Systematics and Biodiversity **1** (3): 327–337
DOI: 10.1017/S1477200003001221 Printed in the United Kingdom

Issued 16 February 2004

S. Carranza &
E. N. Arnold*
Department of Zoology, The Natural History Museum, London, Cromwell Road, London, SW7 5BD, UK

submitted April 2003
accepted July 2003

History of West Mediterranean newts, *Pleurodeles* (Amphibia: Salamandridae), inferred from old and recent DNA sequences

Abstract MtDNA sequences (396 bp cytochrome *b* and 369 bp 12S rRNA) from recent material and old museum specimens indicate *Pleurodeles poireti* and *P. waltl* form independent clades with 7.76% genetic divergence. Within *P. poireti*, populations from Djebel Edough, NE Algeria are very distinct with 6.12% genetic divergence from the remainder and may deserve separate species status. Away from Djebel Edough, *P. poireti* consists of three distinct clades (coastal NW Tunisia; central N Algeria; Constantine plus inland NW Tunisia) with a maximum genetic divergence of only 1%. *P. waltl* contains two clades with 2.96% genetic divergence, one in SE and E Spain plus north Morocco, the other in Portugal and SW and central Spain. *Pleurodeles* probably invaded NW Africa from SW Europe during the Messinian Salinity Crisis, when land contact was first established at 5.6 Ma, and then interrupted at 5.3 Ma. Molecular clocks, calibrated in the assumption that the latter event separated *P. waltl* and *P. poireti*, suggest that *Pleurodeles* diverged from its sister taxon, *Tylototriton*, at about 8.6–10 Ma. Djebel Edough *P. poireti* separated at about 4.2 Ma, perhaps through isolation on a temporary, now 'fossil', island initiated by the Messinian crisis. Differentiation in remaining *P. poireti* may have been caused by Pleistocene climatic fluctuations, while bifurcation in *P. waltl* appears to have taken place in the Pliocene approximately between 3.2 and 2 Ma. This species reached Morocco very recently, perhaps as a result of human introduction. Use in *Pleurodeles* of the slower divergence rates estimated in some other salamandrids results in a less parsimonious historical hypothesis that does not fit known geophysical events.

Key words ancient mtDNA, *Pleurodeles*, *Tylototriton*, *Chelotriton*, dispersal, land bridge, Messinian, fossil island, Djebel Edough

Introduction

In this paper we review the available fossil and other evidence for the relationships and minimum age of the West Mediterranean salamandrid newt genus *Pleurodeles*, and use mtDNA sequence to investigate the detailed phylogeny of the group. The two sources of information are then employed to speculate about the history of *Pleurodeles* including the origin of its present biogeographical pattern.

Current taxonomy of *Pleurodeles*

This genus has two currently recognized species. *P. waltl* is large (up to 300 mm in total length; Pasteur, 1958), with usually 15 presacral vertebrae, a tubercular process on rib 3 (and often traces of this structure on ribs 1 and 2), and sharp rib tips that project through a row of glandular swellings on the flanks. *P. waltl* is common and widely distributed in the southern two-thirds of the Iberian Peninsula and occurs with less abundance in northern Morocco, where it is found in the area delineated by Tangier, Casablanca and Alhoceima (Bons & Geniez, 1996). The second *Pleurodeles* species, *P. poireti*, is small (up to about 200 mm in total length; Pasteur, 1958), with 14 presacral vertebrae, no obvious tubercular processes on the ribs which are not sharp, and no glandular swellings on the flanks. It occurs in northern Tunisia and Algeria extending westwards as far as Oran.

Relationships and possible age of *Pleurodeles*

Morphology and mitochondrial DNA sequences (a total of about 1000 bp of 12S rRNA, 16S rRNA and valine transfer RNA) indicate that among living forms, the sister taxon of

*Corresponding author. Email: ena@nhm.ac.uk

Pleurodeles is the species of *Tylototriton* in South-East Asia, including the type species of that genus, *T. verrucosus* (Titus & Larson, 1995). Within the Salamandridae, *Pleurodeles* and *Tylototriton* form an assemblage with a number of European fossil taxa (Group II of Estes, 1981), which is characterized by extensive sculptured dermal bone on the skull and sometimes on the neural spines of the presacral vertebrae, complete frontosquamosal arches on the skull, and long ribs, at least some of which typically have dorsal tubercular processes. One genus in particular, the extinct *Chelotriton*, is very similar to *Tylototriton* and was widespread in Europe from the Eocene to the Pliocene (Estes, 1981; Bailon, 1989). Both these taxa have extensive dermal bone on the skull and forming expanded plaques on the neural spines of the vertebrae. Living *Tylototriton* differ from *Chelotriton* in their smaller body size, an ornamentation of pits and grooves on the cranial osteoderms instead of pustules (though some pitting may also occur in *Chelotriton robustus*; Estes, 1981), typically 15–16 presacral vertebrae instead of 13–14, a tubercular process present only on rib 3, ossified carpus and tarsus in at least large animals, and a narrower head.

Tylototriton has been reported from the middle Eocene of Germany, as *T. weigelti* Herre 1935. Individuals assigned to this taxon appear to have dermal ossification similar to that of modern *Tylototriton*, but the type is very small and all available specimens are incomplete. This means several critical features cannot be checked, including presacral number and degree of ossification of the carpus and tarsus. *T. weigelti* may in fact be a *Chelotriton* (Milner, 2000). The earliest specimens assigned to *Pleurodeles* are quite recent, coming from the Upper Miocene or Lower Pliocene of Spain (Sanchiz, 1977). It has been suggested (Herre, 1941) that the genus is related to *Palaeopleurodeles* Herre 1941 from the Upper Oligocene of Germany, but several critical features cannot be seen in known specimens of *Palaeopleurodeles*, so this association cannot be substantiated (see Estes, 1981; Milner, 2000).

The molecular evidence for the sister relationship of *Pleurodeles* and *Tylototriton* is also supported by shared morphological features that are apparently derived within the Group II salamandrids, including an ossified carpus and tarsus, and sometimes 15 presacral vertebrae. The sister relationship indicates that the exclusive *Pleurodeles* lineage too may possibly have arisen in the Upper Miocene. Its autapomorphies include reduction of osteodermal bone (completely so on the vertebrae) and in the size of the tubercular processes on the ribs. Comparison with *Tylototriton* and other salamandrids indicates that the very large size and sharp ribs of *P. waltl* are derived, as are the following features of *P. poireti*: small size, 14 presacral vertebrae, and lack of tubercular processes and of glandular swellings on the side of the body.

Material and Methods

Samples and DNA extraction

A total of 46 specimens of the family Salamandridae were used in this study (see Table 1 and Fig. 1). They included 19 *P. waltl* from the Iberian Peninsula and Morocco and 23 *P. poireti* from Algeria and Tunisia, while individuals of *Salamandra salamandra morenica*, *S. s. terrestris*, *Tylototriton taliangensis* and *Mertensiella luschani* were used as outgroups. Sequences of the last two species were obtained from Genbank. Ten of the *P. poireti* investigated were acquired between 1878 and 1888 by Ferdinand Lataste who bequeathed them, with the rest of his large herpetological collection, to George A. Boulenger at the Natural History Museum, London, where they were accessioned in 1920. Lataste apparently preserved his specimens in a distilled wine product, probably brandy. To prevent evaporation, he stored them in containers closed with a glass disc attached with putty; this was then covered with parchment that was tied with twine and finally sealed with black paint. In some cases, Lataste's containers have been left unopened to the present day, so the enclosed specimens have remained in what is essentially ethanol instead of being transferred to industrial methylated spirits (IMS), the usual preservative at the Natural History Museum. Because of this there was the possibility that Lataste's sealed specimens might still be viable sources of DNA, which is usually largely or wholly degraded by IMS. To avoid contamination, Lataste's animals were treated as ancient DNA samples. The opening of the bottles, handling of specimens, DNA extraction and PCR amplification were all carried out in isolation, following procedures previously successfully used in other attempts to obtain DNA from old Museum specimens (Carranza *et al*., 1999, 2001; Carranza & Arnold, 2003). Nine of the ten Lataste specimens investigated yielded adequate DNA that was not seriously degraded, even though they were collected well over a century ago. DNA was extracted from both Lataste and recent samples using the Tissue Extraction Kit from Quiagene. Primers used in both amplification and sequencing were: S1F (5′- TTC AAC TAC AAA AAC CTA ATG ACC C - 3′) and cytochrome b2 (Kocher *et al*., 1989) for cytochrome *b*, and 12Sa and 12Sb (Kocher *et al*., 1989) for 12S rRNA.

