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ORIGINAL ARTICLE

Biogeography and evolution of European cave salamanders, *Hydromantes* (Urodela: Plethodontidae), inferred from mtDNA sequences

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ABSTRACT

Aim To infer the phylogenetic relationships and biogeography of *Hydromantes*, with special emphasis on the European taxa. In particular, we aimed to test: (1) the monophyly of the European species and current views on their interrelationships; and (2) previously proposed timings of the separation of European and American *Hydromantes*, and of biogeographically important events within Europe.

Location California and the Western Mediterranean Basin, specifically south-east France, Italy, and the island of Sardinia.

Methods Partial sequences of mitochondrial genes (cytochrome b and 12S rRNA) were obtained from 45 specimens of *Hydromantes*, including all European extant species and subspecies, and two species from California. In addition, a fragment of the mitochondrial 16S rRNA gene was amplified for 16 specimens. Data sets were aligned using ClustalX, and well-supported phylogenetic trees were produced using maximum-likelihood, Bayesian and maximum-parsimony methods. Estimates of divergence times were obtained with the program r8s, the molecular clock being calibrated using the opening of the Strait of Gibraltar, the final event in the Messinian Salinity Crisis of 5.3 Ma.

Results Separation between the American and European clades occurred approximately 13.5 Ma, most probably before or after westward dispersal across the Bering Land Bridge. In Europe, divergence started in the late Miocene, when *Hydromantes (A.) genei* separated from other members of the genus 9 Ma and colonized south-west Sardinia. Movement between the European mainland and Sardinia, by a member of the subgenus *Speleomantes*, occurred in the Messinian Salinity Crisis, after the Mediterranean Basin desiccated almost completely 5.96 Ma. Subsequent widespread aridification fragmented the geographical ranges of *Hydromantes*, which live in cool and humid conditions, resulting in the origin of the six species in the subgenus *Speleomantes*. In contrast, a second period of diversification, in continental Europe 2–1.3 Ma, was probably caused by very cold interludes during the climatic oscillations that characterized the Pleistocene.

Main conclusions The molecular clock used here indicates that the separation of Californian and European *Hydromantes* occurred more recently than previously believed, and the same is true of some subsequent phylogenetic divergences within Europe. Estimated dates for these divergence events are consistent with known geophysical and climatic events that could have caused or facilitated them.

Keywords

Amphibians, *Atylodes*, Bering Land Bridge, *Hydromantes*, Mediterranean Basin, Messinian Salinity Crisis, mitochondrial DNA, phylogeography, Pleistocene glaciations, *Speleomantes*.

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INTRODUCTION

The lungless salamanders that comprise the family Plethodontidae include nearly 70% of the 557 extant species of urodeles (Min *et al.*, 2005), and are distributed predominantly in the Americas (Wake, 1966). The only known Old World members of the family are the recently described *Karsenia koreana* (Min *et al.*, 2005) from south-west Korea and the European cave salamanders. The seven presently recognized species of these occur in the extreme south-east of France, northern and central Italy, and the Mediterranean island of Sardinia, and are most closely related to three species in California assigned to the genus *Hydromantes* Gistel, 1868 (Wake *et al.*, 1978; Lanza *et al.*, 1995; Nascetti *et al.*, 1996; Jackman *et al.*, 1997). In recent decades, the European forms have been referred either to this genus or to *Speleomantes* Dubois, 1984. Use of *Hydromantes* for these species has recently been recommended, with recognition of three subgenera: *Hydromantes* for the Californian species; *Speleomantes* for most European ones; and *Atylodes* for the distinctive European *H. genei* Temminck & Schlegel, 1838 (Wake *et al.*, 2005; see also Crochet, 2007). This treatment is followed here.

As the occurrence of members of *Hydromantes* in Europe makes American plethodontids paraphyletic, it is assumed that the genus dispersed into Europe from there. As yet, however, there is no consensus on the precise origins of the surprisingly disjunct distribution of *Hydromantes* (for a review of this topic see Wake *et al.*, 1978; Lanza *et al.*, 1995). Various suggested dates for entry into the Old World include: the early Eocene (Wake, 1966; Lanza *et al.*, 1995; Delfino *et al.*, 2005; Muller, 2006); the early Oligocene, around the time urodeles of the family Salamandridae are thought to have entered North America from Eurasia (Estes, 1970); near the end of the Oligocene (Wake *et al.*, 1978); the late Miocene or Pliocene (Dunn, 1926); the Pleistocene or even more recently (Schmidt, 1946). All but one of these proposed timings are considerably later than the last continental connection between Europe and North America across the North Atlantic (early Eocene; 50 Ma), so dispersal is usually assumed to have been across the Bering Land Bridge. This route existed between Alaska and Siberia, first being interrupted around 4.8–7.4 Ma but still existing intermittently until 11,000 years ago (Marincovich & Gladenkov, 1999; Sher, 1999). After the Eocene, American lineages entering Siberia would have been able to reach Europe as the intervening Turgai Strait disappeared (Prothero, 1994). Dispersal across the Bering Land Bridge has also been proposed for some other amphibian groups, such as toads (Bufonidae) and tree frogs (Hylidae) in the Oligocene (Borkin, 1999), and for many mammal lineages into the Pleistocene (see for instance Wallace & Wang, 2004).

In recent years there has been considerable work on the taxonomy and relationships of European *Hydromantes*. Their morphology, taxonomy, allozymes and biogeography were investigated by Lanza *et al.* (1995), whose work was complemented by the chromosome and cytogenetic studies of Nardi and his co-workers (Nardi *et al.*, 1986, 2000; Nardi, 1991). Other authors later used the data of Lanza *et al.* (1995) to reassess genetic variability and biogeography (Nascetti *et al.*, 1996; Cimmaruta *et al.*, 1997; Forti *et al.*, 1997). All three American species of *Hydromantes* and three European ones (see Discussion) were included in a study of bolitoglossine plethodontids based on mitochondrial DNA (Jackman *et al.*, 1997). Despite incomplete sampling of the European species and use of only a single fragment of one gene (cytochrome b), the results were largely congruent with those of Nascetti *et al.* (1996). Both analyses indicated that the European cave salamanders are monophyletic, and that *H. (A.) genei* of south Sardinia is the sister taxon of the remaining species, from the neighbouring European mainland and from eastern Sardinia. This pattern of relationship is often taken as indicating that Sardinia was invaded twice by *Hydromantes* (see, for example, Lanza, 1983). However, an equally parsimonious scenario, in terms of journeys, is that there was a single invasion of Sardinia followed by the evolution of the subgenus *Speleomantes* there and a reinvasion of the mainland (Nascetti *et al.*, 1996). Separation of *H. (A.) genei* has been assigned to the Oligocene (Wake *et al.*, 1978; Lanza, 1983), when the microplate that formed Corsica and Sardinia separated from the French–Iberian continental margin, and movement of *Speleomantes* has been assigned to the Messinian Salinity Crisis at the very end of the Miocene.

In spite of all the recent work on *Hydromantes* and long-standing interest in its biogeography, a comprehensive molecular phylogeny based on fragments of more than a single gene is still lacking. This is especially surprising given that estimates of relationships based on DNA have been used to reconstruct the biogeography and evolution of many other groups of Mediterranean amphibians and to revise their taxonomy (see García-París & Jockusch, 1999; García-París *et al.*, 2003; Carranza & Arnold, 2004; Carranza & Wade, 2004; Fromhage *et al.*, 2004; Martínez-Solano *et al.*, 2004; Carranza & Amat, 2005; Mattoccia *et al.*, 2005). We consequently use sequences from three mitochondrial genes to infer phylogenetic relationships within *Hydromantes*, with special emphasis on the European taxa, of which all recognized taxa are considered. The phylogeny is used to test monophyly of the European species and current views on their interrelationships, and to estimate the time of their separation from American *Hydromantes*, and of the two supposed invasions of Sardinia. Results are also combined with other data (Lanza *et al.*, 1995, 2001; Nascetti *et al.*, 1996; Lanza & Leo, 2001) to revise the taxonomy of the European cave salamanders.

