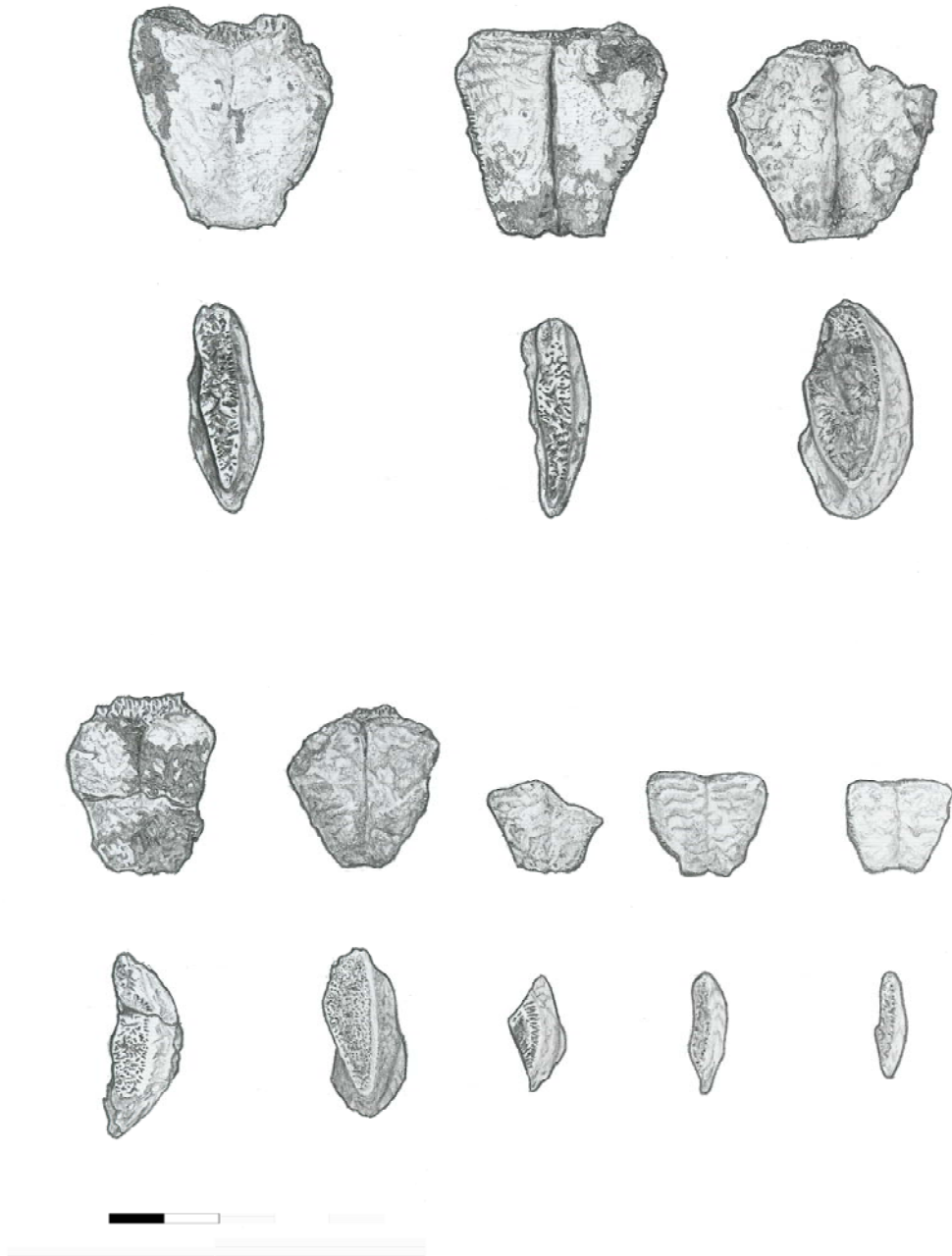


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GENETIC VARIATION WITHIN *Bufo viridis*: EVIDENCE FROM MITOCHONDRIAL 12S AND 16S rRNA DNA SEQUENCES.

