA substitutions followed a Poisson distribution with a constant mean rate in different lineages. The log-likelihood was calculated for ML trees using the model of substitution selected by Modeltest constrained with the null hypothesis of a molecular clock assuming that DNA substitution rates were equal in different lineages (Hypothesis H_0) and allowing for substitution rates to vary among lineages (Hypothesis H_1). The statistics $2\Lambda = \log H_1 - \log H_0$ could be compared with a χ^2 distribution with $(N - 2)$ degrees of freedom, where N is the number of sequences (Huelsenbeck & Rannala, 1997).

RESULTS

Allozyme data

There were 29 polymorphic loci out of the 31 scored; their allele frequencies are shown in the Appendix. The two loci *Hk* and *Pt-4* were monomorphic for the same allele in all samples studied. The genetic relationships among the populations and taxa considered are summarized in the UPGMA dendrogram of Figure 2. The most differentiated taxa were the Corsican *D. montalentii* and the Iberian *D. galganoi* and *D. jeanneae*, while *D. sardus* and *D. pictus s.l.* were genetically closest. The mean values of D_{Nei} between *D. montalentii* and the other taxa ranged from 0.91 to 1.06. The Iberian samples grouped into two subclusters, with a mean D_{Nei} of 0.05 (ranging from 0.02 to 0.08). Population 28 clustered with *D. jeanneae*, in spite of its location on the northern side of the Guadalquivir River, until now considered as the boundary between *D. jeanneae* and *D. galganoi* (Busack, 1986). *D. sardus* was genetically closer to *D. pictus s.l.* (average $D_{Nei} = 0.39$) than it was to the Iberian taxa (average $D_{Nei} = 0.73$). The samples of *D. pictus s.l.* grouped into two subclusters with a mean $D_{Nei} = 0.17$. The first included *D. pictus–auritus* samples, and the second included *D. scovazzi*. The Moulouya River basin is the boundary between these two population groups, showing two fully diagnostic loci (*Aat-1*, *Pt-5*) and another two (*6Pgdh*, *Mdh-1*) with strongly differentiated allele frequencies (see Appendix). Within the *D. pictus–auritus* group (mean $D_{Nei} = 0.04$), populations from south-eastern Sicily (samples 10, 11) and Malta

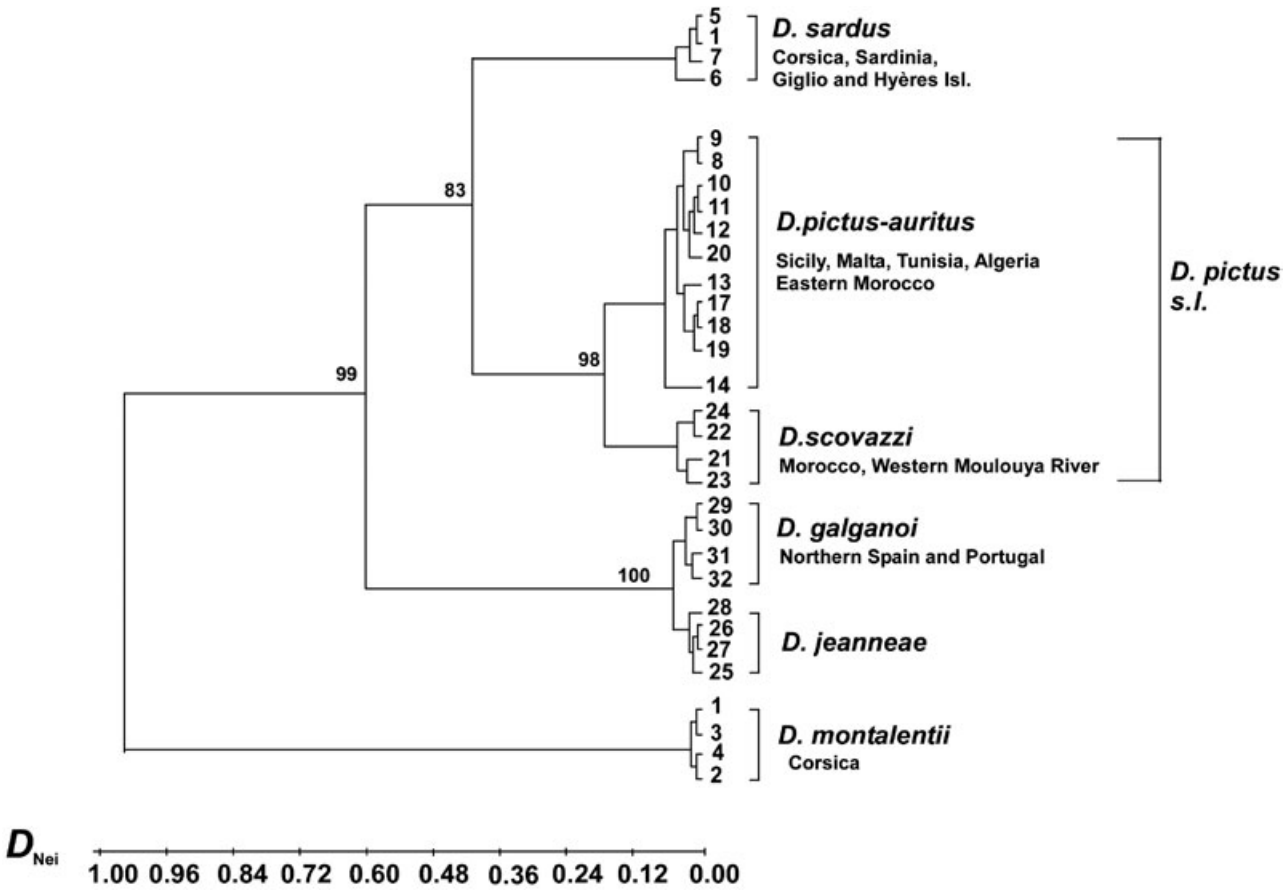


Figure 2. UPGMA dendrogram from Nei's (1972) distances. Bootstrap values are over 100 replicates. Population numbers are those reported in Table 1.

(sample 12) were remarkably homogeneous, as were those from western Algeria (sample 17) and eastern Morocco (samples 18, 19). The sample from Galite Island (sample 14, Tunisia) was the most differentiated ($D_{Nei} = 0.06$), likely as a result of a founder effect.

Haplotypes and nucleotide diversity

Cytochrome b

We obtained 49 cyt *b* sequences that were 384 bp long (128 codons), providing 25 haplotypes. The sequences matched with those available in NCBI (AF 128895–128916; Garcia-Paris & Jockusch, 1999). No insertions or deletions of bases were observed. There were 118 variable sites (representing 140 mutations) over the 384 nucleotides, resulting in 113 parsimony-informative sites (five singletons were observed). The empirical transition-to-transversion (T_i/T_v) ratio was 6.38. As expected from theoretical studies (Yang, 1996) most substitutions were in the third position (80% total substitutions), followed by the first position (13%), while only few substitutions modified the second position (1.6%), resulting in almost all substitutions being silent; we observed seven aminoacidic changes, four of which occurred in *D. montalentii*.

Tajima's *D*-test ($D = 0.54$) for mutation neutrality was statistically not significant ($P > 0.10$). The plot of transitions and transversions vs. the Kimura 2-parameter distances showed the beginning of saturation at values around 0.15–0.18.

To choose the substitution model that best described our data, sequences were analysed using Modeltest vers. 3.06 (Posada & Crandall, 1998). The HKY model (Hasegawa, Kishino & Yano, 1985) was selected, with a gamma shape of 0.19, a T_i/T_v ratio of 6.2 and base frequencies of A = 0.28, C = 0.30, G = 0.14, and T = 0.28.

12S

We obtained 55 sequences for a final alignment of 409 nucleotide positions, providing a total number of 28

haplotypes. There were 72 variable sites (81 mutations) for 58 parsimony-informative sites (14 singletons) and 13 indels. The empirical T_i/T_v ratio was 3.4. Tajima's *D*-test ($D = -0.034$) for mutation neutrality was not significant ($P > 0.10$). The saturation plot of transitions and transversions vs. Kimura 2-parameter distances showed a linear relationship and absence of saturation for this gene.

Modeltest (Posada & Crandall, 1998) selected the Tamura–Nei model with gamma shape parameter (TrN + G; Tamura & Nei, 1993). The gamma shape was 0.39 and base frequencies were: A = 0.32, C = 0.28, G = 0.17, and T = 0.30. Estimates of substitution rates were: A–C = 1, A–G = 3.46, A–T = 1, C–G = 1, C–T = 6.56, and G–T = 1.

