PHYLOGENETIC RELATIONSHIPS OF *IBERUS GUALTIERANUS* AND *I. ALONENSIS* (GASTROPODA: HELICIDAE) BASED ON PARTIAL MITOCHONDRIAL 16S rRNA AND COI GENE SEQUENCES

M.A. ELEJALDE[1], B. MUÑOZ[2], J.R. ARRÉBOLA[3] AND B.J. GÓMEZ-MOLINER[1]

[1]*Departamento Zoología y B.C.A., Facultad Farmacia, Universidad País Vasco, c/Paseo de la Universidad, 7 Vitoria (Álava) 01006, Spain;*
[2]*Departamento Biología Animal 1, Facultad Biología, Universidad Complutense Madrid, Spain;*
[3]*Departamento Fisiología y Biología Animal, Facultad Biología, Universidad Sevilla, Spain*

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ABSTRACT

There is controversy about the phylogenetic relationships between *Iberus gualtieranus* and *I. alonensis*. Some authors consider them as valid species or subspecies while others believe that the flattened shell of *I. gualtieranus* is an ecotypic adaptation to dry karstic environments. Two fragments of the mitochondrial DNA (partial COI and 16S rRNA) were sequenced and used in maximum parsimony, maximum likelihood and neighbour-joining analyses. *Iberus alonensis* show two distinct lineages, one from Almería and the other one from Granada and Jaén-Córdoba regions. *Iberus gualtieranus* populations are recovered as a terminal node within the *I. alonensis* group from Almería. The *I. gualtieranus* clade shows a polytomy and there are no differences between the populations of the three isolated localities where *I. gualtieranus* is currently distributed. This indicates that the geographical isolation of these populations has not resulted in genetic diversification. The results indicate that the population of *I. gualtieranus* from Sierra de Gádor in Almería is the only autochthonous one, while the other two populations originated by historical introductions. On the basis of the differences in shell morphology, together with the presence of a hybrid zone connecting both taxa in nature, and the possibility of obtaining fertile hybrids under laboratory conditions, we conclude that these two taxa represent two subspecies: *Iberus gualtieranus gualtieranus* and *I. gualtieranus alonensis*.

INTRODUCTION

The genus *Iberus* is an endemic of the Iberian Peninsula, comprising 20 morphospecies (Puente, 1994; Arrébola, 1995). *Iberus gualtieranus* (Linnaeus, 1758) and *Iberus alonensis* (Férussac, 1821) are the two morphospecies of greatest economic interest, being much appreciated in gastronomy (Arrébola & Álvarez, 2001; Arrébola, 2002). Moreover, they also have a significant conservation interest with the former considered to be at risk of extinction (Gómez-Moliner *et al*., 2001). *Iberus alonensis*, which has the greatest distribution range of the genus, is found along the eastern half of the Iberian Peninsula, from Andalusia in the south to Catalonia in the north, reaching as far as La Rioja through the Ebro Valley (Puente, 1994). *Iberus gualtieranus* is currently distributed in three small populations in the south of the Iberian Peninsula (Fig. 1): Sierra Elvira (Granada, UTM: 30SVG32), Sierra de Jaén (Jaén, UTM: 30SVG27) and Sierra de Gádor (Almería, UTM: 30SWF07). All three populations are isolated and no natural migration is possible between them. Both taxa show quite different morphologies. *Iberus alonensis* has a rounded, globose shell, covered by a delicate sculpture of radial and spiral lines (Férussac, 1821; Hidalgo, 1875; García San Nicolás, 1957). *Iberus gualtieranus* has a markedly keeled, flattened shell, with strong ornamentation of radial and spiral costulae (Linnaeus, 1758; López-Alcántara *et al*., 1985).