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Abstract:

Bufo viridis is one of the most widespread Old World amphibian species. The presence of diploid, triploid and tetraploid populations within the *B. viridis* complex is unique among amphibians, and has resulted in considerable scientific attention. Surprisingly therefore little is known concerning the phylogeography of the group. In this study 35 individuals including widespread populations were analysed for partial 12S and 16S rRNA mitochondrial DNA. Three divergent lineages were determined; one in North Africa and Sicily, another in Europe, and a third in Sardinia and Mallorca. Relationships between the three lineages were not well supported. A tetraploid individual, typically assigned to *B. danatensis*, nested within the European lineage based on these markers. No evidence for anthropogenic introductions to Mediterranean islands was found, although further sampling is needed to fully determine the origins of the Balearic populations.

Keywords: Phylogeography, *Bufo viridis*, 12S rRNA, 16S rRNA, mitochondrial DNA.

Resum:

Bufo viridis és un dels amfibis europeus amb un àrea de distribució més amplia. La presència de poblacions diploides, triploides i tetraploides dins del complex *B. viridis* és única entre els amfibis i això ha fet que se'l consideri un grup molt interessant des del punt de vista científic. Sorprenentment però, tot i el seu interès la seva filogeografia és encara desconeguda. En aquest estudi 35 individus pertanyents a poblacions ampliament distribuïdes de la Mediterrània Occidental han estat analitzats mitjançant la seqüenciació dels gens mitocondrials 12S i 16S rRNA. Els resultats indiquen que hi ha un mínim de 3 llinatges independents, un al nord d'Àfrica i Sicília, un altre a Europa continental i un tercer a Mallorca i Sardenya. Les relacions filogenètiques entre els tres grups no estan molt ben resoltes.

Paraules clau: Filogeografia, *Bufo viridis*, 12S ARNr, 16S ARNr, ADN mitocondrial.

Introduction

The genus *Bufo* contains more than 250 species, more than half the species of the whole family Bufonidae, and are distributed across Eurasia, Africa and the Americas (Duellmann and Sweet, 1999). Of these *Bufo viridis* has one of the largest ranges. It occurs across Eastern Europe including the southern tip of Sweden, but excluding Western Europe west of the Rhine river and the British Isles. It spreads across the Balkans and Northern Italy, but not Central Italy although it occurs in Southern Italy. It is found in several Mediterranean islands, including Sicily, the Balearics, Corsica, Sardinia and Malta, although some of these populations have been suggested to be due to human introductions (Roth, 1997). It exists in North Africa from coastal Western Sahara through Morocco and Algeria to Western Libya, and into the Middle East and the Arabian Peninsula. It is also found in Central Asia, including Kazakhstan and Tajikistan. Ecologically it is very plastic, being found from sea level to 2400m in light woods, stony areas, coastal dunes, agricultural lands and around human habitations (Arnold, 2002).

Systematics of the whole *Bufo viridis* complex is difficult. Disposition and size of markings is very variable, but without obvious geographic trends thus confounding sub-specific designations. The *B. viridis* complex is the only known amphibian species complex that includes diploid, triploid and tetraploid bisexually reproducing taxa (Stöck *et al.*, 2005). Although tetraploid populations are often assigned to *Bufo danatensis* (e.g. Borkin *et al.*, 2001) and this has been accepted in recent species lists (e.g. Dubois *et al.*, 2005), in Asia complex mixed breeding systems occur (Stöck *et al.*, 2005). Tetraploid forms have also been assigned to *Bufo oblongus* (including *B. o. danatensis*), and *B. pewzowi* (Stöck *et al.*, 2001). Tetraploids differ in morphological and behavioural characters, and cytological data provides evidence for their relatively ancient origin (Odierna *et al.*, 1995), although the mechanism behind their origin is unclear. In Continental Europe and North Africa the situation is less complicated, with most authors accepting the existence of a single, albeit morphologically variable subspecies, *B. viridis viridis*. The subspecies *B. v. balearicus* is confined to the Balearic islands, Corsica and Sardinia (Hemmer and Kadel, 1981).

As a first step towards understanding diversity within the species, fragments of two mitochondrial genes (12S and 16S rRNA) were sequenced for 28 individuals, concentrating on Europe and North Africa, and combined with a few previously published sequences. The aim was that this would help to determine levels of differentiation, possibly also elucidating colonization pathways. By including some island populations the data could also be used to assess if these were likely due to recent anthropogenic introductions.

Materials and Methods.