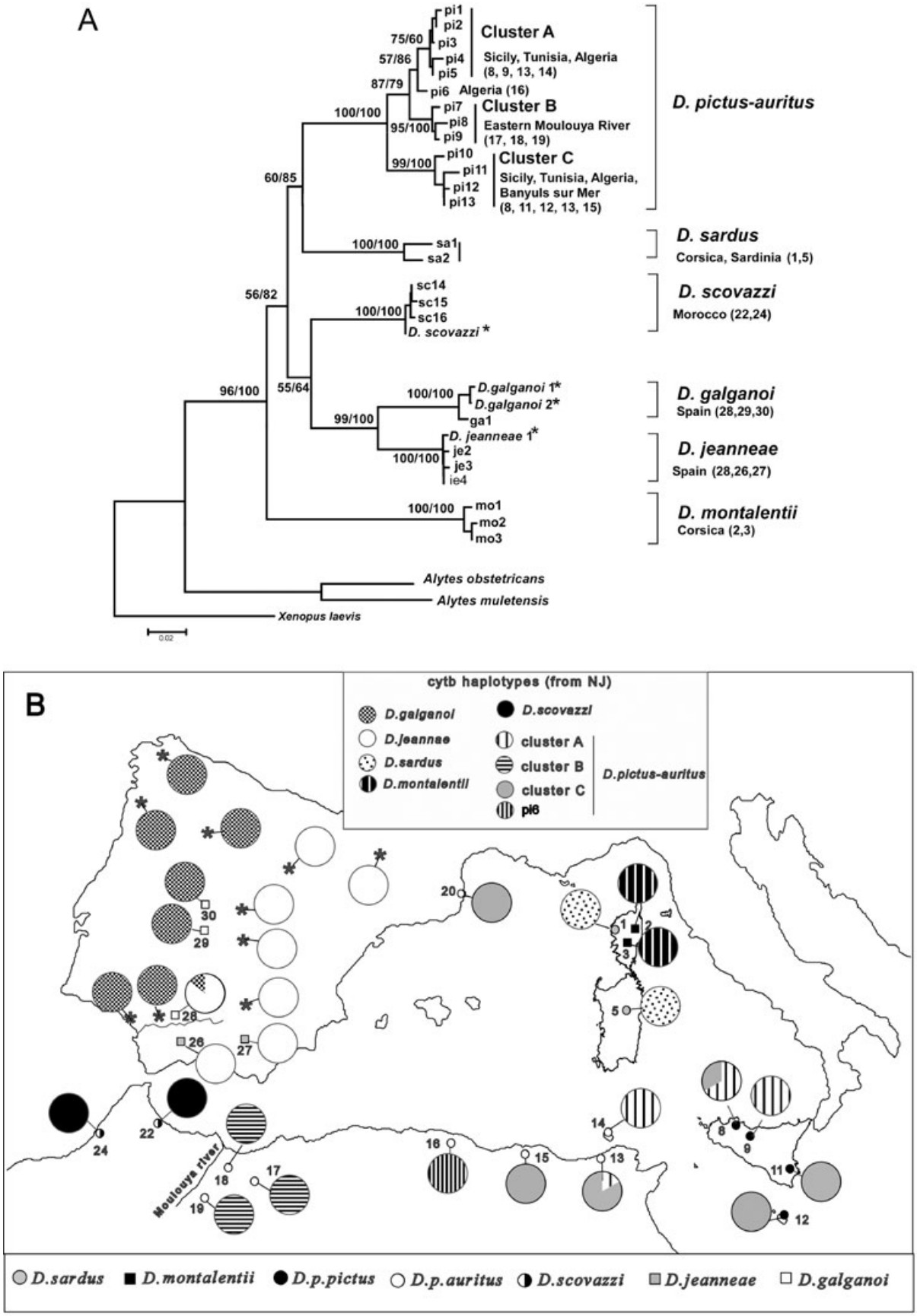
To obtain a reliable sequence alignment (available on request), the secondary structure of the whole 12S gene was considered. *Discoglossus* 12S sequences were aligned with those of *Bos taurus* (Springer & Douzery, 1996). The secondary and tertiary structure of ribosomal rRNA genes is highly conserved allowing for the comparison between such different organisms. All the stems observed in *B. taurus* were detected in the *Discoglossus* 12S structure and any mutations did not affect the secondary structure, except in stems 17 and 18, which were characterized by high variability in nucleotide substitutions and some indels (Fig. 3). MP phylogenetic analyses were carried out either excluding the variable positions (gaps: 19 sites for stems 17, 18, 17′ and 18′), or taking all sites into consideration. The topologies obtained were congruent but the latter option gave the most consistent bootstrap values, confirming the reliability of the alignments obtained.

Geographic structuring and phylogenetic analysis

The statistic of skewness of tree-length distribution g1 (Hillis & Huelsenbeck, 1992) showed a significant phylogenetic signal (at the 1% level) in both cyt *b* (g1 = –0.89) and 12S (g1 = –0.80), allowing us to perform NJ, ML, and MP analyses on both markers, either individually or combined (793 bp). This was possible because the partition homogeneity test (Huelsenbeck *et al*., 1996) indicated no significant conflict in phylogenetic signal among genes ($P > 0.67$). At first the NJ method using Kimura 2-parameter distances for cyt *b* and 12S was implemented using all the haplotypes found (plus another 22 cyt *b* sequences taken from NCBI, Garcia-Paris & Jockusch, 1999), to identify the clusters within and between taxa. Selected haplotypes representing each of these clusters were used to carry out further analyses.

The topologies obtained, regardless of the marker and algorithm used, showed a single difference in the placement of western Moroccan *D. scovazzi* (the position of *D. scovazzi* was also the only difference with respect to the allozyme UPGMA shown in Fig. 2). The first topology (Figs 3A, 4A) showed *D. pictus s.l.* as a polyphyletic taxon, with *D. scovazzi* as the sister taxon of the Iberian *D. galganoi* and *D. jeanneae*. This topology resulted from cyt *b* data, regardless of the algorithm used, and from methods based on genetic distances (NJ and ML) applied to combined data (cyt *b* plus 12S). However, the consistency of this topology differed depending on the various phylogenetic methods: bootstrap values were close to 50% for NJ and MP and close to 80% for ML. The second topology (Fig. 4B) showed *D. pictus s.l.* as a paraphyletic taxon, with *D. scovazzi* clustering with a group including *D. sardus* and the *D. pictus–auritus* samples. This topology resulted from 12S (bootstrap values all above 80%) and from combined data under MP analysis. Looking at the rough data, populations of *D. scovazzi* appeared well differentiated from those of the *D. pictus–auritus* group (14% divergence for cyt *b* and 6% for 12S, Table 2) and showed the same level of divergence from the other *Discoglossus* taxa, except *D. montalentii* (12.2–14.9% for cyt *b*, 5.9–6.4% for 12S, Table 2). The samples of the *D. pictus–auritus* group showed the majority of the recorded haplotypes without a geographically structured distribution (Figs 3B, 4). Only the samples near the Moulouya River Basin (samples 17–19) had their own haplotypes and consequently clustered separately (Fig. 3A, Cluster B). The slow-evolving 12S showed the same pattern, grouping together the populations of the *D. pictus–auritus* group, as sister taxa of the Eastern Moulouyan populations (samples 17–19). Both topologies grouped the Iberian *Discoglossus* taxa into two differentiated clusters of haplotypes, with a divergence of 8.7% for cyt *b* sequences, and of 3% for the more conservative 12S. Haplotypes of *D. galganoi* and *D. jeanneae* were both present in central-northern Spain and were found in syntopy in the Sierra Morena area (sample 28), with a higher frequency of the southern type (86% on the seven specimens studied).

In Corsica, both topologies indicated two well-differentiated clusters of haplotypes, with a divergence of 18% for cyt *b* sequences and of 9% for 12S, corresponding to *D. montalentii* and *D. sardus* populations. In the latter species the haplotypes from different islands (Corsica, Sardinia and the Tuscan Archipelago) showed poor genetic differentiation (2% for cyt *b* and about 1% for 12S). As for allozymes, *D. montalentii* exhibited the highest genetic divergence for both mitochondrial genes (17–20% for cyt *b* and 8–9% for 12S, Table 2) and clustered separately (Figs 3A, 4).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pi2
pi3
pi4
pi5
pi6
Cluster A
Sicily, Tunisia, Algeria
(8, 9, 13, 14)
Algeria (16)
pi7
pi8
pi9
Cluster B
Eastern Moulouya River
(17, 18, 19)
pi10
pi11
pi12
pi13
Cluster C
Sicily, Tunisia, Algeria,
Banyuls sur Mer
(8, 11, 12, 13, 15)
D. pictus-auritus
75/60
57/86
87/79
100/100
95/100
99/100
60/85
sa1
sa2
D. sardus
Corsica, Sardinia (1,5)
100/100
sc14
sc15
sc16
D. scovazzi *
D. scovazzi
Morocco (22,24)
56/82
100/100
96/100
55/64
D.galganoi 1*
D.galganoi 2*
ga1
D. galganoi
Spain (28,29,30)
100/100
99/100
D. jeanneae 1*
je2
je3
ie4
D. jeanneae
Spain (28,26,27)
100/100
mo1
mo2
mo3
D. montalentii
Corsica (2,3)
Alytes obstetricans
Alytes muletensis
Xenopus laevis
0.02
B
cytb haplotypes (from NJ)
D.galganoi
D.jeannae
D.sardus
D.montalentii
D.scovazzi
cluster A
cluster B
cluster C
pi6
D.pictus-auritus
Moulouya river
D.sardus
D.montalentii
D.p.pictus
D.p.auritus
D.scovazzi
D.jeanneae
D.galganoi

Figure 3. Genetic relationships of *Discoglossus* taxa based on cyt *b* sequences. A, Neighbour joining (NJ) tree based on Kimura 2-parameter distance with bootstrap values (NJ/maximum likelihood (ML)) over 1000 replicates; B, geographical distribution of the haplotype frequencies. The haplotypes were grouped according to the clusters identified in the NJ tree (A). Population numbers as in Table 1. Asterisks identify haplotypes found by Garcia-Paris & Jockusch (1999). Haplotype codes: pi, *D. pictus-auritus*; sc, *D. scovazzi*; sa, *D. sardus*; ga, *D. galganoi*; je, *D. jeannae*; mo, *D. montalentii*.

