Molecular Phylogenetics and Evolution 51 (2009) 399–404

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Short Communication

Phylogenetic relationships of Sardinian cave salamanders, genus *Hydromantes*, based on mitochondrial and nuclear DNA sequence data

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ARTICLE INFO

Article history:
Received 5 September 2008
Revised 12 November 2008
Accepted 10 December 2008
Available online 24 December 2008

1. Introduction

The Tyrrhenian island of Sardinia is known for its high level of endemism (Oosterbroek and Arntzen, 1992; Grill et al., 2007). Beside the importance of this island as a refuge during the last glaciations, little is known about the origin and relationships of Sardinian species (reviewed in Grill et al., 2007). The origin of some Sardinian lineages is attributed to the isolation of the Sardo-Corsican plate (e.g. Lanza, 1983; Caccone et al., 1994; Oliverio et al., 2000; Omodeo and Rota, 2008), others to the Messinian salinity crisis (e.g. Marra, 2004) or to more recent events such as the severe climate changes from the early Pliocene and throughout the Pleistocene (Grill et al., 2007 and references therein). It still remains unclear which of these events are more relevant to explain the current distribution of the European members of the genus *Hydromantes*. Despite their common name, *Hydromantes* are not restricted to cave habitats, or even to limestone substrates, but are also found on exposed sites, even on granites and volcanic rocks (Lanza et al., 2005). Their range is therefore not directly restricted by these aspects of geology or even by plant cover. Humid subterranean hiding places, such as the space between the rocks of a rockslide, do seem to be correlated with their presence (personal observation). The salamanders of the genus *Hydromantes* have their highest diversity in Sardinia, being represented by five species, versus only three species in mainland Europe, and three in the western USA. Recently, Vieites et al. (2007) have brought forward a compelling hypothesis for the highly disjunct distribution of the members of the genus *Hydromantes* between the western US and Europe by suggesting dispersal through the Bering land bridge. The eight European species are distributed in southeastern France, north and central Italy and throughout Sardinia. Previous phylogenetic works based on immunological data and allozymes (Wake et al., 1978), allozymes (Nascetti et al., 1996), morphology (Lanza and Caputo, 1995), morphology, caryology and cytochrome *b* sequences (Jackman et al., 1997) and cytochrome *b*, 12S rRNA and 16S rRNA sequences (Carranza et al., 2007) did not fully resolve the relationships among the European species of *Hydromantes*. Some of these studies (e.g., Nascetti et al., 1996; Nardi, 1991; but see also Carranza et al., 2007) support the hypothesis of *H.* (*Atylodes*) *genei*, which inhabits the southwest of Sardinia, as the most basal European species. The eastern Sardinian species (*H.* (*Speleomantes*) *flavus*, *H.* (*S.*) *supramontis*, *H.* (*S.*) *imperialis* and *H.* (*S.*) *sarrabusensis*) are thought to be more closely related to the mainland species (*H.* (*S.*) *strinatii*, *H.* (*S.*) *ambrosii* and *H.* (*S.*) *italicus*). Several biogeographic hypotheses have been put forward to explain this pattern (reviewed in Lanza et al., 2005). All these biogeographic hypotheses hinge on the basal position of *H.* (*A.*) *genei*, which is generally considered to have split off from the ancestral stock well before the other species.

The recent publication of Carranza et al. (2007) addresses the phylogenetic relationships between the European species based on three mitochondrial genes, but could not resolve *H.* (*A.*) *genei* unambiguously as the most basal taxon, or the sister group relationship between the mainland species and the eastern Sardinian species. Also the placement of *H.* (*S.*) *sarrabusensis* could not be

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1055-7903/$ - see front matter
doi:10.1016/j.ympev.2008.12.011

unambiguously resolved. Increased taxon sampling and enlargement of the dataset have been shown to increase phylogenetic resolution (Pollock et al., 2002; Brinkmann and Philippe, 2008). Therefore, in an effort to resolve these relationships, in the current study we extended the existing dataset considerably by adding two nuclear genes and additional mitochondrial sequence data, as well as greatly extend the number of Sardinian populations represented in the dataset from eight to 16 populations. Our data confirm the previous findings by Carranza et al. (2007), but despite the larger dataset, several relationships among the European members of the genus *Hydromantes* still remain poorly resolved.