The number and geographic locations of the specimens used in this study are given in Table 1. We also included several previously published sequences: *B. viridis* from Austria (Graybeal, 1997), Armenia (Liu *et al.*, 2000) and Ukraine (Pauly *et al.*, 2004), *B. danatensis* from China (Liu *et al.*, 2000), and as outgroups *Bufo lemairii* (Cunningham and Cherry, 2000) and *Bufo bufo* (Pauly *et al.*, 2004). Total genomic DNA was extracted from small pieces of tadpoles or adult toes. Primers used in both amplification and sequencing

were 12Sa and 12Sb and 16SL and 16SH from Kocher *et al.* (1989). Amplification conditions were the same as described by Harris (2001). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Sequences were aligned using Clustal W (Thompson *et al.*, 1994) and adjusted by hand. The 12S rRNA and 16S rRNA were, respectively, 348 and 528 base pairs long. Only two insertions/deletions within the 16S rRNA were postulated in order to align all sequences.

Table 1. Sampling localities of *Bufo viridis* sequenced for this study.

Code	Sampling Locality
Mor1	Al Jadida, Morocco
Mor2	Arba, Morocco
Mor3	Azrou, Morocco
Mor4	Ras-El-Mar, Morocco
Mor5	Oulad Brahim, Morocco
Mor6	Laayoune, Western Sahara
Mor7	Taza, Morocco
Mor8	Debdou, Morocco
Mor9	Azrou, Morocco
Mor10	Ras-El-Mar, Morocco
Mor11	Ras-El-Mar, Morocco
Mor12	Taza, Morocco
Mor13	Debdou, Morocco
Tun1	Bou Salem, Tunisia
Tun2	Bou Chebka, Tunisia
Tun3	Jbel Bour, Tunisia
Tun4	Bou Chebka, Tunisia
Tun5	Dam Bou, Tunisia
Tun6	Ain Draham, Tunisia
Sic	Palermo, Sicily, Italy
Sar1	Sardinia, Italy
Sar2	Sardinia, Italy
Mal	Mallorca, Balearic Islands,
Tur1	Turkey
Tur2	Turkey
Tur3	Turkey
Gre1	Greece
Gre2	Greece
Hun	Hungary
SCDeveli0	??
Egy	Egypt

Combined sequences were imported into PAUP* 4.0b10 (Swofford, 2003) for phylogenetic analysis. For the phylogenetic analysis of the combined data, maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference were used. The

approach outlined by Huelsenback and Crandall (1997) was used to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada and Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree using ML (Felsenstein, 1981) with random sequence addition (10 replicate heuristic search with TBR – branch swapping). The MP analysis was also carried out with random sequence addition (100 replicate heuristic search, TBR branch swapping), and support for nodes was estimated using the bootstrap technique (Felsenstein, 1985) with 1000 replicates. The Bayesian analysis was implemented using MrBayes 3.0b4 (Huelsenback and Ronquist, 2001), which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. Bayesian analysis were conducted with random starting trees, run 0.5×10^6 generations, and sampled every 1000 generations using a general-time-reversible model of evolution with a gamma model of among site rate variation. In both searches stationarity of Markov chain was determined as the point when sampled log likelihood values plotted against generation time reached a stable mean equilibrium value; “burn-in” data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. The sequences were also joined in median networks (Bandelt *et al.*, 1999).