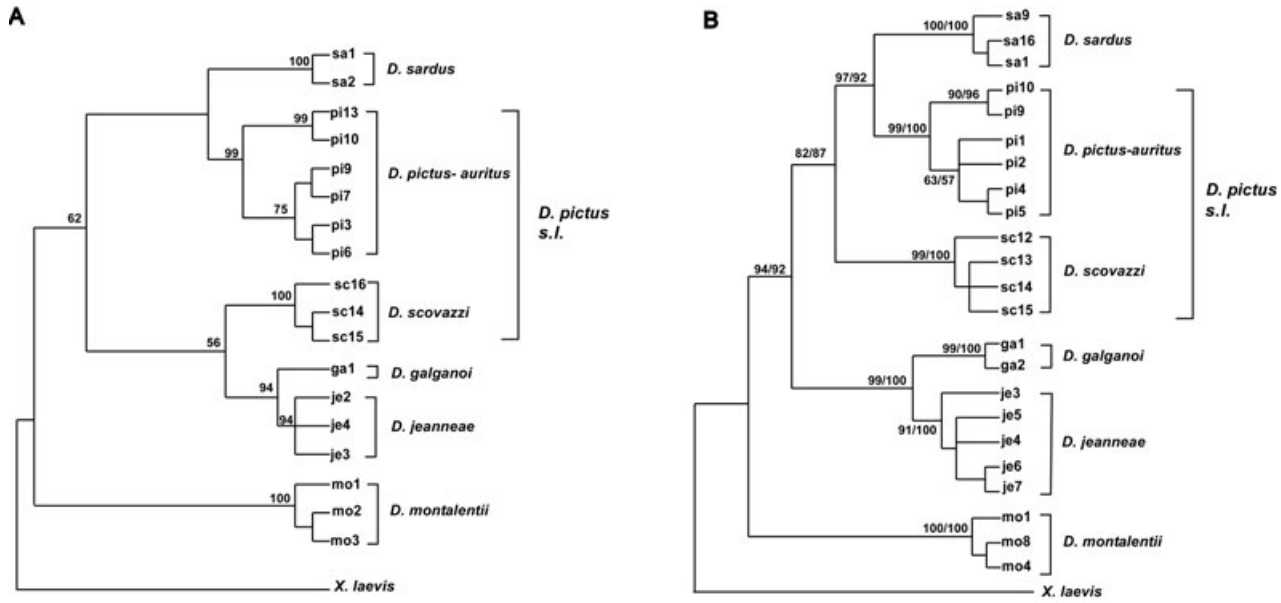

Figure 4. Maximum parsimony (MP) topologies based on cyt *b* sequences (A) and on 12S/combined data (B). Bootstrap values over 1000 replicates are shown for > 50% supported nodes. Haplotype codes as in Figure 3.

Table 2. Kimura 2-parameter distances calculated among the taxa and clusters identified with neighbour-joining method

Taxon	1	2	3	4	5	6	7	8	9	10
1. *Discoglossus montalentii*	–	0.095 (0.015)	0.104 (0.015)	0.098 (0.015)	–	0.069 (0.013)	0.085 (0.023)	0.086 (0.014)	–	0.198 (0.014)
2. *D. sardus*	0.187 (0.023)	–	0.048 (0.011)	0.051 (0.011)	–	0.060 (0.012)	0.061 (0.011)	0.074 (0.013)	–	0.198 (0.023)
3. *D. pictus pictus* Cluster A	0.201 (0.026)	0.139 (0.019)	–	0.014 (0.005)	–	0.060 (0.012)	0.061 (0.012)	0.064 (0.012)	–	0.204 (0.022)
4. *D. p. auritus* Cluster B	0.192 (0.024)	0.128 (0.019)	0.03 (0.008)	–	–	0.061 (0.012)	0.061 (0.013)	0.068 (0.024)	–	0.210 (0.023)
5. *D. p. auritus* Cluster C	0.201 (0.026)	0.139 (0.02)	0.054 (0.011)	0.067 (0.012)	–	–	–	–	–	–
6. *D. scovazzi*	0.192 (0.024)	0.138 (0.019)	0.139 (0.02)	0.147 (0.022)	0.149 (0.021)	–	0.059 (0.012)	0.064 (0.011)	–	0.189 (0.022)
7. *D. galganoi*	0.208 (0.027)	0.155 (0.022)	0.176 (0.024)	0.157 (0.022)	0.198 (0.026)	0.137 (0.020)	–	0.03 (0.008)	–	0.205 (0.024)
8. *D. jeanneae*	0.178 (0.025)	0.150 (0.021)	0.164 (0.021)	0.157 (0.021)	0.173 (0.023)	0.122 (0.019)	0.087 (0.015)	–	–	0.202 (0.024)
9. *Alytes*	0.297 (0.030)	0.258 (0.027)	0.260 (0.028)	0.250 (0.028)	0.270 (0.029)	0.241 (0.027)	0.233 (0.028)	0.261 (0.030)	–	–
10. *Xenopus*	0.249 (0.028)	0.265 (0.029)	0.244 (0.029)	0.252 (0.029)	0.240 (0.029)	0.275 (0.031)	0.267 (0.031)	0.245 (0.027)	0.244 (0.028)	–

Below the diagonal are the values obtained for cytochrome *b*, above it are those for 12S.
Standard error values obtained with bootstrap method over 1000 replicates are in parentheses.

Table 3. Test of topological differences using parametric bootstrapping over 100 sequence replicates

Marker	Constraints evaluated	MP trees statistics	No. trees found	Score of best trees	Length difference	*P*
Cyt *b* (polyphyly)		CI = 0.6541 HI = 0.3459 RI = 0.8111 RC = 0.5306	2	266		
	Constraint 1 (monophyly)			268	2	0.23
	Constraint 2 (paraphyly)			267	1	0.17
12S (paraphyly)		CI = 0.8146 HI = 0. 1854 RI = 0.9049 RC = 0.7371	16	178		
	Constraint 1 (monophyly)			184	6	< 0.001
	Polyphyly (cyt b topology)			183	5	< 0.001

Differences in length between the best maximum parsimony (MP) tree and the trees obtained under different constrained topologies are reported, with *P*-values, to evaluate monophyly (constraint 1), paraphyly (constraint 2) or polyphyly for *Discoglossus pictus s.l.*

MP tree statistics reported for the best MP tree are: CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

Table 4. Test of topological differences using likelihood-based methods of Kishino–Hasegawa (KH) and Shimoidaira–Hasegawa (SH) for alternative trees

Molecular marker	Tree topology	–ln L	Diff –ln L	KH test (*P*)	SH test (*P*)
Cyt b	NJ tree (polyphyly)	2701.90 (best)			
	1 (monophyly)	2708.88	6.979	0.190	0.094
	2 (paraphyly)	2708.53	6.632	0.212	0.103
12S	NJ tree (polyphyly)	1430.19 (best)			
	1 (monophyly)	1441.94	11.750	0.035*	0.021*
	2 (paraphyly)	1442.05	11.870	0.043*	0.026*

The neighbour-joining (NJ) tree topology is tested against UPGMA and maximum parsimony (MP) topologies.

Values of likelihoods (– lnL), differences of likelihood scores between the alternative topologies (Diff –ln L) and the *P*-values for KH and SH tests are reported. *Indicates topologies significantly worse than the best tree ($P < 0.05$).