2. Materials and methods

2.1. Specimen selection

A total of 66 European *Hydromantes* specimens were used in this study. Of these 33 samples are the same as used in the study by Carranza et al. (2007), while the other 33 samples were collected in Sardinia in April 2007 (Table 1). We included a total of 27 populations. Sardinian species were represented by samples from across the range of each species from a total of 16 populations (Fig. 1). A maximum of three specimens from every sampling of each population were randomly selected to be included in this study (Table 1). Samples taken from sites at very close proximity were counted as a single population.

2.2. DNA extraction, PCR and sequencing

DNA was extracted from tail tips preserved in 99% ethanol using a DNeasy® blood and tissue kit (Quiagen). Primers for one fragment of the 12S rRNA gene and one fragment of the 16S rRNA gene were 12SA-L and 12SB-H and 16SA-L and 16SB-H, used as in Palumbi et al. (1991). Primers for cytochrome *b* were designed based on an alignment of *Hydromantes* sequences available in GenBank. These were Cyt-BYf1 (5′-CAARTCCTYACCGGRYTATTT-3′) as the forward primer and Cyt-BYr1 (5′-TTCGRTKTTTTGADGTRTTTA-3′) as the reverse primer. This fragment was amplified using an initial denaturation step of 2 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 52 °C for 30 s, and extension at 72 °C for 90 s. Two nested pairs of primers to amplify a single 1400 bp fragment of Rag-1 were designed based on an alignment of available salamander Rag-1 sequences from GenBank. The first PCR with forward primer Rag-1Yf1 (5′-CAGATTTTCCAGCCYTTACAYGC-3′) and reverse Rag-1Yr1 (5′-CCATTTCATAAAACTTGGACTGC-3′). The second PCR with forward primer Rag-1Yf2 (5′-CTWCCAGGMTAYCAYCYVTTYG-3′) and reverse primer Rag-1Yr2 (5′-CGGAAACGTCTGAACAGYTTC-3′). PCR conditions for these Rag-1 primers and the BDNF primers (Van der Meijden et al., 2007) were those as described for Rag-1 and Rag-2 in Van der Meijden et al. (2005). PCR was performed in 25 μl reactions using REDTaq Polymerase Readymix (Sigma, Taufkirchen, Germany). PCR products were purified via spin columns (Qiagen). Sequencing was performed directly using the corresponding PCR primers on an ABI3730XL sequencer. New sequences were combined with existing sequences taken from GenBank in the final dataset and additional five closely related plethodontid species and the salamandrid *Salamandra* were used as nested outgroups for the phylogenetic analyses (Table 1). New sequences were deposited in GenBank (accession numbers in Table 1).

2.3. Phylogenetic analysis

Chromatograms were checked by eye using FinchTV 1.4 (Geospiza.com) and the sequences were subsequently aligned using the Muscle alignment program version 3.6 (Edgar, 2004) using the default settings (best accuracy). The resulting alignments were checked by eye, but were not found to require additional editing. The final aligned dataset was deposited in the TreeBase database (Study accession number S2274).

Missing genes were coded as "missing" (?) in the concatenated dataset (see Table 1). In Bayesian Inference (BI) and Maximum Parsimony (MP) analyses of large multi-gene data sets of hylid frogs, no relationship was found between completeness of the sequence data of a taxon, and the support values the taxon receives (Wiens et al., 2005), suggesting that the limited amount of missing data in our concatenated alignment is unlikely to distort the phylogenetic results. Furthermore, the placement of taxa with missing data in our analysis was congruent for well supported branches (bootstrap support >80%) between the single gene analyses in which the taxon was represented by a complete sequence, and the combined dataset, suggesting that omission of one or more genes from the combined set did not influence the placement of particular taxa (data not shown).