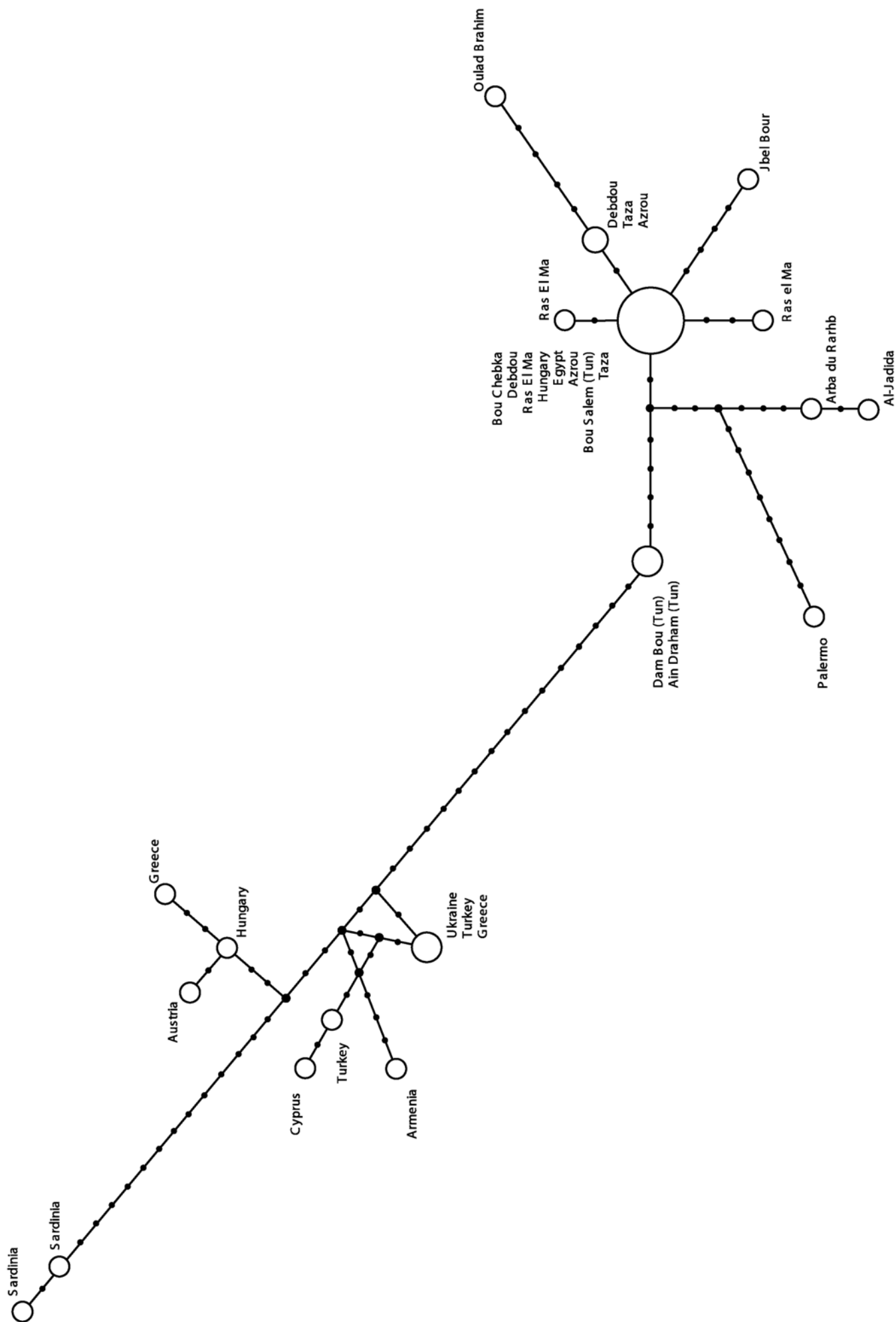
Results

In total, 38 combined sequences of which 31 were generated for this study, were included for a total of 876 base pairs; independent ML, MP and Bayesian analyses produced congruent estimates of relationships (Fig. 1). The most appropriate model for the combined data was the GTR model with an estimate of invariable sites and a discrete approximation of the gamma distribution. The ML heuristic search using this model found 22 trees of $-\ln 2323$. Bayesian analysis produced a similar estimate of relationships. For MP 55 characters were informative, and the MP search found 234 trees of 203 steps. The 50% bootstrap consensus tree derived from the MP analysis did not differ from the phylogram derived from the Bayesian analysis, but was less well resolved (Fig. 1). Major divisions from the phylogenetic analysis are also presented in the networks (Fig. 2).

Figure 2. Median Joining Networks of the two main clades estimated from the phylogenetic analysis. Solid circles imply inferred missing haplotypes. Size of circles is proportional to number of specimens that have this particular haplotype.

Figura2.- Xarxa d'haplotips mitocondrials dels tres clades principals recuperats a les anàlisis filogenètiques. Els cercles negres indiquen haplotips que no s'han mostrejat o que han desaparegut de la població. Els cercles blancs indiquen els diferents haplotips mitocondrials trobats i la seva mida és proporcional al nombre d'individus que tenen aquest haplotip determinat.