Testing competing phylogenetic hypotheses: Parametric Bootstrapping and Likelihood-Based Tests

To test the monophyletic condition of *D. pictus s.l.* vs. paraphyly or polyphyly, parametric bootstrapping and likelihood-based tests (KH and SH) were carried out. Although theoretically different, both methods showed the *D. pictus–auritus* group to be closer to *D. sardus* than it was to *D. scovazzi* (Tables 3, 4). For cyt *b*, neither constraint 1 (monophyly of *D. pictus s.l.)* nor constraint 2 (monophyly of *D. pictus s.l.* plus *D. sardus*) could be rejected, while for 12S, constraint 1 was rejected with a confidence lower than 0.001 (parametric bootstrapping, Table 3) and equal to 0.035 (KH test, Table 4).

Comparison with other molecular datasets

Fromhage *et al*. (2004) presented a phylogeny on *Discoglossus*, *Alytes* and *Bombina* based on partial 12S (a fragment not investigated in this study) and 16S mitochondrial genes. They found a sister-group

relationship between *D. scovazzi* and *D. galganoi*/*D. jeanneae* and between *D. sardus* and *D. pictus*. This topology coincides with that presented in Figures 3A and 4A and, as we found also, was variably supported under the various methods used (bootstrap values from 41% to 96%). Finally, the same topology resulted from combining our data (one sequence per taxon per geographical area) with those published in GenBank by Fromhage *et al*. (2004) (Accession numbers AY333680–724), with bootstrap values ranging again from 56% (MP) to 98% (NJ) under different methods.

Application of molecular clock

Table 5 shows the values of genetic distance between *Discoglossus* taxa, obtained using different markers (allozymes and 12S) and their times of divergence. Two different evolutionary rates have been proposed for allozymes. The first is $0.2D_{Nei}$/Myr (Nei, 1975), used for *Discoglossus* by Lanza *et al*. (1986), while the second is $0.1D^*_{Nei}$/ Myr, calibrated on non-hybrid species of the *Rana esculenta* complex (Beerli *et al*., 1996). The latter fitted better with Mediterranean palaeogeographical events, with the reopening of the Gibraltar Straits being the vicariance palaeogeographical event that promoted the isolation between Iberian and Moroccan taxa 5.33 Mya. This in turn allowed for the calibration of 12S Kimura 2-parameter distances, providing an evolutionary rate of 0.0112/Myr/pair, in agreement with other reports for this gene (0.01–0.03/Myr/pair; Fu, 1998; Harris, Arnold & Thomas, 1998; Lin, Chen & Lue, 2002). Enforcing a molecular clock on 12S using *X. laevis* as a root resulted in a tree with a log-likelihood not significantly worse at the 1% level ($\log LH_0 = -1399.98411$; $\log LH_1 = -1378.57922$; d.f. = 25; $P = 0.16$), so that the molecular clock hypothesis could not be rejected. No calibrations were applied to cyt *b*, showing saturation signs.

DISCUSSION

Contrasting patterns of mitochondrial and nuclear markers in *Discoglossus*

Nuclear and mitochondrial markers showed contrasting patterns of genetic relationships. The mitochondrial markers showed similar levels of genetic divergence among *D. scovazzi*, *D. pictus*, *D. sardus* and the Iberian *D. galganoi–D. jeanneae* (Figs 3, 4), while the nuclear markers linked *D. scovazzi* with *D. pictus* (Fig. 2), and showed *D. galganoi* and *D. jeanneae* as very close ($D_{Nei} = 0.05$).

A possible cause for these conflicting data could be lineage sorting of ancestral polymorphisms, which is expected to be faster for mtDNA genes as their population size is one quarter that of nuclear genes (Moore, 1995). Thus, mitochondrial genes will achieve fixation of sequence differences four times faster than will nuclear genes, causing an underrating of nuclear genetic distances. Another possible cause is male-biased dispersal: gene flow occurring mainly via migrating males would lower nuclear genetic distances, but not mitochondrial ones. This is, however, little known in frogs (see, for example, Lampert *et al*., 2003). Female heterogamy in *Discoglossus* (Morescalchi, 1973) may also be responsible for biased introgression of nuclear genes, as suggested by Garcia-Paris & Jockusch (1999). According to Haldane's rule, hetero-

Table 5. Genetic distances and times of divergence between *Discoglossus* taxa with their standard deviations

	Genetic distance			Time of divergence		
	Allozymes		12S			
	D_{Nei}	D^*_{Nei}	K2 parameter	I 0.2/ Myr	II 0.1/ Myr	12S 0.0112/ Myr
D. montalentii vs. other taxa	1.004 ± 0.060	1.041 ± 0.067	0.090 ± 0.013	5.02 ± 0.30	10.41 ± 0.67	8.03 ± 1.16
D. scovazzi vs. *D. galganoi*	0.494 ± 0.032	0.524 ± 0.032	0.060 ± 0.005	2.47 ± 0.16	5.24 ± 0.32	**5.33**
D. scovazzi vs. *D. pictus pictus–auritus*	0.171 ± 0.012	0.181 ± 0.017	0.060 ± 0.012	0.85 ± 0.06	1.83 ± 0.17	5.36 ± 1.07
D. scovazzi vs. *D. sardus*	0.518 ± 0.043	0.544 ± 0.049	0.059 ± 0.012	2.59 ± 0.21	5.44 ± 0.49	5.26 ± 1.07
D. sardus vs. *D. p. pictus–auritus*	0.394 ± 0.044	0.411 ± 0.049	0.053 ± 0.008	1.97 ± 0.22	4.11 ± 0.49	4.73 ± 0.71
D. galganoi vs. *D. jeanneae*	0.047 ± 0.018	0.048 ± 0.021	0.030 ± 0.008	0.23 ± 0.09	0.48 ± 0.21	2.68 ± 0.71
D. pictus vs. *D. p. auritus*	0.037 ± 0.019	0.046 ± 0.021	0.005 ± 0.002	0.37 ± 0.19	0.46 ± 0.21	0.45 ± 0.17

Times of divergence were estimated according to the calibrations of: (I) Nei (1975) applied on D_{Nei} (Nei, 1972); (II) Beerli *et al.* (1996) applied on D^*_{Nei} (Hillis, 1984); 12S evolutionary rate was calibrated on the reopening of Gibraltar Straits (5.33 Mya; Krijsgman *et al.*, 1999, evidenced in bold) and applied on K2 parameter distances.

gametic females of hybrid origin likely have lower fitness (Coyne & Orr, 1989), explaining the spread of nuclear genes only. This mechanism, however, can act only in contact zones.

The presence of ancestral polymorphisms was evidenced in our data by the finding of alleles shared between highly divergent taxa (*D. montalentii* vs. the other taxa, Appendix), making lineage sorting of ancestral polymorphisms the most likely explanation. However, lineage sorting is not expected to produce any coherent geographical pattern, although we observed a lowering of nuclear with respect to mitochondrial levels of differentiation only between parapatric and neighbouring taxa (*D. scovazzi* vs. *D. pictus* and *D. galganoi* vs. *D. jeanneae*). Therefore, it is likely that all the cited phenomena could have combined in producing the observed patterns.

Evolutionary and biogeographical scenarios for the western Mediterranean *Discoglossus*

Although a number of different biogeographical scenarios have already been proposed for *Discoglossus* taxa, they were based only on allozymes (Lanza *et al*., 1986) or on mitochondrial data (Fromhage *et al*., 2004). In this study for the first time mitochondrial markers and allozymes have been studied on the same samples, tracking both ancient and recent events of the genus.