MATERIALS AND METHODS

Samples, DNA extraction and PCR amplification

A total of 45 specimens of the genus *Hydromantes* were used in this study (see Table 1 & Fig. 1). They included two representatives of the subgenus *Hydromantes* from California, six of the subgenus *Atylodes* from south-west Sardinia, and 37 of the subgenus *Speleomantes* from Sardinia, France and mainland

Table 1 Details of material and new sequences used in this study. The number after the species name refers to the locality shown in Fig. 1.

Taxon	Locality	Accession numbers Cyt b/12S/16S	Code
Hydromantes (H.) brunus	California (USA)	NC_006345 (mt genome)	
Hydromantes (H.) platycephalus	California (USA)	U89612/DQ283227	
Hydromantes (A.) genei -1	Sanità, Fluminimaggiore, Cagliari, Sardinia (Italy)	EU117017/EU116957/EU117001	E10076.5
Hydromantes (A.) genei -2	Sanità, Fluminimaggiore, Cagliari, Sardinia (Italy)	EU117018/EU116958/EU117000	E10076.6
Hydromantes (A.) genei -3	Grotta su Mannau, Sardinia (Italy)	EU117019/EU116959/EU116999	E3056.8
Hydromantes (A.) genei -43	Monte Tasua, Carbonia, Sardinia (Italy)	EU117020/EU116960	E1037.1
Hydromantes (A.) genei -44	Monte Tasua, Carbonia, Sardinia (Italy)	EU117021/EU116961	E1037.2
Hydromantes (A.) genei -45	Monte Tasua, Carbonia, Sardinia (Italy)	EU117022/EU116962	E1037.3
Hydromantes (S.) flavus -4	Conca 'e Crapa, Sardinia (Italy)	EU117002/EU116942/EU116995	E3056.6
Hydromantes (S.) flavus -5	Conca 'e Crapa, Sardinia (Italy)	EU117003/EU116943/EU116996	E3056.7
Hydromantes (S.) flavus -6	Conca 'e Crapa, Sardinia (Italy)	EU117004/EU116944/EU116994	E3056.5
Hydromantes (S.) supramontis -7	Grotta su Bentu, Sardinia (Italy)	EU117005/EU116945	E3056.12
Hydromantes (S.) supramontis -8	Grotta su Bentu, Sardinia (Italy)	EU117006/EU116946/EU116997	E3056.13
Hydromantes (S.) supramontis -9	Grotta su Bentu, Sardinia (Italy)	EU117007/EU116947/EU116998	E3056.14
Hydromantes (S.) supramontis -10	Punta Cuccuttos, Urzulei, Ogliastra, Sardinia (Italy)	EU117008/EU116948	E10076.7
Hydromantes (S.) supramontis -11	Punta Cuccuttos, Urzulei, Ogliastra, Sardinia (Italy)	EU117009/EU116949	E10076.8
Hydromantes (S.) supramontis -12	Punta Cuccuttos, Urzulei, Ogliastra, Sardinia (Italy)	EU117010/EU116950	E10076.9
Hydromantes (S.) imperialis sarrabusensis -13	Mount Sette Fratelli, Burcei, Cagliari, Sardinia (Italy)	EU117011/EU116951	E10076.1
Hydromantes (S.) imperialis sarrabusensis -14	Mount Sette Fratelli, Burcei, Cagliari, Sardinia (Italy)	EU117012/EU116952	E10076.2
Hydromantes (S.) imperialis sarrabusensis -15	Mount Sette Fratelli, Burcei, Cagliari, Sardinia (Italy)	EU117013/EU116953	E10076.3
Hydromantes (S.) imperialis imperialis -16	Ulassai, Sardinia (Italy)	EU117014/EU116954/EU116991	E3056.9
Hydromantes (S.) imperialis imperialis -17	Ulassai, Sardinia (Italy)	EU117015/EU116955/EU116993	E3056.10
Hydromantes (S.) imperialis imperialis -18	Ulassai, Sardinia (Italy)	EU117016/EU116956/EU116992	E3056.11
Hydromantes (S.) strinatii -19	W. Monte Groppi, Genova, Liguria (Italy)	EU117023/EU116963/EU116988	E3056.4
Hydromantes (S.) strinatii -20	W. Monte Groppi, Genova, Liguria (Italy)	EU117024/EU116964	E25106.3
Hydromantes (S.) strinatii -21	W. Monte Groppi, Genova, Liguria (Italy)	EU117025/EU116965	E25106.4
Hydromantes (S.) strinatii -22	Rio Tonno, Vallbrevenna, Genova, Liguria (Italy)	EU117028/EU116968	E2306.17
Hydromantes (S.) strinatii -23	S. Bartolomeo, Savignone, Genova, Liguria (Italy)	EU117027/EU116967	E2306.9
Hydromantes (S.) strinatii -24	S. Bartolomeo, Savignone, Genova, Liguria (Italy)	EU117026/EU116966	E2306.11
Hydromantes (S.) strinatii -25	Trabocchetto, Pietra Ligure, Savona, Liguria (Italy)	EU117029/EU116969/EU116989	E2306.20
Hydromantes (S.) strinatii -26	Monte Pietravecchia, Pigna, Imperia, Liguria (Italy)	EU117032/EU116972/EU116990	E2306.18
Hydromantes (S.) strinatii -27	Maritime Alps (France)	EU117031/EU116971	E14076.1
Hydromantes (S.) strinatii -28	Maritime Alps (France)	EU117030/EU116970	E14076.2
Hydromantes (S.) ambrosii bianchii -29	Apuan Alps, Massa-Carrara, Tuscany (Italy)	EU117033/EU116973/EU116986	E3056.2
Hydromantes (S.) ambrosii bianchii -30	Apuan Alps, Massa-Carrara, Tuscany (Italy)	EU117034/EU116974	E251061
Hydromantes (S.) ambrosii bianchii -31	Apuan Alps, Massa-Carrara, Tuscany (Italy)	EU117035/EU116975	E251062
Hydromantes (S.) ambrosii ambrosii -32	Grotta Fornace, Pignone, La Spezia, Liguria (Italy)	EU117042/EU116982	E2306.5
Hydromantes (S.) ambrosii ambrosii -33	Grotta Fornace, Pignone, La Spezia, Liguria (Italy)	EU117043/EU116983	E2306.4
Hydromantes (S.) ambrosii ambrosii -34	Caverna Ossifera, Cassana, La Spezia, Liguria (Italy)	EU117044/EU116984/EU116987	E2306.3
Hydromantes (S.) ambrosii ambrosii -35	Caverna Ossifera, Cassana, La Spezia, Liguria (Italy)	EU117045/EU116985	E2306.1
Hydromantes (S.) ambrosii ambrosii -36	Liguria Region (Italy)	AY728215 (mt partial genome)	HitalCB12
Hydromantes (S.) italicus -37	Sorgenti dell'Esino, Esanatoglia, Ancona, Marche (Italy)	EU117036/EU116976	E2306.12
Hydromantes (S.) italicus -38	Abrutti, Farindola, Pescara (Italy)	EU117037/EU116977	E2306.14
Hydromantes (S.) italicus -39	Monte Tezio, Perugia, Umbria (Italy)	EU117038/EU116978	E2306.8
Hydromantes (S.) italicus -40	Monte Tezio, Perugia, Umbria (Italy)	EU117040/EU116980	E2306.6
Hydromantes (S.) italicus -41	Monte Tezio, Perugia, Umbria (Italy)	EU117039/EU116979	E2306.7
Hydromantes (S.) italicus -42	Cardoso, Stazzema, Lucca, Tuscany (Italy)	EU117041/EU116981	E2306.15
Pleurodeles nebulosus	Larba (Algeria)	AY222504/AY222460	Anc10
Pleurodeles nebulosus	Constantine (Algeria)	AY222462/AY222506	Anc12
Pleurodeles nebulosus	Tabarca (Tunisia)	AY222518/AY222474	E181210
Pleurodeles poireti	Annaba (Algeria)	AY222507 /AY222463	Anc13
Pleurodeles waltl	Extremos (Portugal)	AY222515/AY222471	E10114
Pleurodeles waltl	Perelló (Spain)	AY222531/AY222487	E181224
Salamandra algira tingitana	Djebel El Fahies (Morocco)	AY247732/DQ221227	E1712.7
Salamandra algira algira	Akshur near Talembot (Morocco)	AY247734/DQ221229	E1712.9