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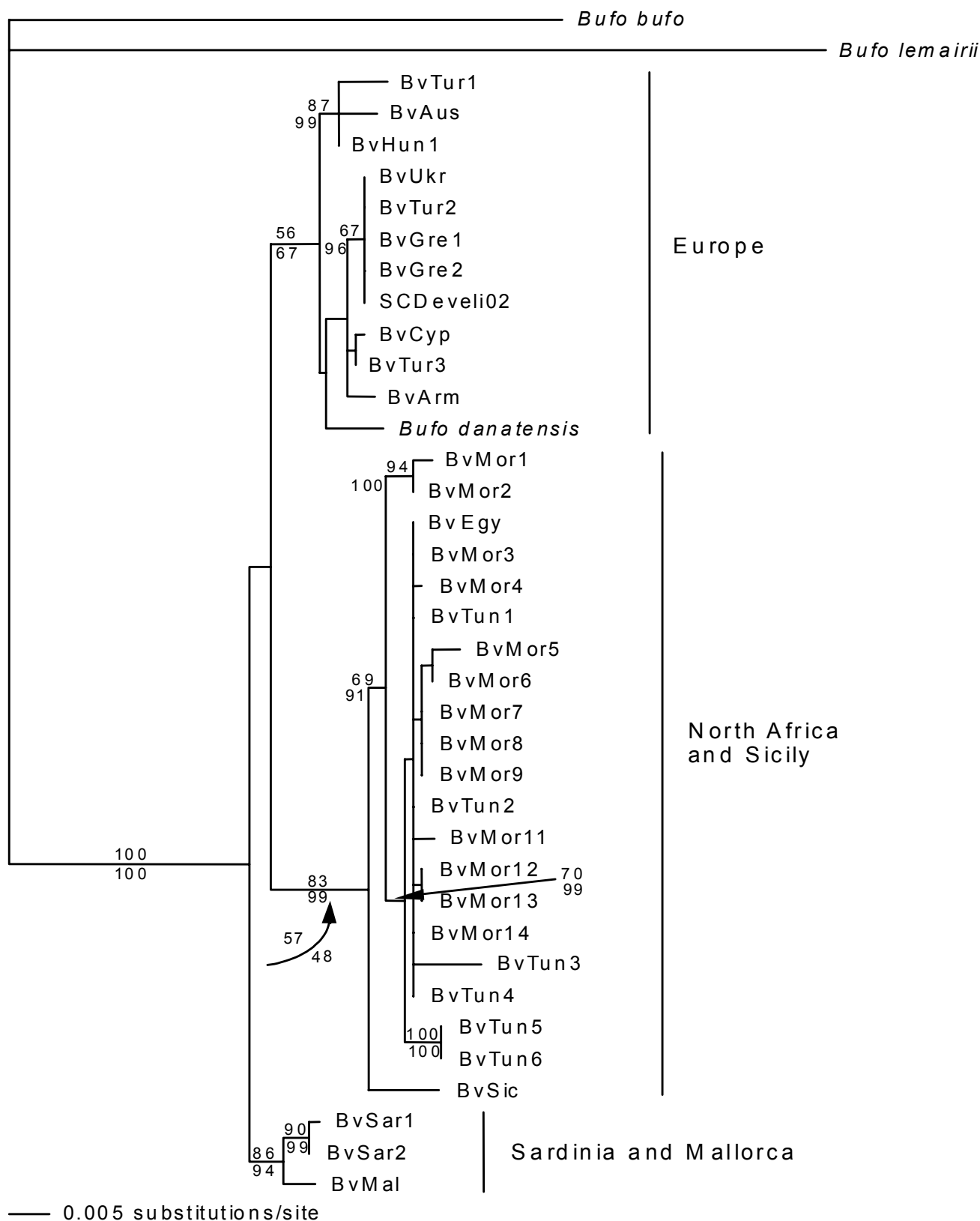


Figure 1. One of two ML trees estimated from the combined mtDNA sequences. Bootstrap values from the MP analysis are given above the nodes, Bayesian Posterior Probabilities below the nodes. The only difference between the methods was the position of the Sardinia and Mallorcan clade, as indicated.

Figura 1: Un dels dos arbres de ML estimat a partir de les dades de 12S y 16S combinades. Els valors de bootstrap resultants de l'anàlisi de MP estan a sobre dels nodes i els valors de probabilitat posterior resultants de l'anàlisi Bayesià a sota dels nodes. L'única diferència entre els mètodes es la posició del clade que inclou Mallorca i Sardenya (indicat en l'arbre).