A homogeneity partition test (Farris et al., 1994) as implemented in PAUP* version 4.0b10 (Swofford, 2002) did not reject homogeneity of the five different markers ($P = 0.17$). Besides an analysis of the combined data set we also performed independent analyses for each gene and for the combined mitochondrial and the combined nuclear genes in the dataset. Phylogeny reconstruction based on the separate and combined datasets was performed using Maximum Likelihood (ML) and BI methods. The best fitting models of sequence evolution were determined by the AIC criterion in Modeltest 3.7 (Posada and Crandall, 1998). ML tree searches were performed using PhyML, version 2.4.4 (Guindon and Gascuel, 2003). Bootstrap branch support values were calculated with 500 replicates for the combined datasets, and 200 replicates in the single gene analyses. The BI analyses of the combined and separate datasets were conducted with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), using models estimated with Modeltest under the AIC criterion, with 2,500,000 generations, sampling trees every 100th generation (and calculating a consensus tree after omitting the first 6250 trees). Log likelihood scores for the remaining trees were graphed in Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer) and checked for appropriateness of the burnin-period. The topologies resulting from the ML and BI analyses of the combined dataset were compared using a Shimodaira–Hasegawa (SH) test as implemented in PAUP* (Swofford, 2002), with RELL optimization and 10000 bootstrap replicates. Between species genetic distances (uncorrected *p*-distances, see Table 2) were calculated based on the cytochrome *b* alignment using MEGA version 4 (Tamura et al., 2007). Tables of genetic distances based on the other genes are available from the authors on request.

3. Results and discussion

The concatenated dataset contained a total of 3494 base pairs (375 bp of 12S rRNA, 449 bp of 16S rRNA, 755 bp of cytochrome *b*, 1230 bp of RAG-1 and 685 bp of BDNF). PCR amplification of some specimens failed for some genes despite several retries (see Table 1). The results of both the ML and BI analyses of the complete dataset as well as a separate mitochondrial and a nuclear dataset yielded similar groupings where branch support was high. Although not all support values were high, both the mitochondrial and nuclear dataset analyses showed all five Sardinian species as monophyletic (Fig. 2).

3.1. Position of H. (A.) genei

The basal position of *H.* (*A.*) *genei* is considered well supported based on, among other things, allozyme data (Wake et al., 1978;

Table 1
Specimens used in this study. Field numbers from Carranza et al. (2007) are listed for reference. Missing sequences are indicated by "missing". *H.* = *Hydromantes*. *A.* = *Atylodes*. *S.* = *Speleomantes*. The *H.* (*A.*) *genei* "A" and "B" refer to the two subclades of that species, as shown in Fig. 2.