Allozyme data were used considering two allozyme calibrations which have been used already on frogs, that by Beerli *et al*. (1996) for the non-hybrid species of the *Rana esculenta* complex and that by Nei (1975), adopted by Lanza *et al*. (1986), for *Discoglossus*, producing the dating reported in Table 5. The mitochondrial data could then help to choose between one clock and the other. However, they showed short internal branches and conflicting topologies for *D. galganoi–jeanneae*, *D. scovazzi*, *D. sardus* and *D. pictus* (Fig. 3). This was not because of the markers used, because a significant phylogenetic signal was detected using g1 statistics, and significant bootstrap values (over 90%) were reported for both intraspecific nodes and deep levels of divergence (Fig. 3A). Also, the same pattern was found by Fromhage *et al*. (2004) studying different mitochondrial markers in the same taxa. Ancient cladogenetic events occurring in close proximity might result in a lack of univocal phylogenetic signals, independent of the marker used (Albertson *et al*., 1999; Chek *et al*., 2001). Therefore, a relatively ancient and almost simultaneous split of these lineages seems a likely explanation and could be connected to a well-known palaeogeographical event: the reopening of the Gibraltar Straits at the end of the Messinian salinity crisis. This could explain a quick rise in geographical barriers after a rapid and wide expansion around the Mediterranean basin. Dating back to 5.33 Mya (Krijgsman *et al*., 1999), this is in agreement with the calibration proposed by Beerli *et al*. (1996). Adopting this molecular clock, *D. montalentii* would have colonized Corsica during the Tortonian epoch (around 10 Mya), when land bridges of unknown duration connected Corsica and Sardinia with the mainland (Orszag-Sperber *et al*., 1993). The spread of *Discoglossus* between Europe and Africa should have occurred at the end of the Messinian salinity crisis (about 6 Mya, Krijgsman *et al*., 1999), when favourable conditions were present: stable landmass bridges, cooling of the climate conditions and changing of Mediterranean hypersaline waters to brackish or freshwater (Bertolani-Marchetti, 1985; Roveri, Bassetti & Ricci Lucchi, 2001; Rouchy *et al*., 2003). The subsequent reflooding of the Mediterranean basin (5.33 Mya) isolated *D. galganoi*, *D. scovazzi*, *D. sardus* and *D. pictus* ancestors. In particular, Rif and Tell–Atlas were separated by an early Pliocene marine transgression in the Rifian gateway (Rouchy *et al*., 2003), in agreement with the finding of the Moulouya River Basin as the boundary between *D. scovazzi* and *D. pictus–auritus*. This valley has already been hypothesized as an important geographical barrier between other Moroccan vs. Algerian–Tunisian taxa of amphibians and reptiles (Mateo, 1990; Mateo *et al*., 1996; Álvarez *et al*., 2000; Harris *et al*., 2002). In this area, subsequent marine regressions and climate oscillations occurred until 1.7 Mya (Thompson & Fleming, 1996) and could have allowed repeated contacts between Moroccan and Algerian *Discoglossus*, as tracked by nuclear genes, indicating a split at about 1.8 Mya (Table 5), when local climatic oscillation ended (Thompson & Fleming, 1996). Mitochondrial genes did not retain traces of these contacts, due to lineage sorting or other phenomena that have been discussed already.

Iberian taxa showed mitochondrial lineages more differentiated compared with nuclear gene pools. Plio-Pleistocenic climatic oscillations and the presence of multiple glacial refugia in the Iberian Peninsula (Gómez & Lunt, in press) could explain this pattern. Mitochondrial genes would have diverged since the Pliocene, while the pattern of nuclear diversity would be related to the spread of genes across contact zones originated after a later Pleistocenic expansion from northern and southern refugia. A recent Pleistocenic range expansion is suggested for *D. pictus* and *D. sardus*, which were genetically homogeneous.

With respect to previously proposed scenarios, ours seems more parsimonious and more capable of reconciling nuclear and mitochondrial data. The scenario proposed by Fromhage *et al*. (2004) dated the isolation of the *D. montalentii* ancestor to about 15 Mya and hypothesized two old events of vicariance for the other

splits: the separation of the Calabro-Peloritan massif from Sardinia for *D. sardus–D. pictus* (5.61 Mya) and the fragmentation of the Betic region for *D. scovazzi–D. galganoi* (9.54 Mya). Even if biogeographically plausible, this scenario could not explain the lower nuclear differentiation with respect to mitochondrial divergence between *D. scovazzi* and *D. pictus*. Also, the scenario proposed by Lanza *et al*. (1986) of Plio-Pleistocenic splits among *D. galganoi*, *D. scovazzi*, *D. sardus* and *D. pictus* seemed poorly parsimonious because unknown land bridges or sporadic dispersal by natural rafting had to be hypothesized between Spain and Morocco.

Taxonomic inferences

Iberian Discoglossus taxa

The Iberian *Discoglossus* was assigned to *D. galganoi* on the basis of allozyme data (Capula *et al*., 1985). Busack (1986) detected a second Iberian taxon in southern Spain, *D. jeanneae*. It was considered a subspecies of *D. galganoi* by Lanza *et al*. (1986), due to the low genetic divergence found between the two taxa at the allozyme level. Data on nuclear DNA content (Fritz, Vences & Glaw, 1994) supported this view, while mtDNA studies were in favour of a specific rank for *D. jeanneae* (Garcia–Paris & Jockusch, 1999).

Our study confirms the lack of genetic divergence between *D. galganoi* and *D. jeanneae* at the allozyme level (neither discriminating nor highly differentiated loci were observed), associated with a differentiation of the mtDNA. The two mitochondrial lineages characterizing *D. galganoi* and *D. jeanneae* coexist in the Sierra Morena area (sample 28), where a contact zone could be hypothesized. This genetic pattern suggests that nuclear genes are still moving across the contact zone and that reproductive isolation has not been achieved. Thus a subspecific status for *D. g. galganoi* and *D. g. jeanneae* seems to be more appropriate, and this is supported by bioacoustic studies (Vences & Glaw, 1996).

D. scovazzi and D. pictus

D. scovazzi populations west of the Moulouya River were genetically distinct from those of *D. pictus* east of the River, indicating a current lack of gene exchange at the level of both mitochondrial and nuclear genes. At the allozyme level, *D. scovazzi* clustered with *D. pictus* (Fig. 2) but the two taxa were well differentiated, having two diagnostic loci (*Aat-1*, *Pt-5*) and another two loci showing strongly differentiated allele frequencies (*6Pgdh*, *Mdh-1*). The mitochondrial markers also attested that *D. pictus s.l.* is not monophyletic, linking *D. scovazzi* either to *D. galganoi* (Figs 3, 4A) or to *D. pictus* plus *D. sardus* (Fig. 4B). Indeed, *D. scovazzi* showed similar levels of mitochondrial differentiation with respect to *D. galganoi*, *D. sardus* and *D. pictus*. These data confirm the specific rank of *D. scovazzi*, as proposed by Fromhage *et al*. (2004).

The little genetic differentiation detected among Algerian and Tunisian *D. auritus* with respect to Maltese and Sicilian *D. pictus* suggested a very recent isolation of Sicilian populations and did not support the necessity of a subspecific rank for *D. p. auritus*. The sample of *D. pictus* from Banyuls sur Mer (sample 20), introduced in recent times by man (Nascetti *et al*., 1986), shared some peculiar genetic features with the Tell–Atlas populations, which can be considered its source.

CONCLUSIONS

The simultaneous investigation of mitochondrial and nuclear markers in *Discoglossus* revealed contrasting patterns in the two datasets. The stochastic sorting of ancestral polymorphism, with a possible contribution of male-biased dispersal, and the establishment of secondary contacts (present or past) in the Iberian Peninsula and Maghreb could have been relevant in generating the extant patterns.

Clustering of taxa characterized by an intermediate level of divergence resulted in short internal branches and nodes with different bootstrap support under different algorithms, even in the presence of a significant phylogenetic signal, suggesting that *Discoglossus* underwent rapid radiation. This phenomenon has been postulated recently for other peri-Mediterranean animal taxa (Tsigenopoulos *et al*., 2003), identifying the responsible palaeogeographical event as the Messinian Lago Mare phase. Also, the genetic relationships of the *Discoglossus* taxa evidenced the Moulouya River basin as an important biogeographical barrier, as recently suggested for Iberian–Maghreb fauna. The taxonomic array of the genus was consequently revised, confirming the specific status of the Moroccan *D. scovazzi* (Fromhage *et al*., 2004) and suggesting a subspecific status for the Iberian *D. galganoi* and *D. jeanneae* until further investigation is carried out in the Sierra Morena contact zone.

Phylogenetic and biogeographical investigation of *Discoglossus* provided an insight into the evolutionary patterns in the western Mediterranean basin and confirmed the necessity of considering both nuclear and mitochondrial markers to reconstruct biogeographical history and to identify species boundaries.

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REFERENCES

Albertson RC, Markert JA, Danley PD, Kocher TD. 1999. Phylogeny of a rapidly evolving clade: The cichlid fishes of Lake Malawi, East Africa. *Proceedings of the National Academy of Sciences, USA* **96:** 5107–5110.

Alcover JA, Sanders E, Sanchiz B. 1984. El registro fosil de los sapos parteros (Anura, Discoglossidae) de Baleares. In: Hemmer H, Alcover JA, eds. *Historia biologica del ferreret (life history of the Mallorcan midwife toad)*. Ciutat de Mallorca: Moll, 109–121.

Alvarez W. 1972. Rotation of the Corsica-Sardinia microplate. *Nature* **235:** 103–105.

Álvarez Y, Mateo JA, Andreu AC, Diaz-Paniagua C, Diez A, Bautista JM. 2000. Mitochondrial DNA haplotyping of *Testudo graeca* on both continental sides of the Straits of Gibraltar. *Journal of Heredity* **91:** 39–41.

Ballard JWO, Whitlock MC. 2004. The incomplete natural history of mitochondria. *Molecular Ecology* **13:** 729–744.

Beerli P, Hotz H, Uzzell T. 1996. Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. *Evolution* **50:** 1676–1687.

Bertolani-Marchetti D. 1985. Pollen paloclimatology in the Mediterranean since the Messinian time. In: Stanley DJ, Wezel FC, eds. *Geological evolution of the Mediterranean basin*. New York: Springer–Verlag, 525–543.