Table 1 Continued

Taxon	Locality	Accession numbers Cyt b/12S/16S	Code
Salamandra s. longirostris	Los Barrios (Spain)	DQ221243/DQ221223	E1712.37
Salamandra infrainmaculata orientalis	W. of Adana (Turkey)	DQ221242/DQ221222	E3009.22
Salamandra s. europaea	El Montseny (Spain)	AY222503/AY222459	E1712.49
Salamandra lanzai	(Italy)	DQ221244/DQ221224	E3009.23

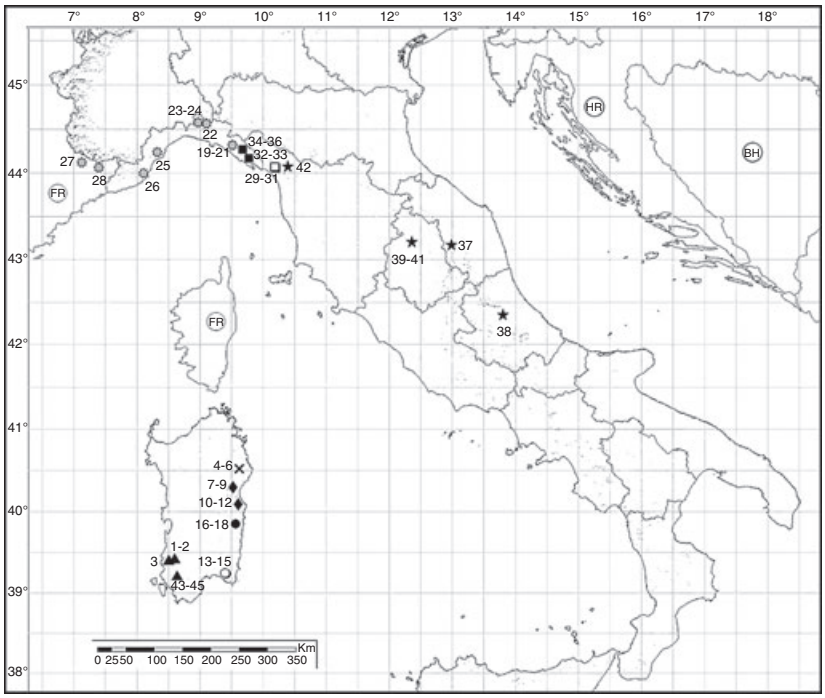

Figure 1 Localities of the samples used in this study. Numbers correspond to specimens listed in Table 1. Sun symbols, *Hydromantes (S.) strinatii*; filled squares, *H. (S.) a. ambrosii*; open square, *H. (S.) a. bianchii*; stars, *H. (S.) italicus*; filled triangles, *H. (A.) genei*; cross, *H. (S.) flavus*; diamonds, *H. (S.) supramontis*; black filled circle, *H. (S.) imperialis imperialis*; open circle, *H. (S.) imperialis sarrabusensis*. FR, France; HR, Croatia; BH, Bosnia-Herzegovina. Italian map modified from Sindaco *et al.* (2006).

Italy. The sampling included representatives of two recently described subspecies: *H. (S.) imperialis sarrabusensis* (Lanza *et al.*, 2001) and *H. (S.) ambrosii bianchii* (Lanza *et al.*, 2005) as well as representatives of the two distinct populations of *H. (A.) genei* from south-west Sardinia (Lanza *et al.*, 1995). DNA was extracted using a DNAasy extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Primers used in both amplification and sequencing were cytochrome *b1* and cytochrome *b2* (Kocher *et al.*, 1989) for the cytochrome *b* (cytb) gene, 12Sa and 12Sb (Kocher *et al.*, 1989) for the 12S rRNA gene, and 16Sar and 16Sbr (Palumbi, 1996) for the 16SrRNA gene. The three gene fragments were amplified by polymerase chain reaction (PCR), and the resultant DNA was sequenced using the same standard protocols and conditions as described in Carranza *et al.* (1999, 2001).

Phylogenetic analyses

DNA sequences were aligned using ClustalX (Thompson *et al.*, 1997) with default parameters (gap opening = 10; gap extension = 0.2). All the cytb sequences had the same length and therefore no gaps were postulated. These sequences were translated into amino acids using the vertebrate mitochondrial code and no stop codons were observed, suggesting that they were probably all functional. Although some gaps were postulated in order to resolve length differences in the 12S rRNA and 16S rRNA gene fragments, all positions could be unambiguously aligned and were therefore included in the analyses.

A saturation analysis for the cytb third codon positions is shown in Fig. 2. The results indicate that not even the cytb third codon transitions were saturated (Fig. 2a), and therefore all positions were included in the phylogenetic analyses. Three methods of phylogenetic analysis were employed for all three independent partitions, and the combined data set and their results compared. These methods were: maximum likelihood (ML), Bayesian analysis, and maximum parsimony (MP). Modeltest v. 3.06 (Posada & Crandall, 1998) was used to select the most appropriate model of sequence evolution for the ML and Bayesian analyses of the independent partitions and the combined data sets, under the Akaike information criterion. The models selected were: TVM+G for the cytb partition (the GTR+G was implemented for the cytb partition in MrBayes); GTR+G for the 12S rRNA partition; HKY+G for

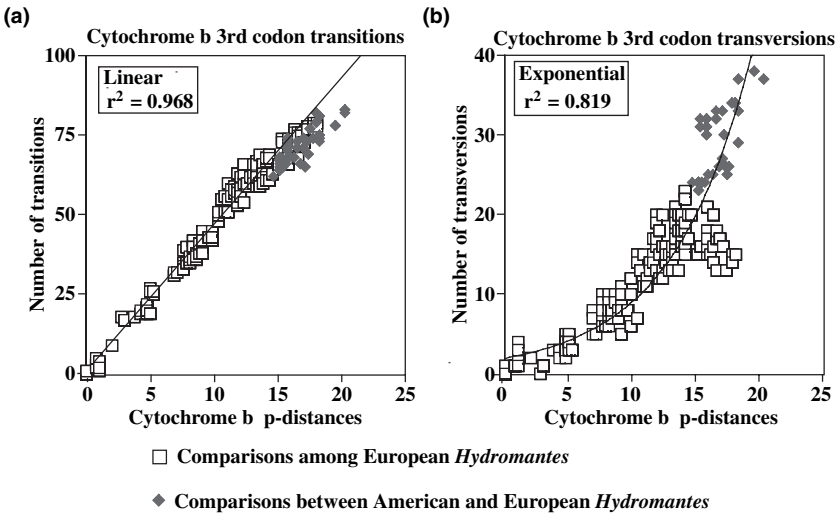

Figure 2 Observed number of transitions (a) and transversions (b) plotted against uncorrected distances for the cytochrome b third codon positions. The empty squares are those transitions (a) or transversions (b) involved in the comparisons among European *Hydromantes*. The filled diamonds are those transitions (a) or transversions (b) involved in the comparison between American and European *Hydromantes*.

the 16S rRNA partition; and GTR+I+G for both the cytb+12S and cytb+12S+16S combined data sets.