Phylogenetic analyses

Two data sets were used in the phylogenetic analyses. Data set I involved all 42 specimens of *Pleurodeles* from Table 1, plus *Mertensiella luschani* from Genbank and included 396 bp of the cytb and 369 bp of the 12S rRNA genes. Data set II involved a representative of each of the six main *Pleurodeles* clades that were discovered by analysis of data set I (highlighted in Figs 1 and 2), plus *S. s. terrestris*, *S. s. morenica*, *M. luschani* and *Tylototriton taliangensis*; it included 346 bp of the cytb gene. Analysis of this second data set was necessary to permit the inclusion of *Tylototriton*, the sister of *Pleurodeles*, because the areas of sequence available for this taxon are different from the other samples. Analysis of data set II provided a check that tree topology of *Pleurodeles* was not changed by inclusion of its closest relative and permitted an estimate to be made of the age of the node at which *Pleurodeles* and *Tylototriton* separated.

DNA sequences were aligned using ClustalX (Thompson *et al*., 1997) with default parameters. No gaps were included in the cytochrome *b* (cytb) alignment and no stop codons were observed when the sequences were translated into amino acids

Taxa	Locality	BMNH number	Collector and Year	Genbank Accession number (Cytb/12S)	Genbank sequence code
S. s. morenica	La Parra (Badajoz)			AY222502/AY222458	E171243
S. s. terrestris	Badalona (Barcelona)			AY222503/AY222459	E171249
P. waltl –1	Extremos (Portugal)			AY222515/AY222471	E10114
P. waltl –2	Aroche; Huelva (Spain)			AY222516/AY222472	E10115
P. waltl –3	San Martín de Valdeiglesias; Avila (Spain)			AY222525/AY222481	E181219
P. waltl –4	Bélmez/Hinojosa; Córdoba (Spain)			AY222534/AY222490	E181229
P. waltl –5	Perelló; Tarragona (Spain)			AY222531/AY222487	E181224
P. waltl –6	Genave; Jaén (Spain)			AY222537/AY222493	E181231
P. waltl –7	Marshes of Charf-la-Kaab, 10 miles from Tanger (Morocco)	Lat. Coll. 1920.1.20.3591	Vaucher, 1887	AY222509/AY222465	Anc18
P. waltl –8	Morhane (Morocco)			AY222512/AY222468	E10111
P. waltl –9	Morhane (Morocco)			AY222513/AY222469	E10112
P. waltl –10	Charf-la-Kaab, Tanger (Morocco) 37.77155S/5.78785W			AY222523/AY222479	E181216
P. waltl –11	Charf-la-Kaab, Tanger (Morocco) 37.77155S/5.78785W			AY222524/AY222480	E181217
P. waltl –12	Near Jerez de La Frontera; Cádiz (Spain)			AY222527/AY222483	E181220
P. waltl –13	Quesada/Jodar, Jaén (Spain)			AY222536/AY222492	E181230
P. waltl –14	Near Jerez de La Frontera; Cádiz (Spain)			AY222528/AY222484	E181221
P. waltl –15	Torregorda; Cádiz (Spain)			AY222529/AY222485	E181222
P. waltl –16	South of Laguna de Medina; Cádiz (Spain)			AY222530/AY222486	E181223
P. waltl –17	Lag. de San Lázaro, Villamanrique de la Condesa; Sevilla (Spain)			AY222532/AY222488	E181225
P. waltl –18	Puerto Alazores; Granada (Spain)			AY222533/AY222489	E181228
P. waltl –19	Tanger (Morocco)			AY222514/AY222470	E10113
P. poireti –20	Annaba, Djebel Edough (Algeria)	Lat. Coll. 1920.1.20.1327	Hagenmüller, 1881	AY222507/AY222463	Anc13
P. poireti –21	Annaba, Djebel Edough (Algeria)	Lat. Coll. 1920.1.20.1369	Hagenmüller, 1881	AY222508/AY222464	Anc14
P. poireti –22	Tabarca (Tunisia)			AY222518/AY222474	E181210
P. poireti –23	Tabarca (Tunisia)			AY222519/AY222475	E181211
P. poireti –24	Tabarca (Tunisia)			AY222520/AY222476	E181212
P. poireti –25	5 Km. West of Nefza (Tunisia)			AY222522/AY222478	E181214
P. poireti –26	5 Km South of Tabarca (Tunisia) 36.90778S/8.74952W			AY222543/AY222499	E18129
P. poireti –27	5 Km South of Tabarca (Tunisia) 36.90778S/8.74952W			AY222542/AY222498	E18128
P. poireti –28	Larba (Algeria)	Lat. Coll. 1920.1.20.3824	Lallemant, 1888	AY222504/AY222460	Anc10
P. poireti –29	Bejaïa (Algeria)	Lat. Coll. 1920.1.20.1750	Hagenmüller, 1882	AY222505/AY222461	Anc11
P. poireti –30	Larba (Algeria)	Lat. Coll. 1920.1.20.1511	Lallemant, 1881	AY222510/AY222466	Anc8
P. poireti –31	Alger (Algeria)	Lat. Coll. 1920.1.20.18	Lasserre, 1878	AY222511/AY222467	Anc9
P. poireti –32	Theniet El-Had (Algeria)	Lat. Coll. 1920.1.20.1529	Bedel, 1884	AY222544/AY222500	E30091
P. poireti –33	Theniet El-Had (Algeria)	Lat. Coll. 1920.1.20.2539	Bedel, 1884	AY222545/AY222501	E30092
P. poireti –34	Constantine (Algeria)	Lat. Coll. 1920.1.20.1504	Henon, 1881	AY222462/AY222506	Anc12
P. poireti –35	Jendouba (Tunisia) 36.4941S/8.76945E			AY222540/AY222496	E18126
P. poireti –36	Near Fernana (Tunisia) 36.64087S/8.69703E			AY222541/AY222497	E18127
P. poireti –37	Dam Bou Heurtma (Tunisia) 36.6739S/8.75785E			AY222535/AY222491	E18123
P. poireti –38	Dam Bou Heurtma (Tunisia) 36.6739S/8.75785E			AY222538/AY222494	E18124
P. poireti –39	Ain Draham (Tunisia) 36.70915S/8.68623E			AY222521/AY222477	E181213
P. poireti –40	Dam Bou Heurtma (Tunisia) 36.6739S/8.75785E			AY222526/AY222482	E18122
P. poireti –41	Dam Bou Heurtma (Tunisia) 36.6739S/8.75785E			AY222517/AY222473	E18121
P. poireti –42	Dam Bou Heurtma (Tunisia) 36.6739S/8.75785E			AY222539/AY222495	E18125

Table 1 Details of material and sequences used in the present study. Numbers after the species names refer to localities shown in Fig. 1. BMNH – Natural History Museum, London; Lat. Coll – Lataste Collection.