There is controversy about the taxonomic validity of these two taxa. Several authors considered them to be valid species (García San Nicolás, 1957; Aparicio, 1983a,b; Aparicio & Ramos, 1988; Ortiz de Zárate, 1991), while others regarded them as subspecies of a polytypic species on the basis of the absence of anatomical differences in the reproductive system, as well as the presence of intermediate forms in contact zones (Kobelt, 1904; Boettger, 1913; Cobos, 1979). Moreover, some authors considered them to be merely ecological forms of the same species (López-Alcántara *et al*., 1983; Alonso *et al*., 1985). After detailed morphological studies, López-Alcántara *et al*. (1985) concluded that *I. gualtieranus* and *I. alonensis* should be considered ecotypes, and that the *gualtierianus* form evolved from the *alonensis* form in three independent events as a result of adaptation to its special environment: a calcareous dolomitic substrate in the initial phases of karstic erosion with numerous narrow fisures and a vegetation composed mainly of saxicole communities, in areas with a warm temperate climate of the thermomediterranean-xeromediterranean type (Alonso *et al*., 1985).

The aim of this work is to clarify the phylogenetic relationships of *I. gualtieranus* and *I. alonensis*, as well as to describe the relationships of the three populations of *I. gualtieranus* to each other. The resolution of these relationships is of interest in order to develop action plans within the *Program for Conservation and Sustainable Snail Exploitation in Andalusia* (the most extensive and 'helicicola' traditional region in Spain) (Arrébola, 2002). Molecular methods, and specifically those using DNA sequence, are useful for species distinction and population characterization (Avise, 1994,2000) particularly when morphological evidence is not conclusive. During the last decade, many studies have investigated molecular variation within and between snail species because this provides an independent source of information about the history of species and populations (Wade, Mordan & Clarke, 2001; Davison, 2002). For this study we have selected two portions of the mtDNA, a fragment of the protein-coding gene COI (subunit I of cytochrome oxidase) and the 16S ribosomal subunit (16S rRNA). They are well known molecular

Correspondence: M.A. Elejalde; e-mail: zobelcaa@vc.ehu.es

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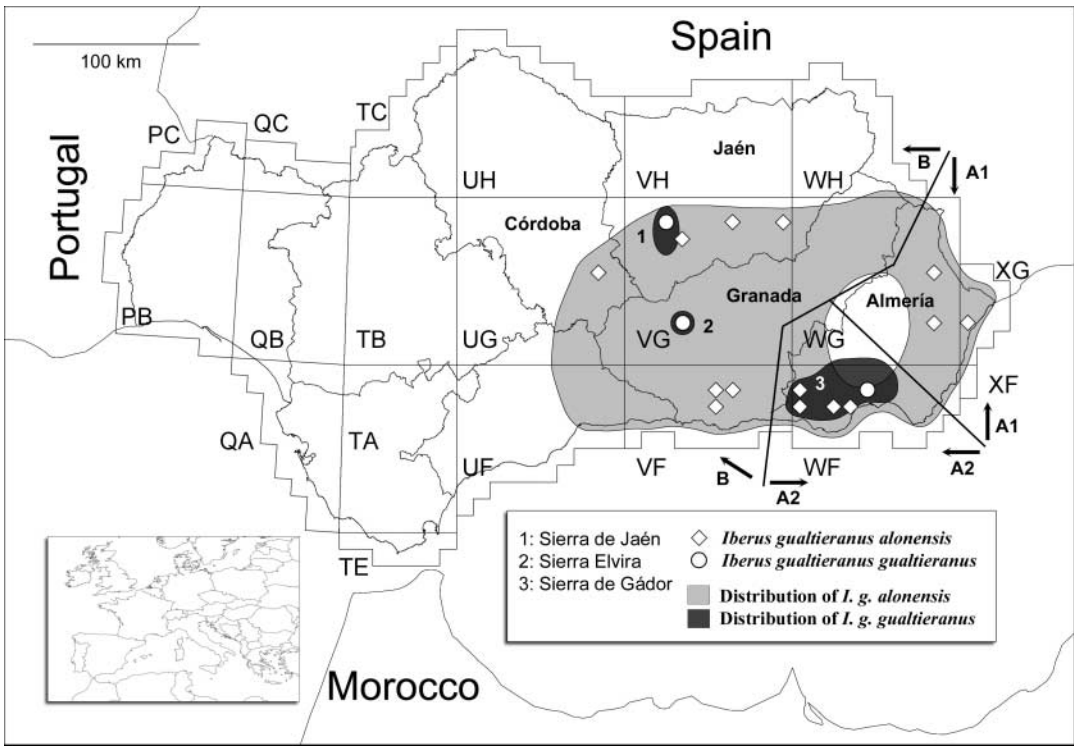