Species name	Specimen number	Locality	Carranza et al. (2007)	12S rRNA	16S rRNA	Cytb	Rag-1	BDNF
H. (A.) genei "B"	1	Monte Tasua, Carbonia, Sardinia (Italy)	E1037.1	FJ602107	missing	FJ602276	FJ602342	FJ602217
H. (A.) genei "B"	2	Monte Tasua, Carbonia, Sardinia (Italy)	E1037.2	FJ602108	missing	FJ602277	missing	missing
H. (A.) genei "B"	3	Monte Tasua, Carbonia, Sardinia (Italy)	E1037.3	FJ602109	missing	FJ602278	FJ602343	FJ602218
H. (A.) genei "A"	4	Grotta su Mannau, Fluminimaggiore, Sardinia (Italy)	E3056.8	FJ602106	FJ602164	FJ602275	FJ602341	FJ602216
H. (A.) genei "A"	5	Sanita' , Fluminimaggiore, Sardinia (Italy)	E10076.5	FJ602104	FJ602162	FJ602273	FJ602339	FJ602214
H. (A.) genei "A"	6	Sanita' , Fluminimaggiore, Sardinia (Italy)	E10076.6	FJ602105	FJ602163	FJ602274	FJ602340	FJ602215
H. (A.) genei "A"	7	Mine Terras Nieddas, Fluminimaggiore, Sardinia (Italy)		FJ602094	FJ602152	FJ602266	FJ602329	FJ602205
H. (A.) genei "A"	8	Mine Terras Nieddas, Fluminimaggiore, Sardinia (Italy)		FJ602095	FJ602153	FJ602267	FJ602330	FJ602206
H. (A.) genei "A"	9	Mine Terras Nieddas, Fluminimaggiore, Sardinia (Italy)		FJ602096	FJ602154	missing	FJ602331	FJ602207
H. (A.) genei "A"	10	Mine Su Corovau, Domusnovas, Sardinia (Italy)		FJ602097	FJ602155	FJ602268	FJ602332	FJ602208
H. (A.) genei "A"	11	Mine Su Corovau, Domusnovas, Sardinia (Italy)		FJ602098	FJ602156	FJ602269	FJ602333	FJ602209
H. (A.) genei "A"	12	Mine Su Corovau, Domusnovas, Sardinia (Italy)		FJ602099	FJ602157	missing	FJ602334	FJ602210
H. (A.) genei "B"	13	Grotta Cava Romana, Nuxis, Sardinia (Italy)		FJ602100	FJ602158	FJ602270	FJ602335	FJ602211
H. (A.) genei "B"	14	Grotta dei geotritoni, Nuxis, Sardinia (Italy)		FJ602101	FJ602159	missing	FJ602336	missing
H. (A.) genei "B"	15	Grotta dei geotritoni, Nuxis, Sardinia (Italy)		FJ602102	FJ602160	FJ602271	FJ602337	FJ602212
H. (A.) genei "B"	16	Grotta dei geotritoni, Nuxis, Sardinia (Italy)		FJ602103	FJ602161	FJ602272	FJ602338	FJ602213
H. (S.) flavus	17	Grotta di Nurai, Lula, Sardinia (Italy)		FJ602088	FJ602146	FJ602260	FJ602323	FJ602199
H. (S.) flavus	18	Grotta di Nurai, Lula, Sardinia (Italy)		FJ602089	FJ602147	FJ602261	FJ602324	FJ602200
H. (S.) flavus	19	Grotta di Nurai, Lula, Sardinia (Italy)		FJ602090	FJ602148	FJ602262	FJ602325	FJ602201
H. (S.) flavus	20	Conca 'e Crapa, Lula, Sardinia (Italy)	E3056.6	FJ602091	FJ602149	FJ602263	FJ602326	FJ602202
H. (S.) flavus	21	Conca 'e Crapa, Lula, Sardinia (Italy)	E3056.7	FJ602092	FJ602150	FJ602264	FJ602327	FJ602203
H. (S.) flavus	22	Conca 'e Crapa, Lula, Sardinia (Italy)	E3056.5	FJ602093	FJ602151	FJ602265	FJ602328	FJ602204