Discussion

Although the phylogenetic relationship of *B. viridis* to other *Bufo* is poorly supported (Pauly *et al.* 2004) the diversity of cytogenetic forms in Asia suggest this was the origin of the species complex. Our analysis suggests that three divergent lineages exist, separated by 2,8-3,2% for these markers. This is higher than typical intraspecific variation in Asian Bufoinds, but less than interspecific divergences (5-8%, Liu *et al.*, 2000). There is strong geographical structuring between these lineages.

The first lineage includes all North African samples, from Egypt to Morocco, and also an individual from Palermo, Sicily. Although the basal split separates the Sicilian individual from the North African samples, otherwise there is no obvious evidence for substructuring within this lineage. Thus while some individuals from Egypt, Morocco and Tunisia share the same haplotype there are also several divergent haplotypes. Such a pattern is congruent with a history of periodic isolation of populations interlaced with periods of considerable gene flow. *Bufo viridis* is a relatively large amphibian that can travel considerable distances during their 4-5 year lifespans (Langton, 1989). However, the climate of North Africa has oscillated considerably across this evolutionary time frame, with the Sahara desert repeatedly expanding and contracting that would be expected to isolate and rejoin populations. Thus our results are not unexpected given the geological history of the region and the ecology of the organism. On the other hand, *Rana saharica*, also a prevalent amphibian in North Africa, showed very little genetic diversity across this region using these markers (Harris *et al.*, 2003). Possibly the greater ecological plasticity of *B. viridis* enabled a larger number of populations to survive drier periods.

Interestingly the sample from Palermo, Sicily, is part of the otherwise solely North African lineage. Its divergence from any known haplotype from elsewhere does not suggest an anthropogenic introduction, although greater sampling would be needed to rule out this possibility. However, the widespread distribution of *B. viridis* in Sicily, Malta and Southern Italy is congruent with an ancient transmarine colonization from North Africa. It also explains the absence of *B. viridis* in Central Italy, with Southern Italy being colonized from North Africa while Northern Italy was presumably colonized by the separate European lineage.

Variation within the second, European, lineage is high, and shows evidence of considerable gene flow, with individuals from Turkey, Greece and the Ukraine sharing haplotypes. The occurrence of divergent haplotypes in Central Europe may indicate the existence of northern glacial refugia, as found in *B. calamita* (Rowe *et al.*, 2006), or may be due to extensive post-glacial gene flow involving divergent haplotypes from southern refugia. More sampling in southern Europe will be needed to assess this. Of particular interest, a sample from the tetraploid form, *B. danatensis* (Liu *et al.*, 2000) is clearly embedded within this lineage. An assessment of relationships between the cytogenetically distinct forms and the diploid *B. viridis* is outside the scope of this study, but clearly warrants further investigation.

A third lineage includes samples from Sardinia and Mallorca. Assessment of if Mallorca was colonised naturally, or if specimens were introduced anthropogenically from Sardinia would require further sampling. The different haplotypes determined in this study gives no evidence of an anthropogenic introduction, but to determine this greater sampling would be needed. Similar cases where distinct genetic lineages within islands have later been shown to be multiple anthropogenic introductions from diverse source populations have already been reported in *Hemidactylus* geckos (Jesus *et al.*, 2001; Carranza *et al.*, 2006). This distinct and well supported clade confirms the existence of the subspecies *B. v. balearicus* from a genetic point of view.

The similar levels of variation within the European and North African lineages is congruent with the hypothesis that these areas were colonized at approximately the same time from Asia. Based on rough molecular clocks estimated for these gene regions (0,69 % change per lineage per million years) and applied in other Bufonids (Macey *et al.*, 1998; Kutrup *et al.*, 2006), the divergence between the two lineages may have started approximately 2,1 million years ago. If this were the case, the lineage in Sardinia would also have diverged around this time, and thus would represent an trans-marine colonization since Sardinia has been isolated from the Continental land mass much longer than this. Trans-marine journeys in amphibians are rare, but do occur (e.g. Vences *et al.*, 2003). There is no evidence that the predominantly European and African lineages now co-exist, but the report of extremely high allozymic variation in populations from the Middle East (Dessauer *et al.*, 1975) would fit with this area being a contact zone that deserves further sampling.