Figure 1. Geographical distribution of the samples. White diamonds: *Iberus gualtieranus alonensis*; white circles, *I. gualtieranus gualtieranus*. Accurate localities are given in Table 1. The two-letter codes indicate the UTM squares identification (100 × 100 km) in Andalusia.

markers for molluscs (Lydeard & Lindberg, 2003), and show the highest evolutionary rates of the mitochondrial genome (Hillis *et al*., 1996). Both fragments have been used in many systematic studies of Gastropoda (e.g. Davis *et al*., 1998; Douris *et al*., 1998; Schander *et al*., 2002; Parmakelis *et al*., 2003; Grande *et al*., 2004). They are useful markers for the analysis of the phylogenetic relationships at the species level and have shown their utility in the resolution of the phylogenetic relationships of closely related species and populations of several Helicoidea taxa (Davison, 2000; Guiller *et al*., 2001; Pfenninger & Posada, 2002; Pfenninger, Posada & Magnin, 2003; Gittenberger, Piel & Groenenberg, 2004; Steinke, Albrecht & Pfenninger, 2004).

MATERIAL AND METHODS

Samples

Samples were collected from different localities in Andalusia (Fig. 1). They included 12 specimens of *I. gualtieranus* from all three localities where it is known to occur. They were collected from the centre of each population in order to avoid the genetic introgression by hybridization with *I. alonensis* in the contact zones. In addition, we analysed 16 specimens of *I. alonensis* from East Andalusia collected from localities in between those of *I. gualtieranus*, as well as in the surrounding areas. *Otala lactea* and *Pseudotachea splendida* were used as outgroups. Whenever possible, museum-preserved samples were used (preserved in 70% ethanol). Additional specimens were sampled live, killed by freezing to avoid DNA degradation by drowning (Schander & Hagnell, 2003) and preserved in absolute ethanol. The samples studied are listed in Table 1. Voucher specimens have been deposited in the following collections: Universidad de Sevilla, Biología (USB); Universidad Complutense de Madrid, (UCM); Universidad País Vasco (UPV).

DNA isolation

Total DNA was extracted from the foot of each snail using the CTAB method (Sokolov, 2000). The muscular tissue was cut into small pieces and mixed in CTAB buffer preheated to 60°C. After incubation at 47°C for 2 days, total cellular DNA was isolated using phenol:chloroform:isoamylalcohol extraction (25:24:1). DNA was then precipitated with sodium acetate and absolute ethanol (1:50) overnight. The pellet was washed twice with 70% and 100% ethanol and air-dried. Finally, it was diluted in autoclaved double-distilled water.

DNA amplification and sequencing

A fragment of approximately 442 bp was amplified from the 16S rRNA gene by polymerase chain reaction (PCR) with the universal primers described by Palumbi *et al*. (1991): 16Sar-L (5′-CGCCTGTTTATCAAAAACAT-3′), 16Sbr-H (5′-CCGG TCTGAACTCAGATCACGT-3′). Also, approximately 712 bp of the COI gene was amplified by PCR with the universal primers developed by Folmer *et al*. (1994): LCO 1490 (5′-GG TCAACAAATCATAAAGATATTGG-3′), HCO 2198 (5′-TA AACTTCAGGGTGACCAAAAATCA-3′).