H. (S.) imperialis	23	Lago Omodeo, Ula Tirso, Sardinia (Italy)		FJ602119	FJ602174	FJ602288	FJ602352	FJ602228
H. (S.) imperialis	24	Lago Omodeo, Ula Tirso, Sardinia (Italy)		FJ602120	FJ602175	FJ602289	FJ602353	FJ602229
H. (S.) imperialis	25	Grotta Sa Turru, S. Nicolò Gerrei, Sardinia (Italy)		FJ602121	FJ602176	FJ602290	FJ602354	FJ602230
H. (S.) imperialis	26	Grotta Sa Turru, S. Nicolò Gerrei, Sardinia (Italy)		FJ602122	FJ602177	FJ602291	FJ602355	FJ602231
H. (S.) imperialis	27	Grotta Sa Turru, S. Nicolò Gerrei, Sardinia (Italy)		FJ602123	FJ602178	FJ602292	FJ602356	FJ602232
H. (S.) imperialis	28	Grotta Sa Rutta 'e Linus, Perdasdefogu, Sardinia (Italy)		FJ602110	FJ602165	FJ602279	FJ602344	FJ602219
H. (S.) imperialis	29	Grotta Sa Rutta 'e Linus, Perdasdefugu, Sardinia (Italy)		FJ602111	FJ602166	FJ602280	FJ602345	FJ602220
H. (S.) imperialis	30	Grotta Sa Rutta 'e Linus, Perdasdefugu, Sardinia (Italy)		FJ602112	FJ602167	FJ602281	FJ602346	FJ602221
H. (S.) imperialis	31	Grotta de Is Lianas, Ulassai, Sardinia (Italy)		FJ602113	FJ602168	FJ602282	FJ602347	FJ602222
H. (S.) imperialis	32	Grotta de Is Lianas, Ulassai, Sardinia (Italy)		FJ602114	FJ602169	FJ602283	FJ602348	FJ602223
H. (S.) imperialis	33	Grotta de Is Lianas, Ulassai, Sardinia (Italy)		FJ602115	FJ602170	FJ602284	FJ602349	FJ602224
H. (S.) imperialis	34	Grotta Orroli, Osini, Sardinia (Italy)		FJ602116	FJ602171	FJ602285	FJ602350	FJ602225
H. (S.) imperialis	35	Grotta Orroli, Osini, Sardinia (Italy)		FJ602117	FJ602172	FJ602286	FJ602351	FJ602226
H. (S.) imperialis	36	Grotta Orroli, Osini, Sardinia (Italy)		FJ602118	FJ602173	FJ602287	missing	FJ602227
H. (S.) imperialis	37	Ulassai, Sardinia (Italy)	E3056.9	FJ602127	FJ602179	FJ602296	missing	missing
H. (S.) imperialis	38	Ulassai, Sardinia (Italy)	E3056.10	FJ602128	FJ602180	FJ602297	FJ602360	FJ602236
H. (S.) imperialis	39	Ulassai, Sardinia (Italy)	E3056.11	FJ602129	FJ602181	FJ602298	FJ602361	FJ602237
H. (S.) sarrabusensis	40	Mount Sette Fratelli, Burcei, Cagliari, Sardinia (Italy)	E10076.1	FJ602124	missing	FJ602293	FJ602357	FJ602233
H. (S.) sarrabusensis	41	Mount Sette Fratelli, Burcei, Cagliari, Sardinia (Italy)	E10076.2	FJ602125	missing	FJ602294	FJ602358	FJ602234
H. (S.) sarrabusensis	42	Mount Sette Fratelli, Burcei, Cagliari, Sardinia (Italy)	E10076.3	FJ602126	missing	FJ602295	FJ602359	FJ602235
H. (S.) supramontis	43	Grotta Su Bentu, Oliena, Sardinia (Italy)		FJ602133	FJ602190	FJ602310	FJ602370	FJ602247
H. (S.) supramontis	44	Grotta Su Bentu, Oliena, Sardinia (Italy)		FJ602134	FJ602191	FJ602311	FJ602371	FJ602248