Busack SD. 1986. Biochemical and morphological differentiation in Spanish and Moroccan populations of *Discoglossus* and the description of a new species from southern Spain (Amphibia, Anura, Discoglossidae). *Annals of Carnegie Museum* **55:** 53–61.

Camerano L. 1878. Osservazioni intorno agli Anfibi Anuri del Marocco. *Atti Della Accademia Delle Scienze Di Torino* **13:** 542–558.

Capula M, Nascetti G, Lanza B, Bullini L, Crespo EG. 1985. Morphological and genetic differentiation between the Iberian and the other west Mediterranean *Discoglossus* species (Amphibia Salentia Discoglossidae). *Monitore Zoologico Italiano* **19:** 69–90.

Chek AA, Lougheed SC, Bogart JP, Boag PT. 2001. Perception and history: molecular phylogeny of a diverse group of neotropical frogs, the 30-chromosome *Hyla* (Anura: Hylidae). *Molecular Phylogenetics and Evolution* **18:** 370–385.

Cocuzza T. 1975. Structural pattern of Sardinia. In: Ognibene L, Parotto M, Praturlon A, eds. *Structural model of Italy*. Quaderni de 'La Ricerca Scientifica', 90. Rome, Italy: CNR, 183–201.

Coyne JA, Orr HA. 1989. Two rules of speciation. In: Otte D, Endler JA, eds. *Speciation and its consequences*. Sunderland, MA: Sinauer Associates, 180–207.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* **19:** 11–15.

Emerson BC, Ibrahim KM, Hewitt GM. 2001. Selection of evolutionary models for phylogenetic hypothesis testing using parametric methods. *Journal of Evolutionary Biology* **14:** 620–631.

Fritz B, Vences M, Glaw F. 1994. Comparative nuclear DNA content in *Discoglossus* (Amphibia, Anura, Discoglossidae). *Zoologischer Anzenger* **233:** 135–145.

Fromhage L, Vences M, Veith M. 2004. Testing alternative vicariance scenarios in western Mediterranean discoglossid frogs. *Molecular Phylogenetics and Evolution* **31:** 308–322.

Fu J. 1998. Toward the phylogeny of the family Lacertidae: implications from mitochondrial DNA 12s and 16s gene sequences (Reptilia: Squamata). *Molecular Phylogenetics and Evolution* **9:** 118–130.

Garcia-Paris M, Jockusch EL. 1999. A mitochondrial DNA perspective on the evolution of Iberian *Discoglossus* (Amphibia: Anura). *Journal of Zoology, London* **248:** 209–218.

Gasc JP, Cabela A, Crnobrnja-Isailovic J, Dolmen D, Grossenbacher K, Haffner P, Lescure J, Martens H, Martínez Rica JP, Maurin H, Oliveira ME, Sofianidou TS, Veith M, Zuiderwijk A. 1997. *Atlas of amphibians and reptiles in Europe*. Paris: Collection Patrimoines Naturels.

Giribet G, Wheeler WC. 1999. On gaps. *Molecular Phylogenetics and Evolution* **13:** 132–143.

Goebel AM, Donneley JM, Atz ME. 1999. PCR primers and amplification methods for 12s ribosomal DNA, the control region, cytochrome oxidase I and cytochrome b in bufonids, and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution* **11:** 163–199.

Gómez A, Lunt DH. In press. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N, eds. *Phylogeography in southern European refugia: evolutionary perspectives on the origins and conservation of European biodiversity*. Dordrecht, the Netherlands: Kluwer Academic Publishers.

Guldbrandtsen B, Tomiuk J, Loeschcke V. 2000. POPDIST: a program to calculate population genetic distance and identity measures, vers. 1.1.1. *Journal of Heredity* **91:** 178–179.

Harris DJ, Arnold EN, Thomas RH. 1998. Relationships of lacertid lizards estimated from mitochondrial DNA sequences and morphology. *Proceeding of the Royal Society of London, Series B* **265:** 1939–1948.

Harris DJ, Carranza S, Arnold EN, Pinho C, Ferrand N. 2002. Complex biogeographical distribution of genetic variation within *Podarcis* wall lizard across the Strait of Gibraltar. *Journal of Biogeography* **29:** 1257–1262.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **21:** 160–174.

Heron-Royer F. 1888a. Essai sur la transmission de la coloration chez les Batraciens Anoures. *Bulletin de la Societé Zoologique de France* **13:** 205–206.

Heron-Royer F. 1888b. À propos du *Discoglossus auritus*. *Bulletin de la Societé Zoologique de France* **13:** 220.

Hillis DM. 1984. Misuse and modification of Nei's genetic distance. *Systematic Zoology* **33:** 238–240.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* **83:** 189–195.

Hsü K, Montandert L, Bernouilli D, Cita MB, Erikson A, Garrison RE, Kidd RB, Melieres F, Muller C, Wright R. 1977. History of the Mediterranean salinity crisis. *Nature* **267:** 399–403.

Huelsenbeck JP, Hillis DM, Jones R. 1996. Parametric bootstrapping in molecular phylogenetics: applications and performance. In: Ferraris JD, Palumbi SR, eds. *Molecular zoology: advances, strategies, and protocols*. New York: Wiley-Liss, 19–45.

Huelsenbeck JP, Rannala B. 1997. Phylogenetic methods come of age: testing hypotheses in an evolutionary context. *Science* **276:** 227–232.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequence. *Journal of Molecular Evolution* **16:** 111–120.

Kishino H, Hasegawa M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution* **29:** 170–179.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceeding of the National Academy of Sciences, USA* **86:** 6196–6200.

Kotsakis T. 1981. Gli anfibi e i rettili del Pleistocene del Lazio (Italia centrale). *Geologica Romana* **20:** 57–67.

Kotsakis T. 1982a. Presence du genre *Discoglossus* Otth (Discoglossidae, Anura, Amphibia) dans le Villafranchien de l'île de Crète. *Geologica Romana* **21:** 185–189.

Kotsakis T. 1982b. Les amphibiens et les reptiles du Villafranchien de l'Italie. *Proceedings of the International Colloquium le Villafanchien Méditerranéen, Lille*, 83–91.

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS. 1999. Chronology, causes and progression of the Messinian salinity crisis. *Nature* **400:** 652–655.

Kumar SK, Tamura S, Jakobsen IB, Nei M. 2001. MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics* **17:** 1244–1245.

Lampert KP, Rand AS, Mueller UG, Ryan MJ. 2003. Fine-scale genetic pattern and evidence for sex-biased dispersal in the túngara frog, *Physalaemus pustulosus*. *Molecular Ecology* **12:** 3325–3334.

Lanza B, Nascetti G, Capula M, Bullini L. 1984. Genetic relationships among West Mediterranean *Discoglossus* with the description of a new species (Amphibia Salientia Discoglossidae). *Monitore Zoologico Italiano* **18:** 133–152.

Lanza B, Nascetti G, Capula M, Bullini L. 1986. Les Discoglosses de la région Méditerranéenne occidentale (Amphibia; Anura; Discoglossidae). *Bulletin Société Herpétologique de France* **40:** 16–27.

Lin SM, Chen CA, Lue KY. 2002. Molecular phylogeny and biogeography of the grass lizards genus *Takydromus* (Reptilia: Lacertidae) of East Asia. *Molecular Phylogenetics and Evolution* **22:** 276–288.

Mateo JA. 1990. Taxonomy and evolution of the North African ocellated lizard, *Lacerta pater* (Lataste, 1880) (Sauria: Lacertidae). *Bonner Zoologische Beiträge* **41:** 203–212.

Mateo JA, López-Jurado LF, Guillaume CP. 1996. Variabilité électrophoretique et morphologique des lézards ocellés (Lacertidae): un complexe d'espèces de part et d'autre du détroit de Gibraltar. *Comptes Rendus Academie Des Sciences, Paris* **319:** 737–746.

Monsen KJ, Blouin MS. 2003. Genetic structure in a montane ranid frog: restricted gene flow and nuclear-mitochondrial discordance. *Molecular Ecology* **12:** 3275–3286.

Moore WS. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution* **49:** 718–726.

Morescalchi A. 1973. Amphibia. In: Chiarelli B, Capanna E, eds. *Cytotaxonomy and vertebrate evolution*. London: Academic Press, 233–348.

Moritz C, Schneider CJ, Wake D. 1992. Evolutionary relationships within *Ensatina escholtzii* complex confirm the ring species interpretation. *Systematic Biology* **41:** 273–291.

Nascetti G, Capula M, Lanza B, Bullini L. 1986. Ricerche elettroforetiche sul genere *Discoglossus* (Amphibia, Anura, Discoglossidae). *Bollettino Di Zoologia* **53:** 57.

Nei M. 1972. Genetic distance between populations. *American Naturalist* **106:** 283–292.

Nei M. 1975. *Molecular population genetics and evolution*. New York: Elsevier.