Bayesian analyses were performed with MrBayes v. 3.0b4 (Huelsenbeck & Ronquist, 2001). For the combined data sets (cytb+12S and cytb+12S+16S), each gene had its own independent model of evolution (see above) and model parameters. Four incrementally heated Markov chains with the default heating values were used. All analyses started with randomly generated trees and ran for 2×10^6 generations, with sampling occurring at intervals of 100 generations, producing 20,000 trees. For each independent analysis, the log-likelihood values of all trees saved were plotted against the generation time. After verifying that stationarity had been reached, both in terms of likelihood scores and parameter estimation, the first 3,500 trees in the cytb+12S data set and the first 4000 trees in the cytb+12S+16S data set were discarded, and independent majority-rule consensus trees were generated from the remaining (post-burning) trees. The frequency of any particular clade of the consensus tree represents the posterior probability of that node (Huelsenbeck & Ronquist, 2001); only values above 95% were considered to indicate that nodes were significantly supported (Wilcox *et al.*, 2002).

ML analyses were performed with both paup* v. 4.0b10 (Swofford, 1998) and phyml (Guindon & Gascuel, 2003). MP analyses were performed with paup* v. 4.0b10 alone. Both ML and MP analyses performed in paup* included heuristic searches involving tree bisection and reconnection (TBR) branch swapping with 10 and 100 random stepwise additions of taxa, respectively. Gaps were included as a fifth state. Reliability of the MP and ML trees was assessed by bootstrap analysis (Felsenstein, 1985), involving 1000 replications for both ML (phyml method only) and MP analyses.

Topological incongruence among partitions was tested using the incongruence length difference (ILD) test (Michkevich & Farris, 1981; Farris *et al.*, 1994). In this test, 10,000 heuristic searches were carried out after the removal of all invariable characters from the data set (Cunningham, 1997). To test for incongruence among data sets, we also used a reciprocal 70% bootstrap proportion (Mason-Gamer & Kellogg, 1996) or a 95% posterior probability threshold. Topological conflicts were considered significant if two different relationships for the same set of taxa were both supported with bootstrap values ≥ 70% or posterior probability values ≥ 95%.

Topological constraints to test alternative topologies were constructed using MacClade v. 4.0 (Maddison & Maddison, 1992) and compared with optimal topologies using the Shimodaira-Hassegawa (SH) (Shimodaira & Hasegawa, 1999) test implemented in paup* v. 4.0b10 (Swofford, 1998). Basic sequence statistics and genetic distances were calculated with the program mega v. 2.1 (Kumar *et al.*, 2001).

The computer program rrtree (Robinson-Rechavi & Huchon, 2000) was used to run a relative-rate test on the sequences used for calibration to find out if there were any sequences that evolved at a significantly different rate.

Estimation of divergence times

In order to estimate divergence times between lineages, the computer program r8sb v. 1.6.4 was used (Sanderson, 1997, 2002). This program implements several methods for estimating absolute rates of molecular evolution, ranging from standard maximum-likelihood ones to more experimental semiparametric and nonparametric methods, which relax the stringency of the clock assumptions using smoothing methods. One of the advantages of this program is that, through a cross-validation test, it allows the user to explore the fidelity with which any of these methods explains the branch-length variation (Sanderson, 2002). This procedure removes each terminal branch in turn, estimates the remaining parameters of the model without that branch, predicts the anticipated number of substitutions on the pruned branch, and reports the performance of these predictions as a cross-validation score, which allows the user to select the method that best explains the branch-length variation (Sanderson, 2002).

To calibrate the clock, use of available fossil plethodontids was considered. A single vertebra of *Hydromantes* is known from the

Miocene of Slovakia, dated by stratigraphy to 12.5–15 Ma (Venczel & Sanchíz, 2005). It has been tentatively assigned to the European *Hydromantes*, partly because of its geographical situation (continental Europe). However, the single vertebra found is damaged, and such a small element could possibly be an intrusion. The genera *Plethodon* and *Aneides* have been reported from early Miocene deposits 23 Ma (Tihen & Wake, 1981). Again, however, identification is based only on vertebrae, and these may not be fully diagnostic. Therefore, use of this palaeontological information for calibrating a molecular clock of the group is not advisable, especially when phylogenies indicate that vertebrae are sometimes ecologically labile in plethodontids (Parra-Olea & Wake, 2001).

Instead of these fossils we have used the genetic divergence of two salamander lineages, each of which appears to have undergone vicariance when the Strait of Gibraltar, between the Atlantic Ocean and the Mediterranean Sea, opened. This event terminated the Messinian Salinity Crisis at the very end of the Miocene, one of the best studied and well-dated geological events in the Earth's history (Hsü *et al.*, 1977; Hsü, 1983; Blondel & Aronson, 1999; Krijgsman *et al.*, 1999; Duggen *et al.*, 2003). About 5.96 Ma, the marine gateways that existed between the Atlantic Ocean and the Mediterranean were closed by the uplift of the Gibraltar arch, which formed a land bridge between the Iberian Peninsula and north-west Africa. This event terminated the continuous influx of waters from the Atlantic to the Mediterranean that had existed before. Without this input, sea level fell by over 1000 m in less than 1000 years, desiccating areas of the floor of the Mediterranean Basin, some of which later became covered by freshwater deposits. The Messinian Salinity Crisis ended exactly 5.3 Ma, when the Gibraltar arch collapsed, opening the Strait of Gibraltar and allowing the entire Mediterranean Basin to refill (perhaps filling in less than 100 years). This event broke the land connection between south-west Europe and north-west Africa that had existed for more than 600,000 years (Blondel & Aronson, 1999; Krijgsman *et al.*, 1999).

A number of amphibian and other terrestrial vertebrate groups with long fossil histories in Europe have distinctive taxa in the south-west of the continent. Several of these possess sister groups in north-west Africa, and phylogenies indicate that they invaded the area from Europe. The amphibian sister pairs show similar divergences in mtDNA. This is true of members of two salamandrids, *Pleurodeles* (Carranza & Arnold, 2004) and *Salamandra* (Escoriza *et al.*, 2006), and of three anurans, *Alytes* (Martínez-Solano *et al.*, 2004), *Discoglossus* (Zangari *et al.*, 2006) and *Pelobates* (García-París *et al.*, 2003). The repeated phylogenetic pattern and similarity in degree of divergence makes it likely that the separation of all sister pairs was caused by the same vicariance event. Entry into north-west Africa and the vicariance event itself probably resulted from the Messinian Salinity Crisis, as some of these taxa also colonized some Mediterranean islands at the same time (Martínez-Solano *et al.*, 2004; Zangari *et al.*, 2006). The land bridge and desiccation of the Mediterranean floor would have permitted entry to north-west Africa, and the formation of the Strait of Gibraltar could have caused the vicariance. The strait is likely to have been a very effective barrier to the forms concerned. With very few exceptions (Vences *et al.*, 2003), such as tree frogs, which may be transported on vegetation carried by currents or storms, amphibians do not cross significant saltwater barriers, being extremely sensitive to the effects of salt on their osmotic balance (Duellman & Trueb, 1986). As the strait is approximately 20 km wide, with a strong surface current that would make any potential journey even longer, passage by amphibians across it is improbable. The Messinian period was the first and only time that south-west Europe and north-west Africa made terrestrial contact, and the end of this period 5.3 Ma was the only known event that could have caused the vicariance indicated in the five groups of amphibians.

The only alternative possibility is that the groups concerned reached north-west Africa via a more eastern land connection that has existed more or less continuously between Eurasia and Africa since the mid-Miocene. This route resulted from Arabia, which formed the north-eastern edge of the African plate, colliding with the Turkish plate 15–19 Ma (Rögl, 1999). However, there is no evidence in the form of fossils or relict populations that close relatives of the north-west African amphibian taxa considered here ever occurred outside south-west Europe (Carranza & Arnold, 2004). Consequently, entry to north-west Africa at the western end of the Mediterranean, followed by vicariance caused by the opening of the Strait of Gibraltar, is much more likely. The latter event is consequently used as a calibration point in the *Pleurodeles* and *Salamandra* lineages included as outgroups in the *Hydromantes* phylogeny.