H. (S.) supramontis	45	Grotta Su Bentu, Oliena, Sardinia (Italy)		FJ602135	FJ602192	FJ602312	FJ602372	FJ602249
H. (S.) supramontis	46	Punta Cuccuttos, Urzulei, Sardinia (Italy)	E10076.7	FJ602139	missing	FJ602316	FJ602375	FJ602253
H. (S.) supramontis	47	Punta Cuccuttos, Urzulei, Sardinia (Italy)	E10076.8	FJ602140	missing	FJ602317	FJ602376	FJ602254
H. (S.) supramontis	48	Punta Cuccuttos, Urzulei, Sardinia (Italy)	E10076.9	FJ602141	missing	FJ602318	FJ602377	FJ602255
H. (S.) supramontis	49	Grotta su Bentu, Oliena, Sardinia (Italy)	E3056.12	FJ602136	missing	FJ602313	FJ602373	FJ602250
H. (S.) supramontis	50	Grotta su Bentu, Oliena, Sardinia (Italy)	E3056.13	FJ602137	FJ602193	FJ602314	FJ602374	FJ602251
H. (S.) supramontis	51	Grotta su Bentu, Oliena, Sardinia (Italy)	E3056.14	FJ602138	FJ602194	FJ602315	missing	FJ602252
H. (S.) ambrosii ambrosii	52	Grotta Fornace, Pignone, La Spezia, Liguria (Italy)	E2306.5	EU116982	FJ602144	FJ602258	FJ602321	FJ602197
H. (S.) ambrosii ambrosii	53	Grotta Fornace, Pignone, La Spezia, Liguria (Italy)	E2306.4	EU116983	FJ602145	FJ602259	FJ602322	FJ602198
H. (S.) ambrosii bianchii	54	Apuan Alps, Massa-Carrara, Tuscany (Italy)	E251061	EU116974	FJ602142	FJ602256	FJ602319	FJ602195
H. (S.) ambrosii bianchii	55	Apuan Alps, Massa-Carrara, Tuscany (Italy)	E251062	EU116975	FJ602143	FJ602257	FJ602320	FJ602196
H. (S.) italicus	56	Sorgenti dell'Esino, Esanatoglia, Ancona, Marche (Italy)	E2306.12	EU116976	FJ602185	FJ602302	FJ602365	FJ602241
H. (S.) italicus	57	Abrutti, Farindola, Pescara (Italy)	E2306.14	EU116977	FJ602186	FJ602303	FJ602366	FJ602242
H. (S.) italicus	58	Monte Tezio, Perugia, Umbria (Italy)	E2306.8	EU116978	FJ602187	FJ602304	missing	FJ602243
H. (S.) italicus	59	Buca delle Fate (Coreglia Antelminelli), Lucca, Tuscany (Italy)		FJ602130	FJ602182	FJ602299	FJ602362	FJ602238
H. (S.) italicus	60	Buca delle Fate (Coreglia Antelminelli), Lucca, Tuscany (Italy)		FJ602131	FJ602183	FJ602300	FJ602363	FJ602239
H. (S.) italicus	61	Tana Buca di Maggiano (Maggiano), Lucca, Tuscany (Italy)		FJ602132	FJ602184	FJ602301	FJ602364	FJ602240
H. (S.) strinatii	62	W. Monte Groppi, Genova, Liguria (Italy)	E3056.4	EU116963	EU116988	FJ602305	FJ602367	FJ602244
H. (S.) strinatii	63	Rio Tonno, Vallbrevenna, Genova, Liguria (Italy)	E2306.17	EU116968	FJ602188	FJ602306	missing	missing
H. (S.) strinatii	64	S. Bartolomeo, Savignone, Genova, Liguria (Italy)	E2306.9	EU116967	missing	FJ602307	missing	missing
H. (S.) strinatii	65	Maritime Alps (France)	E14076.1	EU116971	missing	FJ602308	FJ602368	FJ602245