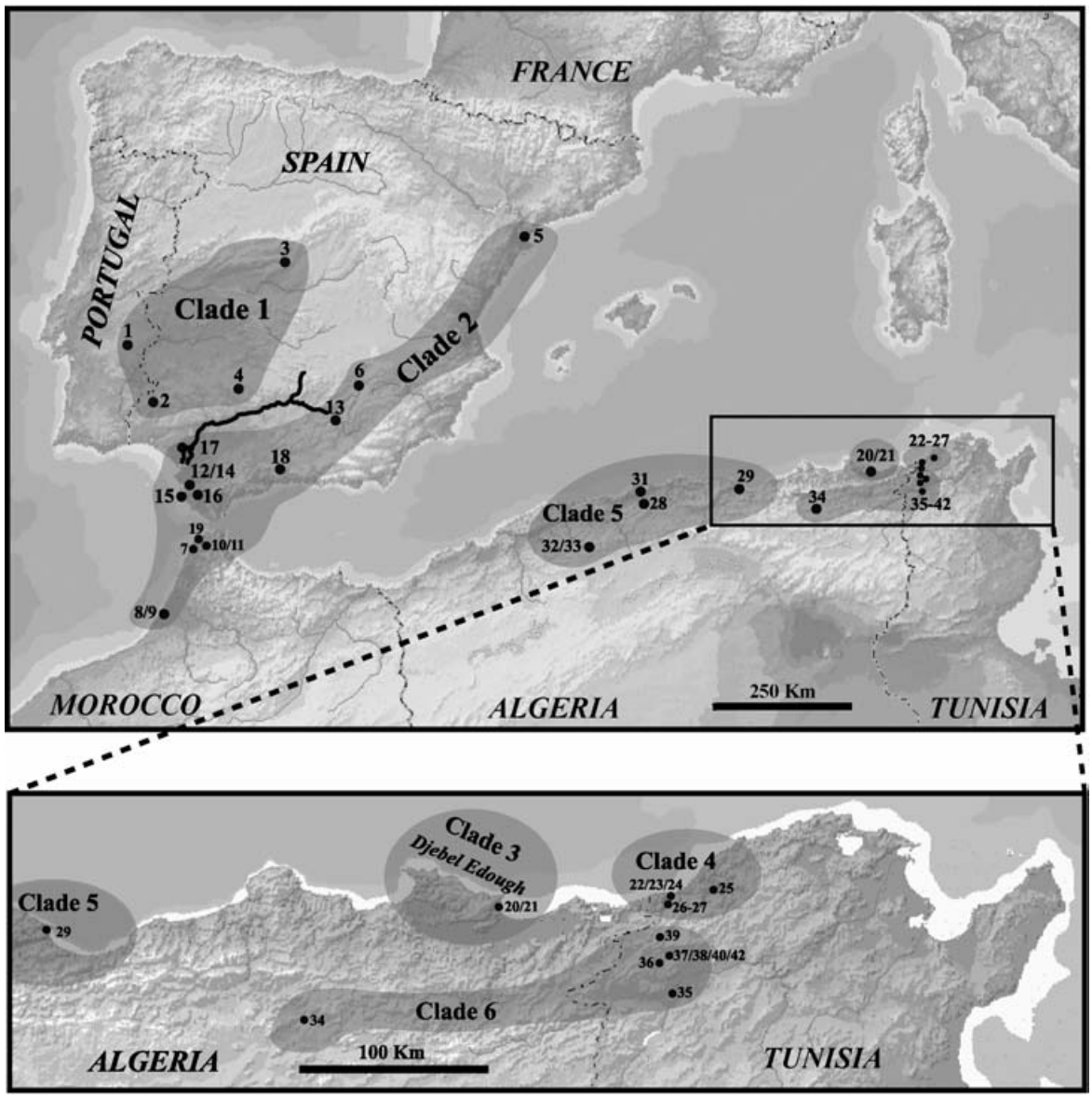

Figure 1 Map of the Iberian Peninsula and North Africa showing localities of *Pleurodeles* samples used in the present study, see Table 1 and Fig. 2 for further details. The Guadalquivir River has been highlighted in black.

using the vertebrate mitochondrial code. This suggests that all the cytb sequences analysed were functional. All the *Pleurodeles* 12S rRNA sequences were of the same length and only two gaps had to be postulated to align the three outgroup sequences with the ingroup.

Three methods of phylogenetic analysis were employed for both data sets and their results compared. These were maximum-likelihood (ML), Bayesian analysis, and Maximum-parsimony (MP). Modeltest (Posada & Crandall, 1998) was used to select the most appropriate model of sequence evolution for the ML and Bayesian analyses, under the Akaike Information Criterion. For both data sets this was the General Time Reversible model (GTR) taking into account the shape of the Gamma distribution (G). Bayesian phylogenetic analyses were performed with MRBAYES v. 2.01 (Huelsenbeck & Ronquist, 2001) using the GTR+G model of sequence evolution (see above) with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. All analyses started with randomly generated trees and ran for 2.5×10^6 generations, with sampling at intervals of 100 generations that produced 25 000 sampled trees. To ensure that the analyses were not trapped on local optima, the data set was run three times independently, each run beginning with a different starting tree. The log-likelihood values of the 25 000 trees in each analysis were plotted against the generation time. All the trees produced prior to reaching stationarity were discarded, making sure that burn-in samples were not retained. Although stationarity was reached very rapidly (data not shown), only the last 5000 trees in each of the three independent analyses were used to estimate separate 50% majority rule consensus trees for these. The frequency of any particular clade, among the individual trees contributing to the consensus tree, represents the posterior probability of that clade (Huelsenbeck &