Another relevant point is that, without additional, independent loci to use in the analyses apart from the mtDNA sequences, the divergence times between taxa could be slightly different from the estimates from mtDNA simply because of variation in the time of coalescence. However, stochastic variation in the coalescence is less relevant when comparing highly divergent taxa and taxa with low effective population sizes.

RESULTS

In order to infer the phylogenetic relationships of *Hydromantes*, three independent data sets were created. Data set 1 contained cytb+12S rRNA sequences of 47 *Hydromantes* (two representatives of the North American subgenus *Hydromantes* were used to root the tree). The results of the ILD test showed that the two gene partitions were congruent ($P = 0.72$), and independent analyses of the two gene partitions confirmed that there were no topological conflicts (Mason-Gamer & Kellogg, 1996). Both mitochondrial fragments were therefore combined for further analyses. Of the 771 bp of the combined data set (398 of cytb and 373 of 12SrRNA), 223 bp were variable (147 bp of cytb and 74 bp of 12S rRNA) and 197 bp were parsimony-informative (128 bp of cytb and 69 bp of 12S rRNA). The results of the ML, MP and Bayesian analyses of Data set 1 are summarized in Fig. 3.

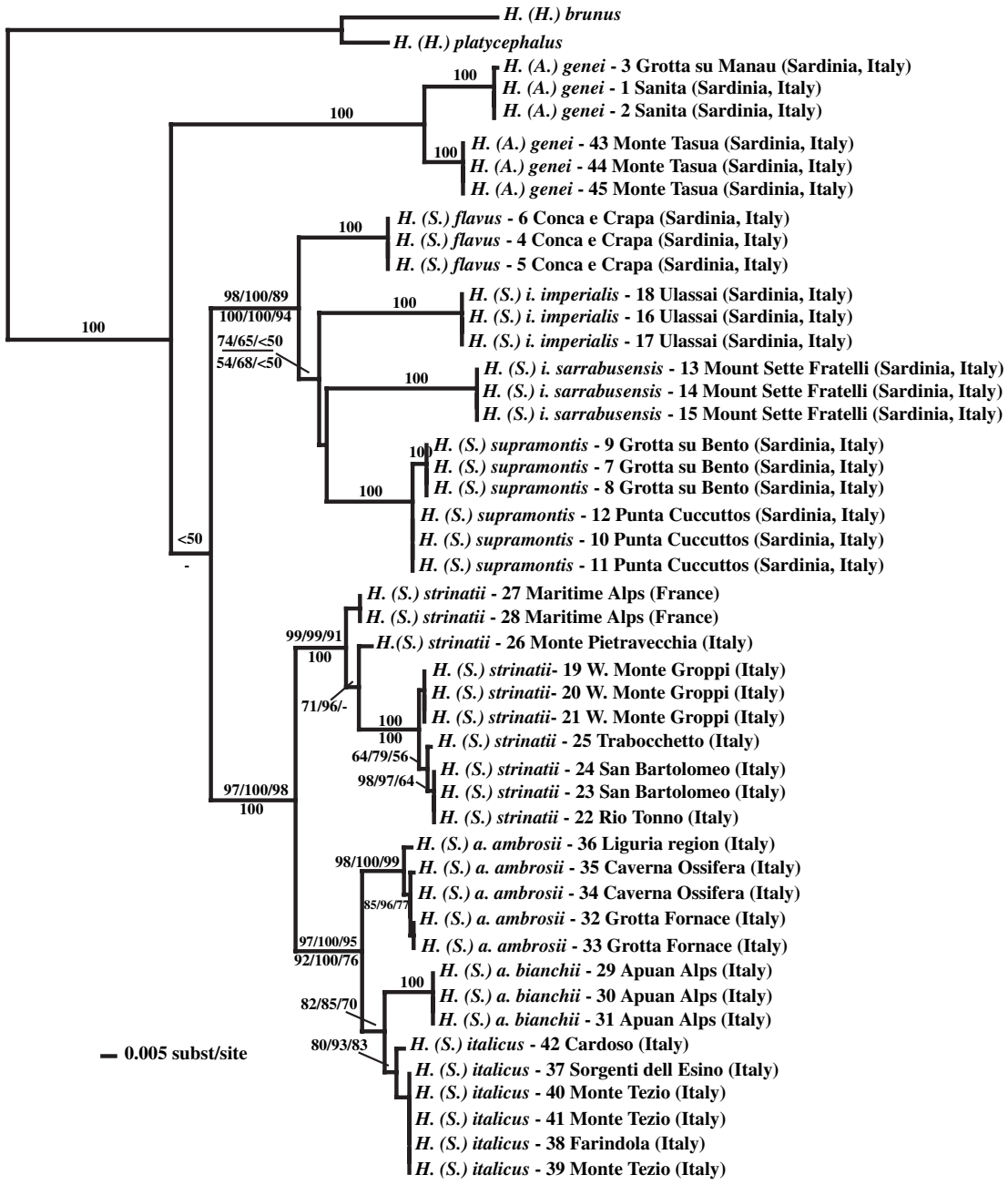

Figure 3 Maximum-likelihood tree (GTR + I + G; −log likelihood = 2988.22406) inferred with Data set 1 (cytb + 12S rRNA). Numbers after species names identify individual samples; their geographic locations are shown in Fig. 1. Figures above branches indicate bootstrap support values for maximum-likelihood analyses (left), posterior probability (pp) values for the Bayesian analyses (centre), and bootstrap support for the maximum-parsimony analysis (right). Numbers below the branches are the same parameters for analyses of Data set 2. A single number above or below a branch indicates that support was the same for all three methods of analysis. The symbol '–' indicates that the particular methods indicated did not support the topology presented in this figure.

Data set 2 included all 16 *Hydromantes* specimens for which three mitochondrial genes (cytb + 12S + 16S) were sequenced; *H. (H.) brunus* (Gorman, 1954) was used to root the tree (see Table 1). As with Data set 1, the results of the ILD test showed that the three gene partitions were congruent ($P = 0.51$), and independent analyses of the three gene partitions confirmed that there were no topological conflicts. Of the 1306 bp analysed (398 bp of cytb; 367 bp of 12S rRNA; and 541 bp of 16S rRNA), 323 bp were variable (132 bp of cytb; 68 bp of 12S rRNA; and 123 bp of 16S rRNA), and 216 bp were parsimony-informative (92 bp of cytb; 46 bp of 12S rRNA; and 78 bp of 16S rRNA). The results of the ML, MP and Bayesian analyses of this data set were very similar to the results obtained with Data set 1, and therefore the bootstrap support and posterior probability (pp) values resulting from the analysis of Data set 2 are incorporated into Fig. 3.

Data set 3 was constructed to calibrate the molecular clock and consisted of cytb+12S rRNA mitochondrial sequences of all the specimens included in Data set 1 plus six representatives of the genus *Pleurodeles*, six representatives of the genus *Salamandra*, and one *Ambystoma t. tigrinum* Dunn, 1940 that was used to root the tree. This data set included 752 bp (398 bp of cytb and 354 bp of 12S rRNA), of which 316 bp were variable (186 bp of cytb and 130 bp of 12S rRNA) and 284 bp were parsimony-informative (171 bp of cytb and 113 bp of 12S rRNA). As with Data set 1, the ILD test suggested that the two partitions were congruent ($P = 0.31$) and that there was no conflict between the trees constructed with each one of the partitions independently (data not shown). As some of the taxa from Data set 3 belong to different families of the order Urodela, a relative rate test was carried out in order to check that all the sequences were evolving at a similar rate. In all comparisons between sequences of representatives of the family Salamandridae (*Salamandra* and *Pleurodeles*) and of the family Plethodontidae (*Hydromantes*), the test did not detect significant differences in the relative rate of evolution ($P > 0.05$). Therefore, the ML tree with branch lengths resulting from the analysis of Data set 3 was used to infer dates with r8s v. 1.6.4 (Sanderson, 1997, 2002). The cross-validation test showed that branch-length variation was explained with the highest fidelity by the Langley-Fitch (LF) method (Langley & Fitch, 1973). Hence, the LF method was run with the Powell algorithm using two calibration points (see Materials and Methods). The inferred cladogram, with dates highlighted in the most relevant nodes, is shown in Fig. 4. The substitution rate inferred from r8s for the concatenated cytb+12S fragments is 0.99% per million years. This is comparable to other rates calculated for exactly the same mtDNA regions in other reptiles and amphibians (Carranza & Arnold, 2004; Carranza *et al.*, 2004).