(*continued on next page*)

Table 1 (*continued*)

Species name	Specimen number	Locality	Carranza et al. (2007)	12S rRNA	16S rRNA	Cytb	Rag-1	BDNF
H. (S.) strinatii	66	Maritime Alps (France)	E14076.2	EU116970	FJ602189	FJ602309	FJ602369	FJ602246
H. shastae				missing	missing	missing	EU275794	EU275875
H. brunus				AY728234	NC006345	AY728234	EU275790	EU275871
H. platycephalus				DQ283227	DQ283227	missing	EU275793	EU275874
Karsenia koreana				missing	missing	missing	AY887135	EU275868
Ensatina eschscholtzii				AY728216	EF107214	AY728216	EU275785	EU275862
Salamandra salamandra				DQ221213	AY336629	AY035819	AY583352	EF453369

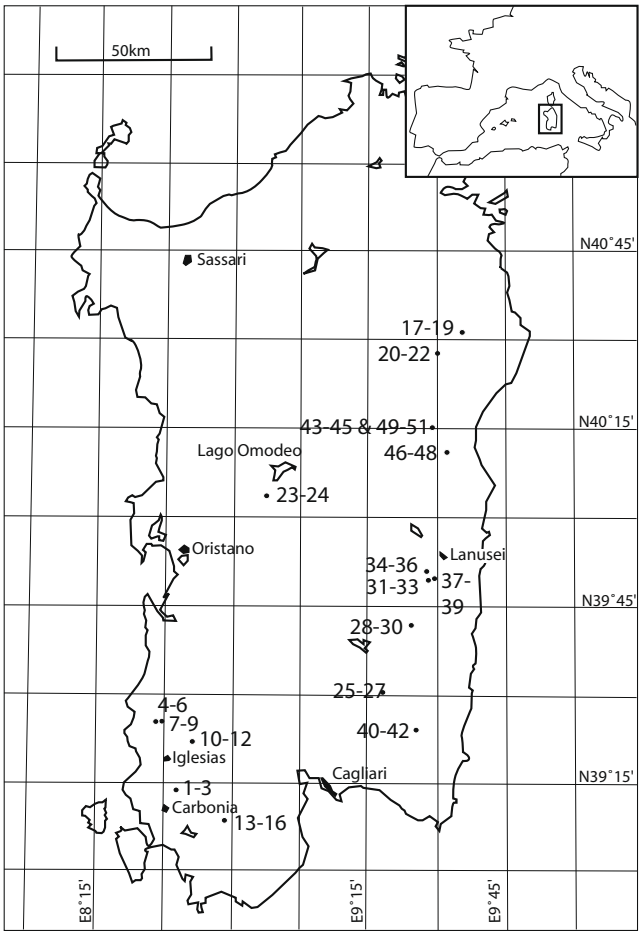

Fig. 1. Sampling localities for all Sardinian species. Numbers correspond to specimen numbers in Table 1. 1–16, *H. (A.) genei*; 17–22, *H. (S.) flavus*; 23–39, *H. (S.) imperialis*; 40–42, *H. (S.) sarrabusensis*; 43–51, *H. (S.) supramontis*.

Nascetti et al., 1996), the lack of differentiated sex chromosomes (Nardi, 1991) and parasites shared by eastern Sardinian species and mainland species, but not by *H. (A.) genei* (Lanza and Caputo, 1995). As in Jackman et al. (1997) and Carranza et al. (2007), the basal position of *H. (A.) genei* could not be unambiguously corroborated in our study despite a much larger dataset, and two additional populations. The BI tree showed *H. (A.) genei* as basal to all other European taxa, whereas the ML tree showed a sister group relationship between *H. (A.) genei* and the mainland species. Neither of these placements were highly supported. A SH test based on the combined dataset showed that neither of the two alternative placements of *H. (A.) genei* could be rejected ($p = 0.43$). In the single gene analyses, only Rag-1 highly supported the sister group relationship between the mainland species and the east Sardinian species to the exclusion of *H. (A.) genei* (93% bootstrap support, data not shown). As support for more derived as well as more basal nodes in the phylogeny based on the combined dataset are well resolved (see Fig. 2), it is possible that the lack of support basally in the clade of *Hydromantes* (*Speleomantes*) may have been the result of a relatively rapid succession of divergences during the cladogenesis of the European species of *Hydromantes*.

3.2. Divergence within H. (A.) genei

Our data corroborate two well supported groups within *H. (A.) genei*, as first resolved based on allozymes by Nascetti et al. (1996). The genetic distance between these two groups is similar to the lowest distances between full species based on the cytochrome *b* sequences available. The *p*-distance for *H. (A.) genei* "A"–*H. (A.) genei* "B" (*sensu* Lanza, 2005) is 0.061, a value identical to the distance between the full species *H. (S.) italicus* and *H. (S.) ambrosii*. These similar distances are consistent with the finding of Carranza et al. (2007), who estimated the divergence time between these two lineages of *H. (A.) genei* to be similar to the divergence times between the mainland species based on a shorter fragment of cytochrome *b* (398 bp) combined with 373 bp of 12S rRNA. The next four lowest genetic distances between full species based on our cytochrome *b* dataset are *H. (S.) sarrabusensis–H. (S.) imperialis* (0.078), *H. (S.) sarrabusensis–H. (S.) flavus* (0.079), *H. (S.) strinatii–H. (S.) ambrosii* (0.080) and *H. (S.) supramontis–H. (S.) flavus* (0.081, see Table 2). We found the average genetic distance between full (European) species to be much higher (0.117).

Specimens 13–16 of *H. (A.) genei* "B" were found at Nuxis (Tattinu), a locality more than 17 kilometers ESE outside the range described for *H. (A.) genei* "B" by Lanza et al. (2005). Nascetti et al. (1996) placed specimens from close to this locality ("Near Nuxus", 5.7 km W, and "Near Pula", 11.5 km SSE of the locality of specimens 13–16) in the *H. (A.) genei* "A" group based on allozyme data.

Table 2
Genetic *p*-distance based on 755 basepairs of the cytochrome *b* gene.