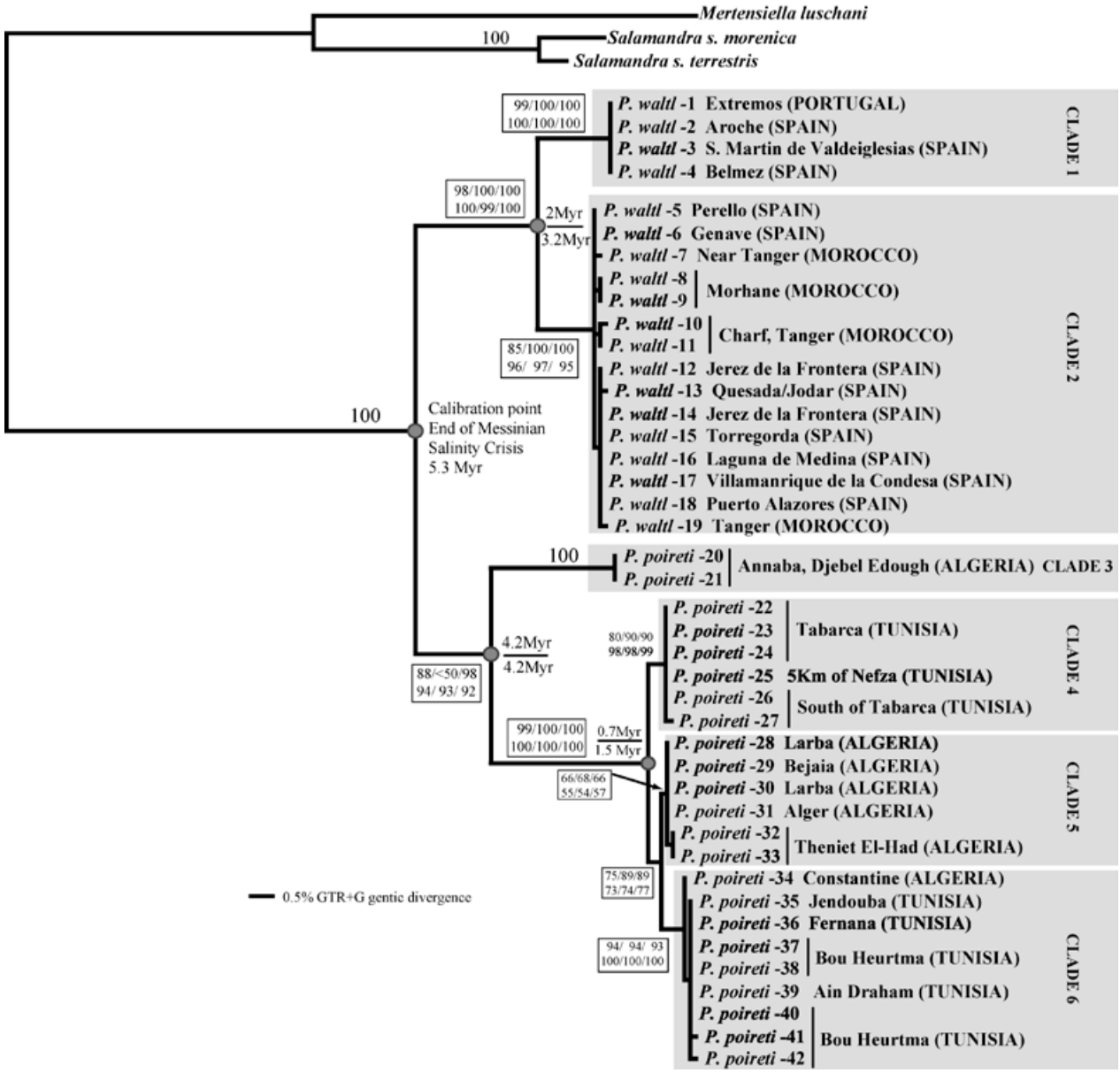

Figure 2 ML tree for *Pleurodeles* (Log likelihood − 2291.95312, GTR+G model of sequence evolution) inferred from data set I. Bootstrap support and posterior probabilities for particular nodes are shown in the boxes, with the figures indicating the percentage support for different analyses. Upper left, bootstrap support derived by ML (GTR+G). Upper middle, bootstrap support derived by MP (ts = tv). Upper right, bootstrap support derived by MP (ts = 1; tv = 6). Lower left, posterior probability values derived from Bayesian analysis (1st replicate). Lower middle, posterior probability values derived from Bayesian analysis (2nd replicate). Lower right, posterior probability values derived from Bayesian analysis (3rd replicate). Estimated dates are given for most important bifurcations, which are marked by filled circles. Dates above the line are derived from Kimura 2-parameter genetic distances, those below are calculated using the NPRS method implemented in r8s (see Material and Methods).

Ronquist, 2001); only values above 95% were regarded as indicating that clades were significantly supported.

Both ML and MP analyses were performed in PAUP* 4.0b10 (Swofford, 1998) and included heuristic searches involving tree bisection and reconnection (TBR) branch swapping with 100 random stepwise additions of taxa. Gaps were included as a fifth state. In the MP analyses of data set I, transversions were given the same weight as transitions and six times that weight. For data set II transversions were given the same weight and nine times that weight. This weighing scheme was applied in order to correct for the observed ts/tv ratio of data sets I and II. Nodal support of the ML and MP results was assessed by bootstrap analysis (Felsenstein, 1985) involving 1000 pseudo-replications. The incongruence length difference (ILD) test (Mickevich and Farris, 1981; Farris *et al*., 1994) was used to check for incongruence between the genes used. In this test, 10 000 heuristic searches were made and invariable characters were removed before starting the analysis (Cunningham, 1997).

Because it was intended to establish approximate dates for events on the phylogeny of *Pleurodeles*, the likelihood ratio test (Huelsenbeck & Crandall, 1997) was used to assess

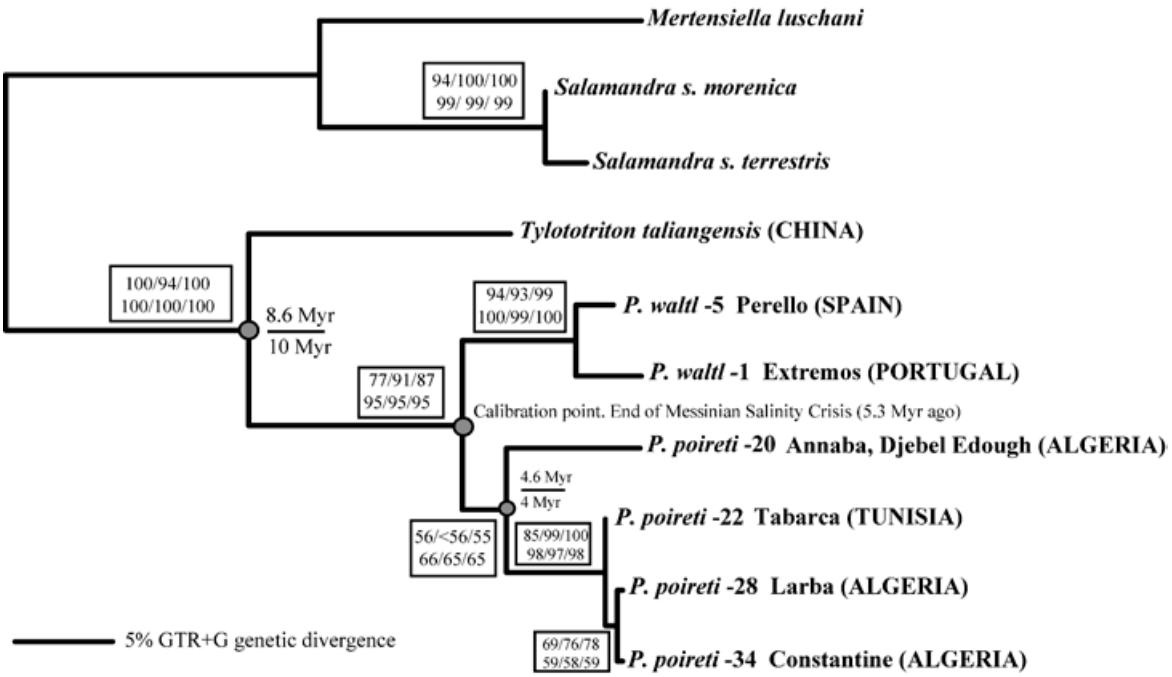

Figure 3 ML tree for *Pleurodeles* (Log likelihood –1244.90457, GTR+G model of sequence evolution) inferred from data set II. Bootstrap support and posterior probabilities for particular nodes are shown in the boxes with the figures indicating the percentage support for different analyses. Upper left, bootstrap support derived by ML (GTR+G). Upper middle, bootstrap support derived by MP (ts = tv). Upper right, bootstrap support derived by MP (ts = 1; tv = 9). Lower left, posterior probability values derived from Bayesian analysis (1st replicate). Lower middle, posterior probability values derived from Bayesian analysis (2nd replicate). Lower right, posterior probability values derived from Bayesian analysis (3rd replicate). Estimated dates are given for some bifurcations, which are marked by filled circles. Dates above the line are derived from Kimura 2-parameter genetic distances, those below are calculated using the NPRS method implemented in r8s (see Material and Methods).

the statistical significance of the difference between the log likelihood of the trees calculated with and without molecular clock assumptions. Absence of significant difference would indicate gene evolution within the phylogeny was clocklike. Divergence times on trees were also estimated using the Nonparametric Rate Smoothing (NPRS) method (Sanderson, 1997) implemented in the program r8s. The topology used to calculate the dates here were the ML trees shown in Figs 2 and 3 with GTR+G branch lengths.