Conclusions

Three distinct mtDNA lineages occur in *B. viridis*; one in North Africa and Sicily, another in the rest of Europe, and one in Sardinia and Mallorca. Divergence between these lineages is considerable, but not at a level typically observed between species. Populations on Mediterranean islands including Sardinia, Sicily and Mallorca show no evidence of being anthropogenically introduced. A Tetraploid individual of *B. danatensis* is part of the European lineage based on analysis of mtDNA sequences. Further sampling, especially in the Middle East and Asia is needed to understand relationships between cytogenetically different forms, and to further determine the current ranges of these distinct lineages.

Acknowledgements.

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NORMES DE PUBLICACIÓ

El Butlletí de la Societat Catalana d'Herpetologia publica estudis d'investigació científica, tècnics o observacionals de qualsevol tema relacionat amb els amfibis i els rèptils, en forma de treballs de recerca, revisions/síntesi, notes i postil·les.

Només s'accepten treballs inèdits i que no es trobin en fase de publicació en cap altra revista. La Societat Catalana d'Herpetologia com editora del Butlletí tindrà tots els drets de publicació. Tots els treballs seran revisats abans d'ésser acceptats, podent proposar-se modificacions o rectificacions. La qualitat, la concisió i l'originalitat s'avaluaran com criteris d'acceptació. La Societat Catalana d'Herpetologia, com editora, no comparteix necessàriament les afirmacions que facin els autors dels articles, així com la correcció del llenguatge que serà responsabilitat dels autors. Es podrà exigir un formulari annex de declaració d'autoria, originalitat i cessió de drets d'autor.

S'enviarà a la Seu de la Societat* dues còpies del manuscrit, tipografiat a espai simple en fulls DIN A-4 deixant marges de 1,5 cm, a més d'un altre en suport informàtic sobre disquet de 3,5", o millor per correu electrònic, amb el text complert, especificant el nom de l'arxiu i programa utilitzat (Preferiblement Write-Windows, Word Perfect 5.0 o Word 6.0) o com fitxer adjunt per correu electrònic. Sempre que la revisió es pugui dur a terme, i siguin d'interès pel soci, els articles es poden escriure en qualsevol idioma, preferentment en català.

Aniran acompanyats d'uns resums al menys en català i en anglès amb les seves corresponents paraules claus en un nombre no superior a 6, en conjunt es recomana no superar les 200 paraules. La redacció es farà càrrec del resum en català en aquells casos d'autors no catalanoparlants. Qualsevol altre resum no hauria d'ultrapassar, amb els dos altres, el 12 % de l'extensió total del treball. El text ha d'estar el menys formatat possible, sense canvis de tipus de lletres, amb arrelament a l'esquerra, amb un sol espai rera dels punts o les comes i sense tabulacions.

Les taules i figures (gràfics i fotografies) hauran d'ésser de bona qualitat, contrast i en un principi es publicaran en blanc i negre, atenent-se a la dimensió màxima en què es presentaran que no podrà ser superior als 12 cm per 18 cm. S'adjuntaran en fulls apart, identificant-les correctament als fulls i dins del treball. Els peus de figura o taula, també en fulls apart, hauran d'ésser comprensibles amb tots els símbols explicats i amb la informació necessària per interpretar correctament sense tenir que recorre al text.

Tots els articles començaran amb el títol, sota els autors, després el lloc de treball o centre si s'escau, al menys una adreça de contacte, seguit s'inseriran els resums i les paraules claus. Totes les pàgines aniran numerades des de la primera. El text de l'article s'estructurarà, segons el seu tipus, com segueix:

De recerca: (Articles d'investigació que es recomana no ultrapassar els 15-20 fulls) que hauran de constar de: Introducció (Serà breu, enfocarà el tema i es fixarà l'objectiu del treball); Material i mètode (Es descriurà amb detall per tal que es pugui avaluar o repetir per altres investigadors. Si cal es farà menció de les normes ètiques i legals seguides); Resultats (Seran concisos, clars, incloent el nombre mínim de taules i figures); Discussió (Es comentarà les troballes pròpies respecte els altres treballs previs, així com les diferències amb altres autors. Les hipòtesis i les especulacions quedaran clarament diferenciades); Agraïments (Si s'escau) i Referències.