The results of the phylogenetic analyses shown in Fig. 3 indicate that, within the European clade, all the continental species form a well-supported monophyletic group with Data sets 1 and 2 and with all the methods of analysis (ML, Bayesian and MP). The continental clade is further subdivided into two well-supported clades consisting of *H. (S.) strinatii* Aellen, 1958 and *H. (S.) italicus* Dunn, 1923 + *H. (S.) abrosii* Lanza, 1955. Variability within *H. (S.) strinatii* is relatively high, with populations from the Maritime Alps in France and Monte Pietravechia in Liguria, Italy, being quite divergent from all the remaining populations of *H. (S.) strinatii* sampled for our study (see Figs 1 & 2 and Table 1). According to the results from Fig. 3, *H. (S.) ambrosii* is paraphyletic, with *H. (S.) italicus* branching inside it. The sister-group relationship between *H. (S.) a. bianchii* and *H. (S.) italicus* is well supported in both ML (82) and MP (70) analyses, but the pp value of the Bayesian analysis for this clade is inferior to 95 (85). The results of the analysis of Data set 1 suggest that the *Hydromantes* from Sardinia are not monophyletic. Instead, the tree from Fig. 3 suggests that the *Hydromantes* of the subgenus *Atylodes* from south-west Sardinia (see Fig. 1 & Table 1) are sister to all the remaining European taxa, although the bootstrap and pp support is very low in all the analyses (Fig. 3). The analyses of Data set 2 did not support a monophyletic insular *Hydromantes* either, but instead placed *H. (A.) genei* as sister to the Continental assemblage (data not shown). A constrained analysis, in which all the *Hydromantes* from Sardinia were forced monophyletic, resulted in a tree that was not significantly worse than the tree presented in Fig. 3 (Shimodaira-Hassegawa test; $P > 0.05$). The four insular taxa of the subgenus *Speleomantes* form a well-supported clade, but the relationships among them are unresolved. The two subspecies of *H. (S.) imperialis* Stefani, 1969 show a similar level of genetic differentiation to that between any other species pair of the subgenus *Speleomantes*.

DISCUSSION

Monophyly of European *Hydromantes*

The results presented in Fig. 3 agree with other evidence that the European species of the genus *Hydromantes* are monophyletic, although one American species was missing in our analyses. The genetic differentiation between American and European *Hydromantes* presented in our study is in agreement with the results of previous molecular studies using albumin immunology, allozymes, mitochondrial DNA, and cytogenetics (Wake *et al.*, 1978; Nardi *et al.*, 1986, 2000; Nardi, 1991; Lanza *et al.*, 1995; Nascetti *et al.*, 1996; Jackman *et al.*, 1997), and is further supported by the morphological, osteological, karyological, cytogenetic and behavioural differences between the two clades (Wake, 1966; Lanza & Vanni, 1981; Nardi, 1991; Lanza *et al.*, 1995).

Timing of the separation of European *Hydromantes*

The molecular clock used here indicates that the split between the American and the European *Hydromantes* occurred approximately 13.5 Ma, during the mid-Miocene. This date is much more recent than several of the previous estimates listed in the Introduction. Certainly, the low level of genetic divergence between American and European *Hydromantes* (see Table S1 in Supplementary Material) and the absence of saturation, even in the third codon positions of the fast-evolving cytb mitochondrial gene (Fig. 2), make it extremely unlikely that separation occurred before the Miocene. Consequently, entrance into Europe before the last continental connection between Europe and North America across the North Atlantic disappeared permanently in the early Eocene (50 Ma) would not have been possible. Instead, migration is likely to have been across the Bering Land Bridge. Such a route is also in agreement with the fact that European *Hydromantes* have their closest relatives in western North America. Exactly how the European and American sections of *Hydromantes* separated is not clear. The speciation event concerned could conceivably have occurred in north-west America or in Eurasia and may possibly have resulted from the widespread extinction that has clearly occurred in the huge region between California

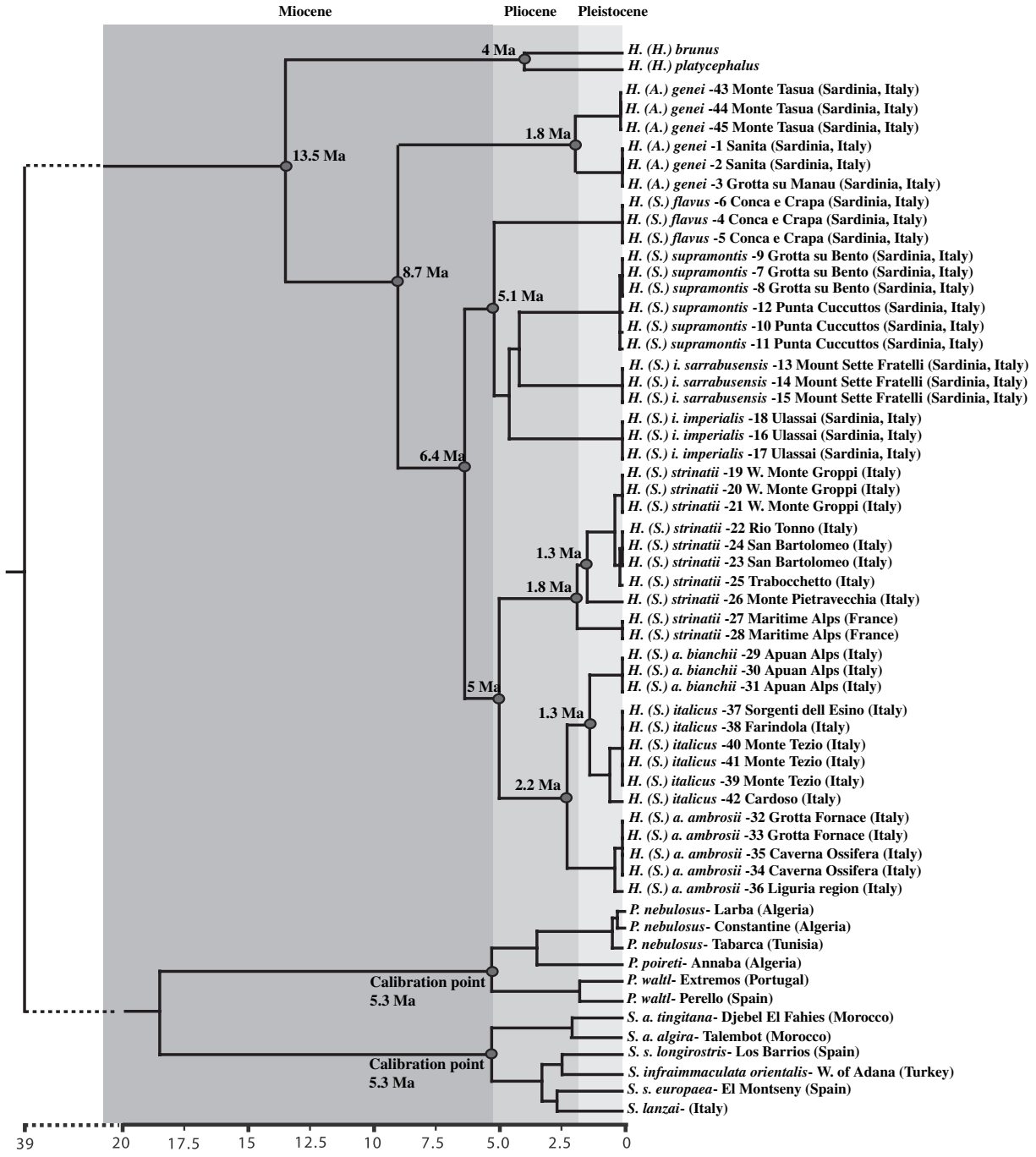

Figure 4 Cladogram of *Hydromantes* based on Data set 3 and program r8s v. 1.6.4 (Sanderson, 2002); see Materials and Methods for details. The scale is calibrated in millions of years. Dashed lines indicate branch lengths and time periods that have been abbreviated.