Results

Analysis of data set I

A total 765 bp (396 bp of cytb and 369 bp of 12S rRNA) were used in the analyses. Of these, 181 bp were variable and 146 parsimony-informative. The ILD test showed that the two gene fragments were congruent (ILD $P > 0.53$) and could consequently be combined in total evidence analyses. The results of these are summarized in Fig. 2. ML and Bayesian analyses produced almost identical topologies that recognized all six major clades highlighted in Fig. 2. The only differences were slight variations in the arrangement of samples within these clades. The two MP analyses, involving different relative weightings of transversions (tv) and transitions (ts) produced trees that recognized all six main clades shown in Fig. 2. Values for the MP trees when uninformative sites were excluded are: MP (tv = 6 ts = 1): two trees of 488 steps, CI (consistency index) = 0.867, RI (retention index) = 0.975; MP (tv = ts): eight trees of 218 steps, CI = 0.794, RI = 0.964.

The most basal bifurcation separates the populations presently placed in *P. poireti* from those assigned to *P. waltl* (7.76% genetic divergence for cytb + 12S rRNA genes). Within the *P. poireti* clade there is a deep division (6.12% genetic divergence) between samples from Annaba (previously Bône) in the Djebel Edough region, on the northeast Algerian coast 70 km from the Tunisian border (clade 3, Fig. 2), and all other material (clades 4–6). The degree of genetic divergence found between Djebel Edough *Pleurodeles* and all the rest of *P. poireti* analysed approaches that separating the two species currently recognized in the genus. It may consequently be appropriate to assign species status to the Djebel Edough population as well, especially as it appears to be morphologically distinct (S. Carranza & E. O. Z. Wade, unpubl. data).

The remaining *P. poireti* form a well-supported monophyletic group made up of three distinct units with a maximum genetic divergence of only 1% (Table 2). Clade 4 contains populations to the north of the Medjerda Mountains in coastal northwest Tunisia; clade 5 ones from central north Algeria, and its sister clade 6 those from Constantine in northeast Algeria (locality 34, Fig. 1) and from inland northwest Tunisia (in and south of the Medjerda Mountains).

	K2P mean genetic distances			Geographical distance between studied localities (Km)	
	Cytb+12S	Cytb	12S	maximum	minimum
Within Clade 1	0.00	0.00	0.00	350	100
Within Clade 2	0.20	0.19	0.21	950	20
Within Clade 3	0.00	0.00	0.00	0	0
Within Clade 4	0.04	0.00	0.09	25	5
Within Clade 5	0.06	0.00	0.12	300	20
Within Clade 6	0.08	0.05	0.12	195	3
Within Morocco	0.31	0.35	0.27	150	4
P. waltl Clades 1–2 *vs P. poireti* Clades 3–6	7.76	10.38	5.06	1500	600
P. waltl Clade 1 *vs P. waltl* Clade 2	2.96	4.60	1.27	727	95
P. poireti Clade 3 *vs P. poireti* Clade 4–6	6.12	8.35	3.82	500	85
P. poireti Clade 4 *vs P. poireti* Clade 5–6	1.00	0.75	1.25	636	18
P. poireti Clade 5 *vs P. poireti* Clade 6	0.82	0.73	0.91	620	142

Table 2 Kimura 2-parameter genetic distances (%) within and between populations (see Fig. 1).

Within *P. waltl* there is also a bifurcation (2.96% genetic divergence). This separates animals from the southeast and east of Spain plus northern Morocco (clade 2) from those in Portugal and southwest and central Spain (clade 1). All the six distinct clades within *Pleurodeles* are genetically quite homogeneous across large geographical distances (see Figs 1, 2 and Table 2).

When the log likelihood value of the ML (GTR+G) tree from Fig. 2 (−2291.95312) was compared with the log likelihood of the same tree constructed under molecular clock assumptions (−2300.7860), there was no significant difference between the two (likelihood ratio test statistic, $-2\log\Delta = 17.6658$, which approximates to a χ^2_{43} distribution under the null hypothesis; $P < 0.05$). The ML tree can therefore be used for estimating dates. The Kimura 2-parameter (K2P) correction was used, to make results comparable with other genetic distances and clock calibrations of other groups previously used by the authors (Carranza *et al*., 2000; Rando *et al*., in press). Relevant K2P genetic distances between the six main clades, and the genetic variability within them are shown in Table 2.

The separation of African *P. poireti* from the essentially Iberian *P. waltl* is used as a calibration point and, for reasons given later, this event is dated at 5.3 M.y. On the basis of the 7.76% difference between the combined cytb and 12S rRNA fragments of the two species, the mean divergence rate is estimated as 1.46% per million years. When the two gene fragments are treated independently, the rates are respectively 1.95% and 0.95%. The Nonparametric Rate Smoothing (NPRS) method produced a similar date to that based on the Kimura 2-parameter genetic distances (Fig. 2) for the split between the Djebel Edough and the other *P. poireti* populations, but dates for later events were distinctly older.

Analysis of data set II

Out of the 346 bp of cytb aligned 111 were variable and 80 parsimony-informative. The results of the phylogenetic analyses are shown in Fig. 3. Maximum Parsimony, Maximum Likelihood and Bayesian analysis all produced identical trees. Among the restricted number of salamandrid taxa included, *Tylototriton* is sister to *Pleurodeles* with high bootstrap support and posterior probability values, in agreement with analyses involving all salamandrid data (Titus & Larson, 1995). Despite the restricted amount of sequence used, the topology within *Pleurodeles* is identical to that obtained from analysis of data set I (Fig. 2). Values for the MP trees excluding uninformative sites are as follows: MP(ts = tv) two trees of 151 steps, CI = 0.669, RI = 0.718; MP(tv = 9, ts = 1) one tree of 423 steps, CI = 0.7, RI = 0.845. The log likelihood of the ML tree from Fig. 3 was not significantly different to the log likelihood of the ML tree inferred under the assumption of a molecular clock ($-2\log\Delta = 4.07$, which approximates to a χ^2_8 distribution under the null hypothesis; $P < 0.05$) and therefore the short cytb fragment could be used to estimate the approximate age of the split between *Tylototriton* and *Pleurodeles*. The calibration point employed was the same as for data set I (see above, and Fig. 2). The rate of molecular evolution for the 346 bp of cytb analysed was 2.0% per million years, slightly higher than the rate calculated for the same gene using data set I. This difference probably results from the different amounts of cytb sequence used in data sets I (396 bp) and II (346 bp).

Discussion

The relationship of *Pleurodeles* successively to *Tylototriton* and to other members of the Group II assemblage of salamandrids, which has been present in Europe as far back as the Eocene, indicates that it arose in Europe and only later spread into its African range. As fossils suggest *Pleurodeles* may have arisen only late in the Miocene, there are two primary routes by which it may have spread into Africa. One is via the land bridge between Europe and Africa that has existed more or less continuously from the mid-Miocene. This arose when the

eastern end of the Mediterranean closed at 19–15 Ma, as a result of the northeast part of the African plate colliding with the Turkish plate (Adams *et al*., 1983; Rögl, 1999), permitting biotic interchange between nearby areas of Eurasia and Africa.

The second route involved a transient land contact at the other extremity of the Mediterranean Sea between the Iberian Peninsula and northwest Africa, right at the end of the Miocene. This was responsible for the event known as the Messinian Salinity Crisis. About 5.59 Ma, tectonic uplift of more than 1000 m along the African and Iberian continental margins formed the Gibraltar arch producing a land bridge. This closed the two marine gateways between the Atlantic Ocean and the Mediterranean Sea that existed in the Miocene (Duggen *et al*., 2003) and isolated the Mediterranean. Without input from the Atlantic, its surface level fell by over 1000 m, perhaps in less than 1000 years (Hsü, 1972; Hsü *et al*., 1973; Blondel & Aronson, 1999; Krijgsman *et al*., 1999*a*). The fall desiccated large areas of the Mediterranean Sea bed which were subsequently partly covered with freshwater sediments brought in by rivers. The end of the Messinian Salinity Crisis at 5.3 Ma was caused by the collapse of the Gibraltar arch which opened the Strait of Gibraltar. This allowed the entire Mediterranean basin to fill again in less than 100 yr (Hsü, 1972; Hsü *et al*., 1973, 1977; Blondel & Aronson, 1999; Krijgsman *et al*., 1999*a*) and broke the land connection which had existed for nearly 300 k.y.