The PCR was performed with 1 μl of each DNA sample in a 25 μl volume (2.5 mM dNTP, 50 mM $MgCl_2$, 10× NH_4 buffer, 20 nM/μl of each primer and 5 units of Taq Polymerase). We used a BioRad iCycler thermal cycler with the following cycling conditions: an initial denaturation step of 2 min at 93°C, followed by 40 cycles of 45 s at 93°C, 1 min at 54°C for COI or 57.7°C for 16 s, and 1 min at 72°C. The cycling ended with a 7 min extension step at 72°C. Reactions were held at 4°C.

Reaction products were run in 1.5% agarose gels, stained with ethidium bromide, to verify the amplifications. Amplicons were sequenced using the dRhodamine Terminator Cycle Sequencing

Table 1. Species, localities, geographical coordinates (using Spanish grid references, UTM), abbreviation and accession numbers of *Iberus* populations used.

Abbreviation	UTM	GenBank accession number	
		COI	16S rRNA
IaAL-01	30SWG82	AY928552	AY928580
IaAL-02	30SXG02	AY928553	AY928581
IaAL-03	30SWG85	AY928554	AY928582
IaAL-04	30SWF07	AY928555	AY928583
IaAL-05	30SWF08	AY928556	AY928584
IaAL-06	30SWF37	AY928557	AY928585
IaAL-07	30SWF27	AY928558	AY928586
IaCO-01	30SUG85	AY928559	AY928587
IaJ-01	30SVG37	AY928564	AY928592
IaJ-02	30SVG37	AY928565	AY928593
IaJ-03	30SVG68	AY928566	AY928594
IaJ-04	30SVG98	AY928567	AY928595
IaGR-01	30SVF57	AY928560	AY928588
IaGR-02	30SVF68	AY928561	AY928589
IaGR-03	30SVF58	AY928562	AY928590
IaGR-04	30SVF68	AY928563	AY928591
IgAL-01	30SWF48	AY928568	AY928596
IgAL-02	30SWF48	AY928569	AY928597
IgAL-03	30SWF48	AY928570	AY928598
IgGR-01	30SVG32	AY928571	AY928599
IgGR-02	30SVG32	AY928572	AY928600
IgGR-03	30SVG32	AY928573	AY928601
IgGR-04	30SVG32	AY928574	AY928602
IgGR-05	30SVG32	AY928575	AY928603
IgGR-06	30SVG32	AY928576	AY928604
IgJ-01	30SVG28	AY928577	AY928605
IgJ-02	30SVG28	AY928578	AY928606
IgJ-03	30SVG28	AY928579	AY928607
Ps-spl	31TCF24	AY937265	AY937266
Ot-lact	30SVF17	AY937263	AY937264

Abbreviations: Ia, *Iberus alonensis*; Ig, *Iberus gualtieranus*; Ps-spl, *Pseudotachea splendida*; Ot-lact, *Otala lactea*; AL, province of Almería; CO, province of Córdoba; J, province of Jaén; GR, province of Granada.

Ready Reaction Kit (Applied Biosystems), in an ABI PRISM Model 3100 Avant Genetic Analyzer.

Data analysis

Sequences were aligned using CLUSTALX version 1.81 (Thompson *et al*., 1997) and then manually adjusted to minimize mismatches. Gaps were treated as missing data and removed from the alignments before phylogenetic analyses. Third codon position data for COI were included in the analyses, as both taxa are very closely related and saturation problems are not expected. The sequence data were partitioned into two data sets: the 16S rRNA and the COI. Subsequently these two data sets were concatenated into a single data set of 1049 nucleotides. The data matrices were subjected to a neighbour-joining (NJ) (Saitou & Nei, 1987), maximum likelihood (ML) (Felsenstein, 1981) and maximum parsimony (MP) (Fitch, 1971) methods of phylogenetic inference. Phylogenetic analyses were conducted in PAUP* 4.0b10 (PPC) (Swofford, 2002).