and Europe. Although the analyses by Min *et al.* (2005) did not support a sister taxa relationship between *Hydromantes* and *Karsenia*, a reanalysis of the Rag-1 sequences of Min *et al.* (2005) suggests that this hypothesis cannot be ruled out (data not shown). If *Karsenia* turns out to be the sister taxon of *Hydromantes*, the two may have entered Eurasia separately, or their common lineage may have done so. In the latter scenario, the speciation event that separated the genera may have taken place in Asia, in which case back migration into America by the lineage leading to the Californian forms is a possibility. At present, there is no way of choosing between these various possibilities, but for all of them the Bering Land Bridge would have been in place to permit the migrations involved (Marincovich & Gladenkov, 1999; Sher, 1999). Spread across mainland Asia to Europe would have been possible around the time American and Eurasian *Hydromantes* separated, as the previously intervening Turgai Strait had disappeared long before (Prothero, 1994). Several other groups moved across

Eurasia during this general period. Among these were salamandrids of the genera *Tylototriton*, *Cynops*, *Pachytriton* and *Paramesotriton* (Carranza & Arnold, 2004; Carranza & Wade, 2004; Weisrock *et al.*, 2005), *Takydromus* lacertids (Arnold *et al.*, 2007) and *Testudo* tortoises and their closest relatives (Parham *et al.*, 2006).

Relationships of main European groups

The phylogenetic analyses presented here (Figs 3 & 4) are congruent with other evidence that *H. (A.) genei* is sister to all the other European cave salamanders. Although support for this relationship in the DNA phylogeny is low, the other evidence is compelling in its variety and extent. It includes thorough analyses of a wide range of allozymic data derived from many specimens (Nascetti *et al.*, 1996), a phylogeny constructed using information from the Hy/Pol III repetitive DNA family (Nardi *et al.*, 2000), and the shared derived presence of morphologically differentiated sex chromosomes of the XX/XY type in the 14th chromosome pair of the subgenus *Speleomantes* (Nardi *et al.*, 1986; Nardi, 1991). Moreover, *H. (A.) genei* has more pericentric heterochromatin and more restriction sites for a particular marker in the ribosomal genes than the other European *Hydromantes*, and there are also differences in the distribution of centromeric satellites on the chromosomes (Nardi, 1991). Finally, mainland and east Sardinian *Speleomantes* share some helminth parasites, whereas different species are found in *H. (A.) genei* (Lanza *et al.*, 1995).

Possible double colonization of Sardinia

The molecular clock suggests that separation of *H. (A.) genei* from other members of the genus occurred approximately 9 Ma. This contrasts with previous estimates of around 27–30 Ma in the late Oligocene (Wake *et al.*, 1978, 2005). The estimated date of separation from mainland relatives is similar to those for the Corsican painted frog, *Discoglossus montalentii* Lanza, Nascetti, Capula & Bullini, 1984 (Zangari *et al.*, 2006) and the Mountain newts, *Euproctus,* of Sardinia and Corsica (Carranza & Amat, 2005). If the possibility of simultaneous transmarine dispersal is discounted, this suggests that all three taxa were involved in the same vicariant event. A land bridge is reported to have been in place at this time, in the Tortonian period, namely 10 Ma (Orszag-Sperber *et al.*, 1993). Another possible mechanism suggested by Lanza (1983) is that a small microplate, the 'Iglesiente block', broke away in the Oligocene from the continental mainland with *H. (A.) genei* on it, and later collided with the south-western part of the main Corsico-Sardinian microplate, but only in the Pliocene. Our results and similar results by Zangari *et al.* (2006) and Carranza & Amat (2005) are more compatible with the first scenario.

The date of the supposed second invasion of Sardinia by *Hydromantes*, involving a member of the subgenus *Speleomantes,* is estimated at 6.4 Ma and is similar to that of the separation of the Tyrrhenian painted frog, *Discoglossus sardus* (Tschudi, 1837), from its nearest mainland relatives (Zangari *et al.*, 2006). The estimates are close enough to the time of the Messinian Salinity Crisis to suggest that invasion was made possible by this event, when the sea bed between the continental mainland and Sardinia desiccated. Such Messinian movement of *Hydromantes* has been postulated previously (Nascetti *et al.*, 1996). Estimated dates of dispersal of other groups from the European mainland to Mediterranean islands are also more or less simultaneous with the Messinian Salinity Crisis (Blondel & Aronson, 1999; Terrasa *et al.*, 2004; Poulakakis *et al.*, 2003; Carranza *et al.*, 2006; Zangari *et al.*, 2006; Arnold *et al.*, 2007). As noted earlier, the alternative possibility, that *Speleomantes* evolved on Sardinia and then invaded the Italian mainland, is equally parsimonious in terms of number of journeys and distances covered. However, such a scenario would imply that the earlier lineages of *Hydromantes* became extinct on the mainland. This seems unlikely, given the great diversity of habitats there. Extinction on the mainland would be unexpected, if *Hydromantes* survived in the generally drier and much less varied environment of Sardinia.

Divergence of *Hydromantes (A.) genei*

Both the mitochondrial DNA data presented here and allozymes (Nascetti *et al.*, 1996; Cimmaruta *et al.*, 1997; Forti *et al.*, 1997) indicate that there are two well-differentiated clades within *H. (A.) genei*, as presently understood. One contains populations from the Carbonia–Barbusi–Monte Tasua zone (specimens 43–45, Figs 1 & 3), and the other the remaining more northerly populations (specimens 1–3; Figs 1 & 3). The molecular clock indicates that these two lineages originated around the Plio-Pleistocene boundary, about the time that divergence occurred in continental lineages of *Speleomantes* (see Fig. 4).

Diversification within the subgenus *Speleomantes*

Diversification of the subgenus *Speleomantes* within eastern Sardinia and continental Europe also commenced close to the Miocene–Pliocene boundary and was similarly likely to have been triggered by the Messinian Salinity Crisis. One of the principal consequences of the desiccation of the Mediterranean Sea was the increase in the aridity of the whole area and its surroundings. For instance, it is said that the Vienna woods were replaced by steppes and that palm trees grew in Switzerland (Blondel & Aronson, 1999). The increase in aridity probably fragmented the geographical distributions of many species associated with moist habitats, including the European *Hydromantes*, and may have been responsible for these species being confined to mountainous regions and caves (Lanza *et al.*, 1995). However, Californian *Hydromantes* are found in similar habitats, suggesting that the European species have not been much modified by the Messinian. Other cases of fragmentation around this time occurred elsewhere in the Mediterranean region. For example, in the Iberian peninsula they include Bosca's newt *Lissotriton boscai* (Lataste, 1879)

(Martínez-Solano *et al.*, 2006), the smooth snake, *Coronella austriaca* Laurenti, 1768 (Santos *et al.*, in press), and various groups of lacertid lizards, including western rock lizards, *Iberolacerta* (Arribas & Carranza, 2004; Carranza *et al.*, 2004), wall lizards, *Podarcis* (Harris *et al.*, 2002; Pinho *et al.*, 2006) and Schreiber's green lizard, *Lacerta schreiberi* Bedriaga, 1878 (Paulo *et al.*, 2001).