Of the two possible routes into northwest Africa, the first is unlikely to have been used by *Pleurodeles*. There is no evidence, either in the form of fossils or relict populations, that this genus ever existed east of the Iberian Peninsula or northwest Africa. In other amphibian groups that occur more widely in the Mediterranean area and have been adequately studied, the closest relatives of northwest African populations are in the Iberian Peninsula, making it the most likely source area. This is true, for example for *Discoglossus* (Garcia-París & Jockusch, 1999), *Alytes* (Arntzen & Garcia-París, 1995), Rana (Beerli *et al*., 1996), *Pelobates* and *Bufo bufo* (S. Carranza & E. N. Arnold, unpubl. data).

For these reasons, it is hypothesized that *Pleurodeles* entered northwest Africa across the Gibraltar land bridge and gene flow between *P. waltl* and *P. poireti* was interrupted when the Mediterranean Sea refilled at 5.3 Ma. This event is used to calibrate the molecular clocks employed in the present study (p. 333). The clock based on data set II indicates that *Pleurodeles* and *Tylototriton* separated at 8–10 Ma, a date compatible with available fossil evidence, which suggests an origin for the clade containing these genera in the Late Miocene.

As parts of the desiccated Mediterranean sea floor were covered with freshwater sediments, the ancestor of the *P. poireti* clade may have been able to cross directly from southeast Spain to western Algeria, rather than via the land bridge into Morocco. Once separated from *P. waltl*, the *P. poireti* clade developed its characteristic morphological autapomorphies (see p. 328) before bifurcating into a very widely distributed monophyletic unit made up of the great majority of *P. poireti* populations (clades 4–6; see Figs 1 and 2), and that on Djebel Edough (clade 3).

The distinct nature of Djebel Edough *Pleurodeles* could have arisen in a number of ways, but the simplest would be by the massif being isolated from the rest of Algeria long enough for speciation to occur. This is possible as the present geography of Djebel Edough indicates it may be a fossil island. Fossil islands consist of areas of land that were once isolated by sea but later became continuous with other usually larger land masses. In the Mediterranean area, they originated at various times and were often inhabited by endemic animals. For example, Monte Gargano, which is now part of the Adriatic coast of Italy, was isolated for a considerable period beginning in the Miocene, during which time a distinctive fauna evolved, including murine rodents, *Microtia* (Giuli & Torre, 1984) and a giant hedgehog, *Deinogalerix* (Butler, 1980). Other islands were formed in the Messinian. Rivers flowing into the Mediterranean in this period cut deep canyons (Said, 1990, 1993) which occasionally separated a coastal area from its hinterland (Hsü, 1972; Hsü *et al*., 1973, 1977; Blondel & Aronson, 1999), for instance if the canyons of adjacent rivers coalesced. When the Mediterranean basin filled again at the end of the Messinian, such coastal areas sometimes became real islands as the encircling canyons filled with salt water. On occasion, the canyons subsequently became choked with fluvial deposits, rejoining the islands to their hinterlands and producing a characteristic topology in which a coastal massif is separated from high ground further inland by rivers and their low-lying plains. This is true for the Djebel Edough massif which is divided from the rest of Algeria by lowlands that contain streams, marshes and the 13 km by 10 km Lake Fzara. However, the molecular clock derived from data set I indicates that the actual separation of the Djebel Edough population occurred at 4.2 Ma, some time after the end of the Messinian Salinity Crisis. So perhaps any canyons around Djebel Edough were not initially entirely continuous and final land connection was only broken rather later.

All the other *P. poireti* populations (clades 4–6) are genetically similar despite having an ancestral lineage that is an estimated 4.2 Ma old. Their high genetic similarity may result from this lineage occupying a very restricted geographical area for most of its history, only spreading and diversifying over north Algeria and Tunisia quite recently. Alternatively, the ancestral lineage may have been widespread since its origin, occupying many of the humid parts of the Maghreb where the only other urodele is *Salamandra algira*. If so, it must have formed an essentially panmictic unit in which genetic homogeneity was maintained by gene flow between at least intermittently continuous populations. On present evidence, it is not possible to decide between these alternatives, although the second seems more plausible.

Whatever its previous history, this lineage underwent divergence in the Pleistocene, separating into three units (clades 4, 5 and 6). As noted, the most divergent populations (clade 4) occur north of the Medjerda mountains (Pasmans *et al*., 2002). They differ genetically by more than 1% from populations in and south of these mountains (clade 6), the nearest of which is less than 18 km away. As well as being genetically distinct, the populations of clade 4 are morphologically differentiated, being significantly smaller than *P. poireti* from further south in Tunisia (Pasmans *et al*., 2002). Clade

6 also extends at least about 200 km westwards into eastern Algeria, to Constantine. It is most closely related (0.82% genetic divergence) to clade 5, which occurs in central north Algeria and has known populations 140–620 km west of Constantine.

It is likely that the Medjerda Mountains acted as a barrier for some time in the Pleistocene (between 0.7–1.5 Ma). They probably interrupted gene flow between clade 4 and the rest of the populations in Tunisia and Algeria that constitute clades 5 and 6, which themselves separated from each other at a rather later date in the Pleistocene. This separation may have been caused by the development of a geographical barrier or may have resulted from one or more of the climatic fluctuations frequent in the western Palaearctic during the Quaternary. These promoted genetic and morphological differentiation by changing the distribution and demography of many species (Hewitt, 1996, 2000; Veith *et al*., 2003). As the maximum geographic distance between clades 5 and 6 is only 120 km (Table 2), it is unlikely that their reciprocal monophyly is due to a sampling artefact. Any populations subsequently found in the intervening area may elucidate the causes of the separation of these clades.

Like the *P. poireti* clade, *P. waltl* also developed its own morphological autapomorphies (see p. 328) and then underwent bifurcation, although this was at a later date, between 3.2 and 2 Ma. The split produced one form in western and central Iberia (clade 1), and another in the southeast and east of Spain (clade 2), which also occurs in northern Morocco. Bifurcations also appear to have taken place within the same area of the Iberian Peninsula in several other groups of amphibians and reptiles including *Salamandra* (Garcia-París *et al*., 1998), *Discoglossus* (Garcia-París & Jockusch, 1999), *Alytes* (Arntzen & Garcia-París, 1995) and *Lacerta lepida* and *Psammodromus* (Carranza & Arnold, unpubl. data).

The near identity of Moroccan *P. waltl* populations with those from southeastern and east Iberia indicates that their ancestors reached Africa comparatively recently and long after the disappearance of the land connection associated with the Messinian Salinity Crisis. Because amphibians are very unlikely to cross salt water, the Moroccan populations may result from anthropogenic introductions that could have been accidental. The Lataste specimen collected in the marshes of Charf-la-Kaab in 1887 by Vaucher (see table 1) and identified as part of clade 2 in our study, confirms that individuals of *P. waltl* genetically very similar to the populations from the southeastern and eastern Iberian Peninsula have been present in Morocco for well over a century.