Orszag-Sperber F, Butterlin J, Clermonte J, Colchen M, Guiraud R, Poisson A, Ricou LE. 1993. Tortonian Palaeoenvironments (11.5–6 Ma) and map. In: Dercourt J, Ricou LE, Vrielynck B, eds. *Atlas tethys, paleoenvironmental maps*. Paris: Gauthier-Villars: 237–239.

Otth A. 1837. Beschreibung einer neuen europäischen Froschgattung. *Discoglossus*. *Neue Denkschriften der Allgemeinen Schweizerischen Gesellschaft für die Gesamten Naturwissenschaften* **1:** 1–8.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G. 1991. *The simple fool's guide to PCR*, *Vers.*2.0. Honolulu: Department Zoology, University Hawaii.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Rambaut A, Grassly NC. 1997. Seq-General. An application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees, Vers. 1.2.5. *Computer Applications in Biosciences* **13:** 235–238.

Rees DJ, Emerson BC, Oromi P, Hewitt GM. 2001. Reconciling gene trees with organism history: the mtDNA phylogeography of three *Nesotes* species (Coleoptera: Tenebrionidae) on the western Canary Islands. *Journal of Evolutionary Biology* **14:** 139–147.

Rokas A, Melika G, Abe Y, Nieves-Aldrey JL, Cook JM, Stone GN. 2003. Lifecycle closure, lineage sorting, and hybridization revealed in a phylogenetic analysis of European oak gallwasps (Hymenoptera: Cynipidae: Cynipini)

using mitochondrial sequence data. *Molecular Phylogenetics and Evolution* **26:** 36–45.

Rouchy JM, Pierre C, Et-Touhami M, Kerzazi K, Caruso A, Blanc-Valleron MM. 2003. Late Messinian to Early Pliocene paleoenvironmental changes in the Melilla Basin (NE Morocco) and their relation to Mediterranean evolution. *Sedimentary Geology* **163:** 1–27.

Roveri M, Bassetti MA, Ricci Lucchi FR. 2001. The Mediterranean Messinian salinity crisis: an Apennine foredeep perspective. *Sedimentary Geology* **140:** 201–214.

Rozas J, Rozas R. 1999. DnaSP, Vers. 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15:** 174–175.

Ruedi M, Auberson M, Savolainen V. 1998. Biogeography of Sulawesian shrews: Testing for their origin with a parametric bootstrap on moleculata data. *Molecular Phylogenetics and Evolution* **9:** 567–571.

Sanchiz B. 1977. Catalogo de los anfibios fosiles de España. *Acta Geologica Hispanica* **12:** 103–107.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* **16:** 1114–1116.

Springer MS, Douzery E. 1996. Secondary structure and patterns of evolution among mammalian mitochondrial 12s rRNA molecules. *Journal of Molecular Evolution* **43:** 357–373.

Swofford DL. 2003. PAUP*: *Phylogenetic analysis using parsimony (* and other methods)*. Beta, Vers. 4.0b 10. Sunderland, Massachusetts: Sinauer Associates.

Swofford DL, Olsen GJ, Waddel PJ, Hillis DM. 1996. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*, 2nd edn. Sunderland: Sinauer, 407–514.

Swofford DL, Selander RB. 1981. Biosys-1: a Fortran programe for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity* **72:** 281–283.

Swofford DL, Selander RB. 1989. *BIOSYS-2. A computer program for the analysis of allelic variation in population genetics and biochemical systematics, release 1.7.* Champaign, Illinois: Illinois Natural History Survey.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:** 585–595.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10:** 512–526.

Thompson RT, Fleming RF. 1996. Middle Pliocene vegetation: reconstructions, paleoclimatic inferences and boundary conditions for climate modelling. *Marine Micropaleontology* **27:** 27–49.

Thompson JD, Gibson TJ, Plewiniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24:** 4876–4882.

Tschudi J. 1837. Appendix. In: Otth A. Bescreibung einer neuen europaischen Froschgattun, Discoglossus. *Neue Denkschriften der Allgemeinen Schweizerischen Gesellschaft Fur die Gesammten Naturwissenscahften* **1:** 7–8.

Tsigenopoulos CS, Durand JD, Ünlü E, Berrebi P. 2003. Rapid radiation of the Mediterranean *Luciobarbus* species (Cyprinidae) after the Messinian salinity crisis of the Mediterranean Sea, inferred from mitochondrial phylogenetic analysis. *Biological Journal of the Linean Society* **80:** 207–222.

Vences M, Glaw F. 1996. Further investigations on *Discoglossus* bioacoustics: Relationships between *D. galganoi galganoi, D. g. jeanneae* and *D. pictus scovazzi*. *Amphibia-Reptilia* **17:** 333–340.

Werner YL. 1988. Herpetofaunal survey of Israel (1950–85), with comments on Sinai and Jordan and on zoogeographic heterogeneity. In: Yom-Tov Y, Tchernov E, eds. *The zoogeography of Israel.* Dordrecht: Dr. W. Junk Publishers, 355–388.

Xia X, Xie Z. 2001. DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity* **92:** 371–373.

Yang Z. 1996. Statistical properties of a DNA sample under the finite-sites model. *Genetics* **144:** 1941–1950.

APPENDIX

Allelic frequencies for the 29 polymorphic loci studied (*Hk* and *Pt-4* were monomorphic) in the 31 *Discoglossus* populations sampled (samples 1–14 and 17–32)

	Population (samples 1–14)														
Allele	m1	2	3	4	5	6	s1	7	8	9	10	11	12	13	14
G3pdh															
90	–	–	0.21	0.07	1.00	1.00	0.50	0.54	–	–	–	–	–	–	–
100	1.00	1.00	0.75	0.93	–	–	0.50	0.46	1.00	1.00	1.00	1.00	1.00	1.00	1.00
110	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–
Ldh-1															
88	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
90	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–
92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
96	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00
105	1.00	1.00	1.00	0.93	–	–	–	–	–	–	–	–	–	–	–
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ldh-2															
90	–	–	–	–	–	–	–	–	–	–	–	–	–	0.43	–
100	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00	0.57	1.00
108	–	–	–	–	–	–	0.06	–	–	–	–	–	–	–	–
113	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mdh-1															
100	–	–	–	–	–	0.62	0.04	–	0.87	0.86	0.45	0.33	0.08	–	–
110	–	–	–	–	–	–	0.07	–	–	–	–	–	0.17	0.44	1.00
113	–	0.09	–	0.29	–	–	–	–	–	–	–	–	–	–	–
120	1.00	0.91	1.00	0.71	1.00	0.38	0.89	1.00	0.13	0.14	0.55	0.67	0.75	0.56	–
125	–	–	–		–	–	–	–	–	–	–	–	–	–	–
Mdh-2															
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
120	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mdhp-1															
84	–	–	–	–	–	–	–	–	0.14	–	–	–	–	–	0.25
92	1.00	0.97	1.00	1.00	–	–	–	–	–	–	–	–	–	0.18	–
100	–	0.03	–	–	1.00	0.44	1.00	1.00	0.06	1.00	1.00	1.00	1.00	0.82	0.75
108	–	–	–	–	–	0.56	–	–	–	–	–	–	–	–	–
Mdhp-2															
86	–	–	–	–	0.09	–	–	–	–	–	–	–	–	–	–
96	–	0.08	–	–	0.91	1.00	0.93	1.00	–	–	–	–	–	0.06	–
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	0.94	1.00
103	1.00	0.92	0.96	1.00	–	–	0.07	–	–	–	–	–	–	–	–
105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
110	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–
Idh-1															
80	–	–	–	–	–	–	–	–	–	–	–	–	–	0.06	–
84	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
90	–	–	–	–	–	–	–	–	0.44	0.50	0.70	0.50	0.42	0.37	1.00
92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
95	–	–	–	–	0.01	–	0.04	–	–	–	–	–	–	–	–
100	–	–	–	–	0.98	0.36	0.88	0.12	0.56	0.50	0.30	0.50	0.58	0.57	–
105	1.00	0.97	1.00	1.00	0.01	0.64	0.08	0.88	–	–	–	–	–	–	–
108	–	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–

APPENDIX *Continued*

	Population (samples 1–14)														
Allele	m1	2	3	4	5	6	s1	7	8	9	10	11	12	13	14
Idh-2															
85	–	0.04	–	–	–	–	–	–	–	–	0.04	–	–	–	–
88	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
93	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00
106	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6Pgdh															
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00
102	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
103	–	–	–	–	1.00	1.00	0.81	1.00	–	–	–	–	–	–	–
105	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
110	–	–	–	–	–	–	0.19	–	–	–	–	–	–	–	–
Sod-1															
90	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
93	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sod-2															
96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Np															
80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
125	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
Aat-1															
85	–	–	–	–	–	–	–	–	–	–	–	–	–	0.06	–
96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	0.75	0.38	0.71	0.57	0.92	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
115	–	–	–	–	0.08	–	0.04	–	–	–	–	–	–	–	–
118	0.25	0.62	0.29	0.43	–	–	–	–	–	–	–	–	–	–	–
Aat-2															
88	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alat															
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00
110	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–
115	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
Ck															
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
106	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
Acph															
88	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–	–
95	1.00	1.00	0.95	1.00	–	–	–	–	–	–	–	–	–	–	–
97	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00
112	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–