A total of six lineages of the subgenus *Speleomantes* originated quite rapidly soon after the Messinian (Fig. 4): four in east Sardinia and two on the continent. The east Sardinian lineages all show a high level of differentiation from each other. This is in contrast to the supposed near identity of two of the continental lineages, *H. (S.) flavus* Stefani, 1969 and *H. (S.) supramontis* Lanza, Nascetti & Bullini, 1986, in which a divergence of only 0.4% in the cytochrome b gene was reported (Jackman *et al.*, 1997). However, the data presented here indicate that the specimens sequenced in that study were in fact individuals of just one species, *H. (S.) supramontis*. The two continental lineages that apparently originated close in time to the Messinian Salinity Crisis are *H. (S.) strinatii,* and the ancestor of populations assigned to *H. (S.) ambrosii* and *H. (S.) italicus*. There was considerable later splitting within these lineages, with four diversification events occurring relatively close to the Plio-Pleistocene boundary.

In *H. strinatii,* the mtDNA phylogeny suggests that there was an early split, estimated at about 1.8 Ma, between the most western populations, in south-east France (samples 27 and 28), and those farther east, in Italy. Among the latter, a later division at 1.3 Ma apparently separated a western population (sample 26 from Monte Pietravecchia) from all those to the east. A disjunction here is also reflected by the low value of gene flow estimated using allozymes and Wright's (1951) method ($Nm = 0.28$; Forti *et al.*, 1997), although there may have been some gene flow across the boundary. In the remaining Italian populations, the most western one (sample 25, from Savona) is separated from the rest (samples 19–24, including that from Genoa) by a gap of over 100 km. In spite of this, all these samples are very similar in their mtDNA, a finding also indicated by the high level of gene flow detected with allozyme data ($Nm = 0.70$; Forti *et al.*, 1997). It has been suggested that the ophiolitic substrate in the intervening area might limit the presence of plethodontids there (Sindaco *et al.*, 2006), but relatively recent contact across it must have occurred.

The mitochondrial DNA phylogeny of the *H. (S.) ambrosii-H. (S.) italicus* clade, which borders *H. (S.) strinatii* to the east, appears to have undergone a division around 2.2 Ma. This separated *H. (S.) a. ambrosii* from a unit consisting of *H. (S.) a. bianchii* and *H.(S.) italicus*. The boundary between the two subspecies assigned to *H. ambrosii* is the valley of the Magra River. Here, the two forms are parapatric and their allozymes show no evidence of gene flow. The two forms differ in four discriminant loci and constitute reciprocally monophyletic units (Forti *et al.*, 1997). Although mtDNA sequence data indicate that *H. (S.) ambrosii* is paraphyletic, this contrasts with interpretations of allozyme data in which *H. (S.) ambrosii* is regarded as monophyletic (Nascetti *et al.*, 1996; Forti *et al.*, 1997). A constrained analysis, in which all the *H. (S.) ambrosii* were forced monophyletic, resulted in a tree that was not significantly worse than the tree presented in Fig. 3 (Shimodaira-Hassegawa test; $P > 0.05$), and therefore indicates that the monophyly of *H. (S.) ambrosii* is not rejected by our data.

The molecular clock suggests that separation of *H. (S.) a. bianchii* and *H. (S.) italicus*, which occurs even further east, took place around 1.3 Ma, but a relatively wide hybrid zone between the two forms has been reported (Forti *et al.*, 1997; Ruggi *et al.*, 2005). Molecular studies of both allozymes and restriction fragment-length polymorphism of mtDNA show that, while allozymes indicate considerable gene flow across the contact zone between the two, the mitochondrial haplotypes characteristic of each do not cross it. As mitochondrial features are inherited exclusively through female lineages, this suggests that hybridization results from movement of males, something that appears to have involved those of *H. (S.) a. bianchii* penetrating the range of *H. (S.) italicus* (Ruggi *et al.*, 2005). The fact that mitochondrial haplotypes of *H. (S.) italicus* have not entered populations assigned to *H. a. bianchii* means that the apparent paraphyly of *H. (S.) ambrosii* indicated by our mtDNA analyses, with *H. (S.) a. bianchii* being more similar to *H. (S.) italicus*, is not an artefact resulting from such spread.

Although *H. (S.) italicus* has the largest geographic distribution of all European members of its genus, extending over 600 km, it has a low level of genetic divergence that is reflected by both allozymes (Nascetti *et al.*, 1996; Forti *et al.*, 1997) and the mtDNA studied here (see Table S1). Even samples from opposite ends of the range (numbers 42 and 48, Fig. 1) show an uncorrected genetic divergence of less than 1% for the cytb. Such uniformity suggests relatively rapid spread, perhaps after a restriction in range that reduced previous genetic diversity.

The complex pattern of diversification in *H. (S.) strinatii* and the *H. (S.) ambrosii-H. (S.) italicus* clade is likely to have been produced by repeated fragmentation and subsequent molecular divergence, often followed by range expansion so that some of the differentiated units contacted each other. Dramatic climatic oscillations particularly in and just before the Pleistocene would have provided a mechanism for these events (Hewitt, 2000). Cold interludes with increased glaciation are likely to have restricted populations of *Hydromantes* to small favourable areas, from which they spread during subsequent relatively warm and humid interludes (Forti *et al.*, 1997). Such distributional changes were widespread in many other animal and plant groups during the Pleistocene (Taberlet *et al.*, 1998; Hewitt, 2000, 2003). Extinctions were also common, and the huge expansion that appears to have taken place in *H. (S.) italicus* may possibly have been facilitated by extermination of previous populations in its present range.

TAXONOMIC ACCOUNT

Status of *Hydromantes imperialis sarrabusensis*

The phylogenetic analyses based on mtDNA (Figs 3 & 4) clearly indicate that the recently described subspecies *H. (S.) imperialis*

sarrabusensis is a long-standing and independent lineage that originated approximately 5 Ma. It also has six diagnostic allozyme loci (*Idh*-2, 6-*Pg dh*, *Gapdh*, *Aat*-2, *Pep C*, *Ca*-4) at the 99% level, out of the 33 loci tested, and is genetically distant from the nominate subspecies (Nei value – Nei, 1972 – of 0.294; Lanza *et al.*, 2001). Moreover, it differs from the nominate subspecies in its smaller body size (maximum total length 111 mm in males and 123 mm in females), and by being scentless (no odour being produced even when handled). Finally, this lineage might also be the only viviparous lineage of *Hydromantes* described to date (Lanza & Leo, 2001), although examination of females with developing young inside them would be needed to confirm this hypothesis. It is also the only European cave salamander for which tail autotomy has been reported (Favelli *et al.*, 2007). In the light of this multiple evidence of its distinctness, *Hydromantes (Speleomantes) imperialis sarrabusensis* is upgraded to full species status as *Hydromantes (Speleomantes) sarrabusensis* stat. nov.

CONCLUDING REMARKS

Hydromantes exhibits an impressive disjunction, with two surviving monophyletic groups of species that are separated by more than 9000 km. The calibration of the molecular clock employed here results in earlier dates for events than previously suggested. Apart from the reasons already given for preferring the calibration used (see above), a further reason is that the estimates of the dates of phylogenetic bifurcations that it produces coincide with climatic and geophysical events that could have caused them. Thus, separation between American and European *Hydromantes* is estimated to have occurred at a time when the Bering Land Bridge was in place; the invasion of the ancestor of *H. (A.) genei* into Sardinia roughly coincides with the presence of a land bridge during the Tortonian period; movement of the subgenus *Speleomantes* between Italy and Sardinia occurred around the time of the Messinian Salinity Crisis, when terrestrial dispersal is likely to have been possible; initial divergence in both continental and Sardinian members of the subgenus *Speleomantes* occurred at a time when this event was causing climatic change likely to fragment distributions; and the later divergence of *Speleomantes* is associated with climatic fluctuations that characterize the Pleistocene epoch. Other calibrations of molecular clocks that result in earlier dating of phylogenetic events show less convincing correlations with known geophysical and climatic events.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

Table S1 Pairwise uncorrected genetic distances (substitutions per site) for 771 bp of the cytochrome b + 12S rRNA mitochondrial genes.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2699.2007.01817.x

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