The calibration used above gives rates of genetic divergence considerably faster than those estimated for other salamandrids. In *Euproctus* newts, the same region of cytb employed here was calibrated against a vicariance event, the Oligocene separation of Corsica and Sardinia from southern France, which is assumed to have isolated the ancestor of the endemic species on these islands from that of their mainland congener. This gave a divergence rate of 0.7% per Myr (Caccone *et al*., 1997). A similar, maximum figure for the cytb fragment was also obtained for the separation of the North American *Taricha* and *Notophthalmus*, using fossils of known stratigraphy for calibration (Tan & Wake, 1995). However, the monophyly of *Euproctus* has not been firmly established and other taxa (*Triturus* spp. and *Neurergus*) may actually be included in the clade (Caccone *et al*., 1994, 1997; Steinfartz *et al*., 2002), raising the possibility that later, unappreciated nodes may really represent the vicariance event. In the second case, the fossils used for calibration of *Notophthalmus* are isolated vertebrae (Estes, 1981), so identity is not necessarily strongly corroborated. There is also the potential problem that such ancient events might not be appropriate for calibrating relatively fast evolving mitochondrial genes that may have become saturated over the periods concerned (Avise, 2000). Finally, *Euproctus* and *Notophthalmus–Taricha* are not very closely related to *Pleurodeles* (Titus & Larson, 1995). This makes calibration based on events within the *Pleurodeles* clade preferable, as rates may vary among distant taxa.

If, in spite of these reservations, the slower rates for the cytb gene are applied to the *Pleurodeles* phylogeny, the initial split between the *P. waltl* and *P. poireti* clades would have occurred at roughly 14 Ma or more. At this time, there was no direct land connection between the Iberian Peninsula and northwest Africa. Southern Spain and Morocco were quite close to each other, with a set of islands that would later become the Betic and Rifian mountains between them. But the islands were separated from the mainlands by two main sea corridors. The Betic corridor in the north divided the Iberian mainland from the "Betic island", and the Rifian corridor in the south divided the "Rifian island" from northern Morocco (Weijermars, 1988, 1991; Krijgsman *et al*., 1999*a*, 1999*b*, 2000; Krijgsman & Langereis, 2000; Duggen *et al*., 2003). The Betic corridor only closed at about 7.2 Ma (Krijgsman *et al*., 1999*a*, 2000), while the Rifian corridor was only obliterated more than 1 m.y. later (Krijgsman *et al*., 1999*a*; Krijgsman & Langereis, 2000).

There is consequently no evidence of events at 14 Ma that would have enabled *Pleurodeles* to extend southwards into an area outside the Iberian peninsula and become isolated there as the ancestral population of *P. poireti*. If this lineage had reached Morocco, there would have been a population of *P. poireti* that became extinct in this area after spreading eastwards into Algeria. An alternative possibility is that *P. waltl* and *P. poireti* separated within the Iberian Peninsula but there is no evidence for this and it would again imply extinction of the original *P. poireti* population.

Applying the slower rate of divergence, the division of the Djebel Edough population from the rest of *P. poireti* would have taken place at approximately 12 Ma, at a time when there was no obvious geophysical mechanism that might have produced this. On the same basis, the separation of the *P. waltl* clade into two lineages would have occurred at roughly 6 Ma around the time of the Messinian Salinity Crisis. It could be hypothesized that the division was caused by *P. waltl* entering Morocco at this period and then being isolated to produce a second form that then reached Spain very recently. If so, it must have spread extremely rapidly and extensively there, perhaps replacing its sister form, which remained in Iberia, over large areas.

In summary, using a slower rate of gene evolution than that calibrated with the Messinian Salinity Crisis, results in a historical hypothesis that is much less parsimonious than the

one outlined earlier in this discussion. It involves additional events including an extinction, two colonizations of North Africa instead of one, and possible partial species replacement. Furthermore, most bifurcations in the phylogeny do not correlate with known geophysical events that could have caused them.

Acknowledgements

We are indebted to J. Roca for technical support during the later stages of this project and to the authors of the "Atlas de Andalucia" (J. P. Gonzalez, J. M. Barnestein and D. Donaire) for all the samples of *Pleurodeles* from Southern Spain used in this study. We would also like to thank Edward Wade, Soumia Fahd, R. Fonoll and David Herbert for their help and advice in various matters concerning this work. We are also very grateful to J. Bartley and C. Griffin who run the sequencing facility at the Natural History Museum, London. This project was supported by grants from the Natural Environment Research Council (NERC) NER/B/S/2000/00714 and NER/A/S/2001/00511.

References

ADAMS, C.G., GENTRY, A.W. & WHYBROW, P.S. 1983. Dating the terminal Tethyan event. In: MEULENKAMP, J. E., Ed., *Reconstruction of Marine Paleoenvironments. Utrecht Micropaleontological Bulletin* **30**, 273–298.

ARNTZEN, J.W. & GARCIA-PARÍS, M. 1995. Morphological and allozyme studies of midwife toads (genus *Alytes*), including the description of two new taxa from Spain. *Contributions to Zoology* **65**, 5–34.

AVISE, J.C. 2000. *Phylogeography, the History and Formation of Species*. Cambridge, MA: Harvard University Press.

BAILON, S. 1989. Les amphibiens et les reptiles du Pliocène Supérieur de Balaruc II (Hérault, France). *Palaeovertebrata-Montpellier* **19**, 7–28.

BEERLI, P., HOTZ, H. & UZZELL, T. 1996. Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. *Evolution* **50**, 1676–1687.

BLONDEL, J. & ARONSON, J. 1999. *Biology and Wildlife of the Mediterranean Region*. Oxford University Press, New York.

BONS, J. & GENIEZ, P. 1996. *Amphibians and reptiles of Morocco (including Western Sahara), Biogeographical atlas*. Asociación Herpetológica Española, Barcelona.

BUTLER, M. 1980. The giant erinaceid insectivore, *Deinogalerix* Freudental, from the Upper Miocene of Gargano, Italy. *Scripta Geologica* **57**, 1–72.

CACCONE, A., MILINKOVITCH, M.C., SBORDONI, V. & POWELL, J.R. 1994. Molecular biogeography – using the Corsica–Sardinia microplate disjunction to calibrate mitochondrial rDNA evolutionary rates in mountain newts (*Euproctus*). *Journal of Evolutionary Biology* **7**, 227–245.

CACCONE, A., MILINKOVITCH, M.C., SBORDONI, V. & POWELL, J.R. 1997. Mitochondrial DNA rates and biogeography in European newts (Genus *Euproctus*). *Systematic Biology* **46**, 126–144.

CARRANZA, S. & ARNOLD, E.N. 2003. Investigating the origin of transoceanic distributions: mtDNA shows *Mabuya* lizards (Reptilia, Scincidae) crossed the Atlantic twice. *Systematics and Biodiversity* **1**, 275–282.

CARRANZA, S., ARNOLD, E.N., THOMAS, R.H., MATEO, J.A. & LÓPEZ-JURADO, L.F. 1999. Status of the extinct giant lacertid lizard *Gallotia simonyi simonyi* (Reptilia: Lacertidae) assessed using mtDNA sequences from museum specimens. *Herpetological Journal* **9**, 83–86.

CARRANZA, S., ARNOLD, E.N., MATEO, J.A. & LÓPEZ-JURADO, L.F. 2000. Long-distance colonization and radiation in gekkonid lizards, *Tarentola* (Reptilia: Gekkonidae), revealed by mitochondrial DNA sequences. *Proceedings of The Royal Society of London Series B Biological Sciences* **267**, 637–649.

CARRANZA, S., ARNOLD, E.N., MATEO, J.A. & LÓPEZ-JURADO, L.F. 2001. Parallel gigantism and complex colonization patterns in Cape Verde scincid lizards *Mabuya* and *Macroscincus* (Reptilia: Scincidae) revealed by mitochondrial DNA sequences. *Proceedings of The Royal Society of London Series B Biological Sciences* **268**, 1595–1603.

CUNNINGHAM, C.W. 1997. Is congruence between data partitions a reliable predictor of phylogenetic accuracy? Empirically testing an iterative procedure for choosing among phylogenetic methods. *Systematic Biology* **46**, 464–478.

DUGGEN, S., HOERNLE, K., VAN DEN BOGAARD, P., RÜPKE, L. & MORGAN, J.P. 2003. Deep roots of the Messinian salinity crisis. *Nature* **422**, 602–606.