	H. (S.) ambrosii	*H. (S.) flavus*	*H. (A.) genei*	*H. (S.) imperialis*	*H. (S.) sarrabusensis*	*H. (S.) italicus*	*H. (S.) strinatii*
H. (S.) flavus	0.123						
H. (A.) genei	0.179	0.132					
H. (S.) imperialis	0.127	0.083	0.161				
H. (S.) sarrabusensis	0.132	0.079	0.163	0.078			
H. (S.) italicus	0.061	0.105	0.169	0.119	0.110		
H. (S.) strinatii	0.080	0.112	0.140	0.119	0.118	0.106	
H. (S.) supramontis	0.152	0.081	0.158	0.084	0.063	0.130	0.114

Fig. 2. Bayesian Inference tree based on all data combined. Numbers at branches represent Bayesian posterior probabilities/Maximum Likelihood bootstrap percentages based on 500 bootstrap replicates. Branches not present in the ML tree are indicated with a dash (''–''). Outgroup taxa not shown.

It therefore seems that *H.* (*A.*) *genei* ''A'' and ''B'' live in close proximity in the southern part of their range. Further studies with a higher sampling density including the localities described in Nascetti et al. (1996) will be necessary to address this issue.

3.3. Position of H. (S.) sarrabusensis

Although resolved to be monophyletic in all analyses, the position of the clade representing *H.* (*S.*) *sarrabusensis* is different

between the ML and BI trees. It is placed with low support sister to the "Eastern Sardinia" group formed by *H.* (*S.*) *flavus*, *H.* (*S.*) *supramontis* and *H.* (*S.*) *imperialis* by the ML analysis, whereas the BI analysis places it as a sister group to the *H.* (*S.*) *imperialis* clade (Fig. 2). A SH test showed these two placements to be not significantly different ($p = 0.23$). More data will be required to resolve the position of this newly distinguished taxon within the highly supported eastern Sardinian group.

3.4. Hydromantes (Speleomantes) imperialis from the Lake Omodeo area

The samples from the locality near Lake Omodeo (specimen numbers 23–24, Fig. 1 and Table 1) at the westernmost border of the range of *H.* (*S.*) *imperialis* are resolved as distinct from other populations with a genetic distance of 0.060 (*p*-distance) in cytochrome *b*. This is considerably higher than the highest genetic distance found between two populations of *H.* (*S.*) *imperialis* excluding these samples (*p*-distance of 0.040, between specimens 28 and 31, Fig. 1). The sampling of *H.* (*S.*) *imperialis* in the current study is highly disjunct (see Fig. 1), including samples only from the eastern and western parts of the distribution range. Therefore, the high genetic distance between the specimens from Lake Omodeo and the remaining *H.* (*S.*) *imperialis* populations could be an artifact of the disjunct sampling in this study, and might be bridged by including samples from intermediate populations. However, a study based on allozyme data by Cimmaruta et al. (1998), which did included some samples from more intermediate localities, showed the populations from near Lake Omodeo to be highly distinct from, and basal to the remaining populations of *H.* (*S.*) *imperialis*. Further studies including a denser sampling across the whole distribution range will be necessary to resolve the genetic structure of this widespread species. Such a study would be particularly necessary to assess the status of the populations near Lake Omodeo which, based on the sampling in the current study, display a genetic distance approaching that between other species of the genus *Hydromantes*.

Acknowledgments

We are thankful to the Italian Ministero dell'Ambiente e della Tutela del Territorio (Prot.DPN-2007-0014603, DPN-2008-0008210, DPN-2006-7546 and 9333-03/04/2007) for issuing the permits for tissue collection. We are grateful to the speleologists of the Gruppo Grotte Ogliastra, Renzo Tocco, Titino Salis, Luciano Cuccu and to Vasco Alderighi for helping with the sampling. Field work has been supported by a grant of the Societas Europea Herpetologica to A.vdM., Y.C., M.M., M.V., S.C. was supported by grant CGL2005-06876/BOS from the Ministerio de Educación y Ciencia, Spain. This is the ISE-M publication *ISEM 2008-128 of Y.C.

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