Revisió / Síntesi: (Anàlisi crítica dels coneixements sobre un tema d'interès actualitzat, poden ser reforçats per dades pròpies. Seguirà una estructura general similar a l'anterior no ultrapassant les 20 planes).

Notes (Treballs eminentment descriptius d'un o més casos o tècniques que suposin una aportació important en el coneixement del tema. S'estructuraran preferentment amb una Introducció, Observació-resultat, Discussió i Referències. Extensió màxima recomanada 10 fulls).

Postil·les: (Discussió de treballs publicats recentment al Butlletí o aportacions d'observacions que per les seves característiques es puguin resumir en un text breu. Es recomana no ultrapassar 3 planes i 10 referències bibliogràfiques).

En tots els casos les referències seran només aquelles esmentades en el text, en forma d'autor en majúscules i l'any. S'ordenaran alfabèticament per autors (en majúscules) i cronològicament (cognoms, inicials dels noms, de tots els autors (any de la publicació): títol original de la referència. Nom complet de la publicació, o abreviació reconeguda, en cursiva, amb editorial i lloc d'edició i editor si s'escau: Número, i pàgines de l'article o nombre total si es tracte d'un llibre. Tot seguit es mostren exemples a seguir, d'un article d'una revista, d'un llibre i d'un capítol d'un llibre:

MALUQUER, J. (1985): Estudi preliminar sobre l'herpetofauna de la Penyalgosa. *Butll. Soc. Cat. d'Ictiol. i Herp.* 11/13: 5-11.

MAYOL, J. (1985): *Rèptils i Amfibis de les Balears*. Manuals d'introducció a la naturalesa, 6. Ed: Moll, Palma de Mallorca. 233 p.

MARTÍNEZ DE CASTILLA, A.; JIMÉNEZ, J. & LACOMBA, I. (1987): Los reptiles de Columbretes, 181-194, *In* Alonso, L. A.; Carretero, J. L. & García, A. M.: *Islas Columbretes. Contribución al estudio de su medio natural*. Monografies 5. Generalitat Valenciana. Conselleria d'Obres Públiques, Urbanisme i Transports. València.

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SUMARI

Treballs de recerca

- Restes fòssils de *Testudo hermanni* al Massís del Garraf.
Joan Daura, Montse Sanz, Oriol Font Cot & Joan Budó 9
- Estudi sobre la depredació i supervivència d'exemplars juvenils de tortuga mediterrània (*Testudo hermanni hermanni*) a la Serra de l'Albera (Pirineu Oriental, Catalunya).
Joan Budó & Xavier Capallera..... 20
- Genetic variation within *Bufo viridis*: evidence from mitochondrial 12S and 16S rRNA DNA sequences.
Vasco Batista, Salvador Carranza, Miguel A. Carretero and D. James Harris..... 24
- Dades sobre el moviment i la dispersió de juvenils de *Salamandra salamandra* (L.), en un bosc de Badalona (Catalunya).
Jesús G. Simón; Xavier Rivera1; Josep A. Melero; Nestor Úrios & Alberto Vilagrosa 34

Treballs de revisió / síntesi

- 104 noves observacions de *Testudo marginata* (Schoepff, 1793) a Grècia i recull bibliogràfic sobre l'espècie a la península hel·lènica.
Jordi Arias Ferreres 41

Notes herpetològiques

- Un cas de supervivència extrema en larves d'*Alytes obstetricans* (Laurenti, 1768) (*Anura, Discoglossidae*).
Salvador Domènech Beain & Asun García-Gascó 51
- Albinismo e hipomelanismo en culebra viperina.
F. Ceacero Herrador, L. Pedrajas Pulido 53
- Nuevo registro altitudinal para *Pelodytes punctatus* en Cataluña.
Arregui, L. & García-París, M...... 56
- Gigantismo larvario en *Salamandra algira* Bedriaga 1883, descripción de un caso
D. Escoriza, M. M. Comas & David Donaire 59

Treballs nomenclaturals

- Noms en català de les tortugues del món recomanats per la Societat Catalana d'Herpetologia.
David Perpiñán..... 67
- Llistat dels amfibis i dels rèptils del paleàrtic occidental, amb una proposta de nom en català.
Societat Catalana d'Herpetologia, Coordinador: Xavier Rivera 78