ESTES, R. 1981. *Gymnophiona, Caudata. Handbuch der Paläoherpetologie* (*Encyclopedia of Paleoherpetology*), 2 (2). Gustav Fischer Verlag, Stuttgart.

FARRIS, J.S., KALLERSJO, M., KLUGE, A.G. & BULT, C. 1994. Testing significance of incongruence. *Cladistics* **10**, 315–319.

FELSENSTEIN, J. 1985. Confidence-limits on phylogenies – an approach using the bootstrap. *Evolution* **39**, 783–791.

GARCIA-PARÍS, M. & JOCKUSCH, E.L. 1999. A mitochondrial DNA perspective on the evolution of Iberian *Discoglossus* (Amphibia: Anura). *Journal of Zoology* **248**, 209–218.

GARCIA-PARÍS, M., ALCOBENDAS, M. & ALBERCH, P. 1998. Influence of the Guadalquivir River basin on mitochondrial DNA evolution of *Salamandra salamandra* (Caudata: Salamandridae) from southern Spain. *Copeia* 173–176.

GIULI, C. DE & TORRE, D. 1984. Species relationships and evolution in the Pliocene endemic faunas of Apricena (Gargano Peninsula – Italy). *Geobios Mémoire Special* (Lyon) **8**, 379–383.

HERRE, W. 1935. Die Schwanzlurche der mitteleocänen (oberlutetischen) Braunkohle des Geiseltales und die Phylogenie der Urodelen unter Einschluss der Fossilen Formen. *Zoologica* (Stuttgart) **33**, 1–85.

HERRE, W. 1941. *Palaeopleurodeles hauffi* nov. gen., nov. spec., ein fossiler Schwanzlurch aus dem Miozän Süddeutschlands. *Zoologischer Anzeiger* **134**, 1–17.

HEWITT, G.M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**, 247–276.

HEWITT, G.M. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405**, 907–913.

HSÜ, K.J. 1972. When the Mediterranean dried up. *Scientific American* **227**, 27–36.

HSÜ, K.J., RYAN, W.B.F. & CITA, M.B. 1973. Late Miocene desiccation of the Mediterranean. *Nature* **242**, 240–244.

HSÜ, K.J., MONTADERT, L., BEERNOUILLI, D., CITA, M.B., ERICKSON, A., GARRISON, R.E., KIDD, R.B., MELIERES, F., MULLER, C. & WRIGHT, R. 1977. History of the Mediterranean salinity crisis. *Nature* **267**, 399–403.

HUELSENBECK, J.P. & CRANDALL, K.A. 1997. Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics* **28**, 437–466.

HUELSENBECK, J.P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755.

KOCHER, T.D., THOMAS, W.K., MEYER, A., EDWARDS, S.V., PAABO, S., VILLABLANCA, F.X. & WILSON, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences USA* **86**, 6196–6200.

KRIJGSMAN, W., GARCES, M., AGUSTÍ, J., RAFFI, I., TABERNER, C. & ZACHARIASSE, W.J. 2000. The Tortonian salinity crisis of the eastern Betics (Spain). *Earth and Planetary Science Letters* **1981**, 497–511.

KRIJGSMAN, W., HILGEN, F.J., RAFFI, I., SIERRO, F.J. & WILSON, D.S. 1999*a*. Chronology, causes and progression of the Messinian Salinity Crisis. *Nature* **400**, 652–655.

KRIJGSMAN, W. & LANGEREIS, C.G. 2000. Magnetostratigraphy of the Zobzit and Koudiat Zarga sections (Taza-Guercif basin, Morocco): implications for the evolution of the Rifian corridor. *Mar. Pet. Geol.* **17**, 359–371.

KRIJGSMAN, W., LANGEREIS, C.G., ZACHARIASSE, W.J., BOCCALETTI, M., MORATTI, G., GELATI, R., IACCARINO, S., PAPANI, G. & VILLA, G. 1999*b*. Late Neogene evolution of the Taza-Guercif Basin (Rifian Corridor, Morocco) and implications for the Messinian Salinity Crisis. *Marine Geology* **153**, 147–160.

MICKEVICH, M.F. & FARRIS, J.S. 1981. The implications of congruence in Menidia. *Systematic Zoology* **30**, 351–370.

MILNER, A.R. 2000. Mesozoic and Tertiary Caudata and Albanerpetontidae. In: HEATWOLE, H. & CARROL, R. L., Eds. *Amphibian Biology*. Surrey Beatty & Sons, Australia, pp. 1412–1444.

PASMANS, F., BOGAERTS, S., DONAIRE, D. & HERBERT, D. 2002. Field notes on *Pleurodeles poireti* in Tunisia. *Zeitschrift fur Feldherpetologie* **9**, 111–114.

PASTEUR, G. 1958. Sur la systématique des espèces du genre *Pleurodeles* (Salamandridés). *Bulletin de la Société des Sciences Naturelles et Physiques du Maroc* **38**, 157–165.

POSADA, D. & CRANDALL, K.A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

RANDO, J.C., CARRANZA, S., MACA-MEYER, N., ARNOLD, E.N. & CABRERA, V.M. In press. Status and relationships of the extinct giant Canary Island lizard *Gallotia goliath* (Reptilia: Lacertidae), assessed using ancient mtDNA from its mummified remains. *Biological Journal of the Linnean Society*.

RÖGL, F. 1999. Mediterranean and Paratethys palaeogeography during the Oligocene and Miocene. In AGUSTÍ, J., ROOK, L. & ANDREWS, P., Eds. *The Evolution of Neogene Terrestrial Ecosystems*. Cambridge University Press, Cambridge, pp. 8–22.

SAID, R. 1990. *Geology of Egypt*. Balkema, Rotterdam.

SAID, R. 1993. *The River Nile: Geology, Hydrology and Utilization*. Oxford, Pergamon.

SANCHIZ, F. 1977. Nuevos anfibios del Neogeno y Cuaternario de Europa. Origen, desarrollo y relaciones de la batracofauna Española. Universidad Complutense, Madrid, tesis doctoral, 3 vols. 863 pp.

SANDERSON, M.J. 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. *Molecular Biology and Evolution* **14**, 1218–1231.

STEINFARTZ, S.U., HWANG, W., TAUTZ, D., ÖZ, M. & VEITH, M. 2002. Molecular phylogeny of the salamandrid genus *Neurergus*: evidence for an intrageneric switch of reproductive biology. *Amphibia–Reptilia* **23**, 419–431.

SWOFFORD, D.L. 1998. PAUP*: phylogenetic analysis using parsimony (and other methods), v 4.0 Sunderland, MA: Sinauer Associates.

TAN, A.M. & WAKE, D.B. 1995. MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata, Salamandridae). *Molecular Phylogenetics and Evolution* **14**, 414–422.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**, 4876–4882.

TITUS, T.A. & LARSON, A. 1995. A molecular phylogenetic perspective on the evolutionary radiation of the salamander family Salamandridae. *Systematic Biology* **44**, 125–151.

VEITH, M., KOSUCH, J. & VENCES, M. 2003. Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae). *Molecular Phylogenetics and Evolution* **26**, 310–327.

WEIJERMARS, R. 1988. Neogene tectonics in the western Mediterranean may have caused the Messinian salinity crisis and associated glacial events. *Tectonophysics* **148**, 211–219.

WEIJERMARS, R. 1991. Geology and tectonics of the Betic zone, SE Spain. *Earth-Science Reviews* **31**, 153–236.

WESTPHAL, F. 1978. *Tylototriton* (Amphibia, Urodela) aus dem Obermiozän von Ohningen. *Neues Jahrbuch für Geologie und Palaeontologie Monatshefte* **8**, 491–501.