APPENDIX *Continued*

	Population (samples 1–14)														
Allele	m1	2	3	4	5	6	s1	7	8	9	10	11	12	13	14
Ada															
96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00
104	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–
110	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
112	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ca															
90	1.00	1.00	0.96	1.00	–	–	–	–	–	–	–	–	–	–	–
97	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mpi-1															
85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
90	–	0.09	–	–	–	–	0.04	–	–	–	–	–	–	–	–
93	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
96	–	–	–	–	0.21	–	–	–	–	–	–	–	–	–	–
98	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	0.91	0.96	1.00	0.79	1.00	0.96	1.00	0.88	1.00	1.00	1.00	1.00	1.00	1.00
102	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–
105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
108	–	–	–	–	–	–	–	–	0.12	–	–	–	–	–	–
Mpi-2															
100	–	–	–	–	–	–	–	–	1.00	1.00	0.94	1.00	1.00	1.00	1.00
103	–	–	–	–	–	–	–	–	–	–	0.06	–	–	–	–
104	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–
Gpi															
93	–	0.06	0.04	0.14	–	0.06	–	0.27	–	–	–	–	0.17	0.14	–
100	1.00	0.94	0.96	0.86	1.00	0.04	1.00	0.73	1.00	1.00	1.00	1.00	0.83	0.86	1.00
Pgm-1															
90	0.25	0.10	–	–	–	–		–	–	–	–	–	–	–	–
95	0.75	0.90	1.00	1.00	0.03	0.92	0.06		–	–	–	–	–	–	–
100	–	–	–	–	0.97	0.08	0.94	1.00	1.00	1.00	0.86	0.83	1.00	0.63	–
105	–	–	–	–	–	–	–	–	–	–	0.14	0.17	–	0.37	1.00
Pgm-2															
85	–	0.15	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	0.85	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pt-1															
96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
98	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pt-3															
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
104	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
Pt-5															
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
108	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–
112	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Acr															
92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
106	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–

APPENDIX *Continued*

	Population (samples 17–32)															
Allele	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
G3pdh																
90	0.03	–	–	–	0.25	0.07	0.06	–	–	–	–	–	–	–	–	–
100	0.97	1.00	1.00	1.00	0.75	0.93	0.94	1.00	1.00	1.00	1.00	0.75	1.00	1.00	1.00	1.00
110	–	–	–	–	–	–	–	–	–	–	–	0.25	–	–	–	–
Ldh-1																
88	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
92	0.06	–	–	0.08	–	–	–	–	–	–	–	–	–	–	–	–
96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	0.94	1.00	1.00	0.92	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
110	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ldh-2																
90	–	0.04	–	0.07	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	0.96	1.00	0.93	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	0.70	0.65	1.00	1.00
108	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
113	–	–	–	–	–	–	–	–	0.03	–	–	–	0.30	0.35	–	–
Mdh-1																
100	0.03	–	–	0.19	–	–	–	–	–	–	–	–	–	–	–	–
110	–	0.04	0.43	0.23	1.00	1.00	1.00	1.00	–	–	–	–	–	–	0.10	0.14
113	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
120	0.97	0.96	0.57	0.58	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	0.90	0.86
125	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mdh-2																
100	0.97	1.00	1.00	1.00	1.00	1.00	0.89	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
110	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
120	–	–	–	–	–	–	0.11	–	–	–	–	–	–	–	–	–
Mdhp-1																
84	–	–	–	–	0.37	–	0.61	–	–	–	–	–	–	–	–	–
92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	0.63	1.00	0.39	1.00	0.93	1.00	1.00	1.00	0.50	0.62	0.75	1.00
108	–	–	–	–	–	–	–	–	0.07	–	–	–	0.50	0.38	0.25	–
Mdhp-2																
86	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
96	–	0.12	–	0.08	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	0.88	1.00	0.92	1.00	0.72	0.94	1.00	–	0.32	–	–	–	–	–	–
103	–	–	–	–	–	–	–	–	1.00	0.68	1.00	1.00	1.00	1.00	1.00	1.00
105	–	–	–	–	–	0.28	0.06	–	–	–	–	–	–	–	–	–
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Idh-1																
80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
84	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.10	–
90	0.14	0.27	0.14	0.38	0.25	0.17	–	–	–	–	–	–	–	–	–	–
92	–	–	–	–	–	–	–	–	–	0.03	0.15	–	0.50	0.76	0.30	0.50
95	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	0.82	0.73	0.57	0.62	0.75	0.83	1.00	1.00	1.00	0.97	0.85	0.92	0.50	0.24	0.60	0.50
105	0.04	–	0.29	–	–	–	–	–	–	–	–	0.08	–	–	–	–
108	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Idh-2																
85	–	–	–	–	0.25	–	–	0.10	–	–	–	–	–	–	–	–
88	–	–	–	–	–	–	–	–	0.73	0.93	1.00	1.00	0.50	0.56	1.00	1.00

APPENDIX *Continued*

	Population (samples 17–32)															
Allele	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
93	–	–	–	–	–	–	–	–	0.27	0.07	–	–	0.50	0.44	–	–
100	1.00	1.00	1.00	1.00	0.75	1.00	1.00	0.90	–	–	–	–	–	–	–	–
106	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6Pgdh																
100	1.00	0.85	1.00	1.00	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
102	–	–	–	–	–	0.06	0.06	0.20	–	–	–	–	–	–	–	–
103	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
105	–	0.15	–	–	1.00	0.94	0.94	0.80	–	–	–	–	–	–	–	–
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sod-1																
90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
93	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sod-2																
96	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
Np																
80	–	–	–	–	0.25	0.43	0.11	0.33	–	–	–	–	–	–	–	–
90	–	–	–	–	–	–	–	–	0.37	0.30	0.07	0.17	0.10	0.07	0.25	0.21
100	1.00	1.00	1.00	1.00	0.75	0.57	0.89	0.67	–	0.13	–	0.16	–	–	–	–
115	–	–	–	–	–	–	–	–	0.63	0.57	0.93	0.67	0.90	0.93	0.75	0.79
125	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aat-1																
85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
96	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
100	1.00	1.00	0.93	1.00	–	–	–	–	–	–	–	–	–	–	–	–
110	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–	–	–
115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
118	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aat-2																
88	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.93
115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.07
Alat																
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ck																
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
106	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Acph																
88	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
95	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
97	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
112	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ada																
96	–	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	0.63	0.30	0.83	0.33	–	–	–	–	–	–	0.20	–

APPENDIX *Continued*

	Population (samples 17–32)															
Allele	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
104	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
112	–	–	–	–	0.37	0.70	0.17	0.67	0.97	1.00	1.00	1.00	1.00	1.00	0.80	1.00
Ca-1																
90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
97	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	0.89	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
105	–	–	–	–	–	–	0.11	–	–	–	–	–	–	–	–	–
Mpi-1																
85	–	–	–	0.22	–	–	–	–	–	–	–	–	–	–	–	–
90	–	–	–	–	–	–	–	–	0.39	0.77	0.81	0.42	–	0.03	–	–
93	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–	–	–
96	–	–	–	–	–	–	–	–	0.07	0.03	–	0.08	–	–	–	–
98	–	–	–	–	–	–	0.17	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	0.75	1.00	1.00	0.77	1.00	0.54	0.13	–	0.42	1.00	0.97	1.00	1.00
102	–	–	–	–	–	–	–	–	–	0.07	0.19	0.08	–	–	–	–
105	–	–	–	–	–	–	0.06	–	–	–	–	–	–	–	–	–
108	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mpi-2																
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
103	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
104	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Gpi																
93	–	–	–	0.09	–	–	–	–	–	0.06	0.06	–	–	0.17	–	–
100	1.00	1.00	1.00	0.91	1.00	1.00	1.00	1.00	1.00	0.94	0.94	1.00	1.00	0.83	1.00	1.00
Pgm-1																
90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
95	–	0.19	0.33	–	–	–	0.06	0.50	–	0.36	0.25	0.92	–	–	0.75	0.14
100	0.20	0.04	–	0.63	1.00	0.89	0.94	0.17	1.00	0.59	0.75	0.08	1.00	1.00	0.25	0.86
105	0.80	0.77	0.67	0.37	–	0.11	–	0.33	–	0.05	–	–	–	–	–	–
Pgm-2																
85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pt-1																
96	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
98	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
Pt-3																
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
104	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pt-5																
100	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–	–
108	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
112	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Acr																
92	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
106	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–