MP was performed using heuristic search (TBR branch swapping: MulTrees options in effect) with 10 random stepwise additions of taxa. A 3:1 transversion (Tv): transition (Ts) weighting scheme was used for the COI fragment. Sites were unweighted in the analysis of 16S rRNA. We used the Akaike Information Criterion (AIC) implemented in MODELTEST 3.06 PPC (Posada & Crandall, 1998) to determine the appropriate model of evolution. ML and NJ analyses were performed in PAUP* using the HKY+G model (Hasegawa, Kishino & Yano, 1985). The robustness of NJ and MP analyses was tested by bootstrapping with 1000 pseudoreplicates. Robustness of ML analysis was tested using 100 pseudoreplicates.

The sequences reported in this article have been deposited in the GenBank database. Accession numbers are in Table 1.

RESULTS

Phylogenetic analyses were based on three different sequence data sets: COI, 16S rRNA and COI and 16S rRNA sequences concatenated.

A fragment of 656 bp (after removing primers) was sequenced from COI. The average nucleotide composition of the coding strand was 26.5% A, 41% T, 14.5% C and 18% G. Of these 443 (67.5%) were invariant sites, 36 (5.5%) were singletons within the dataset, and 177 (27%) were parsimony informative.

A fragment of 393 nucleotides was sequenced from the 16S ribosomal gene. The average nucleotide composition was 30% A, 38.5% T, 14% C and 17.5% G. Of these 272 (69.2%) were invariant sites and 54 (13.7%) were singletons within the dataset, leaving 67 (17%) parsimony informative characters.

The combined length of the two aligned fragments was 1049 sites. Of these 719 (68.5%) were invariant sites and 88 (8.5%) were singletons within the dataset, leaving 242 (23%) parsimony informative characters. Data sets were A-T rich (67.5% COI and 68.5% 16S rRNA) as has been observed in other taxa (Frati *et al*., 2000).

The three methods of phylogenetic inferences (NJ, MP and ML) recovered nearly identical trees for the three sequence data sets. The strict consensus tree with bootstrap values resulting from the NJ analysis of the concatenated data set of the two mtDNA fragments is presented in Figure 2 (MP tree length TL = 910, Consistency Index CI = 0.6890, Retention Index RI = 0.8402).

The phylogenetic analyses showed two major lineages (Fig. 2, A and B). Lineage A was supported by low to moderate bootstrap values (83%, 59% and 61% for NJ, MP and ML, respectively). It included all samples of *I. alonensis* from Almería together with all the *I. gualtieranus* specimens from Andalusia. Lineage B included the *I. alonensis* from Granada, Jaén and Córdoba (100%, 95% and 96% bootstrap values for NJ, MP and ML, respectively). Pairwise sequence divergence between these two major clades ranged from 11.2% to 13.9%. The two main clades within lineage A were supported by high bootstrap values (100%, 100%, 99% for clade A1, and 99%, 97%, 88% for clade A2, for NJ, MP and ML, respectively). The first clade (A1) included the *I. alonensis* samples from NE Almería. The other (A2) included the *I. alonensis* populations from the south of Almería as the sister group of *I. gualtieranus* populations. Pairwise distances of all the *I. alonensis* from Almería ranged from 8.9% to 10.4%. *Iberus gualtieranus* sequences constituted a well supported clade (bootstrap values of 100%, 100% and 94% in NJ, MP and ML, respectively), with all the populations grouped in a polytomy. Pairwise distances between *I. gualtieranus* and *I. alonensis* from the south of Almería ranged from 5.2% to 6.7%. Pairwise distances between all *I. gualtieranus* sequences ranged from 0.0% to 1.1%. They ranged from 0.2% to 1.0% within the Sierra de Gádor population, from 0.0% to 0.8% in Sierra Elvira population and from 0.0% to 0.2% in Sierra de Jaén. *Iberus gualtieranus* did not show monophyletic groups for any of these three populations.

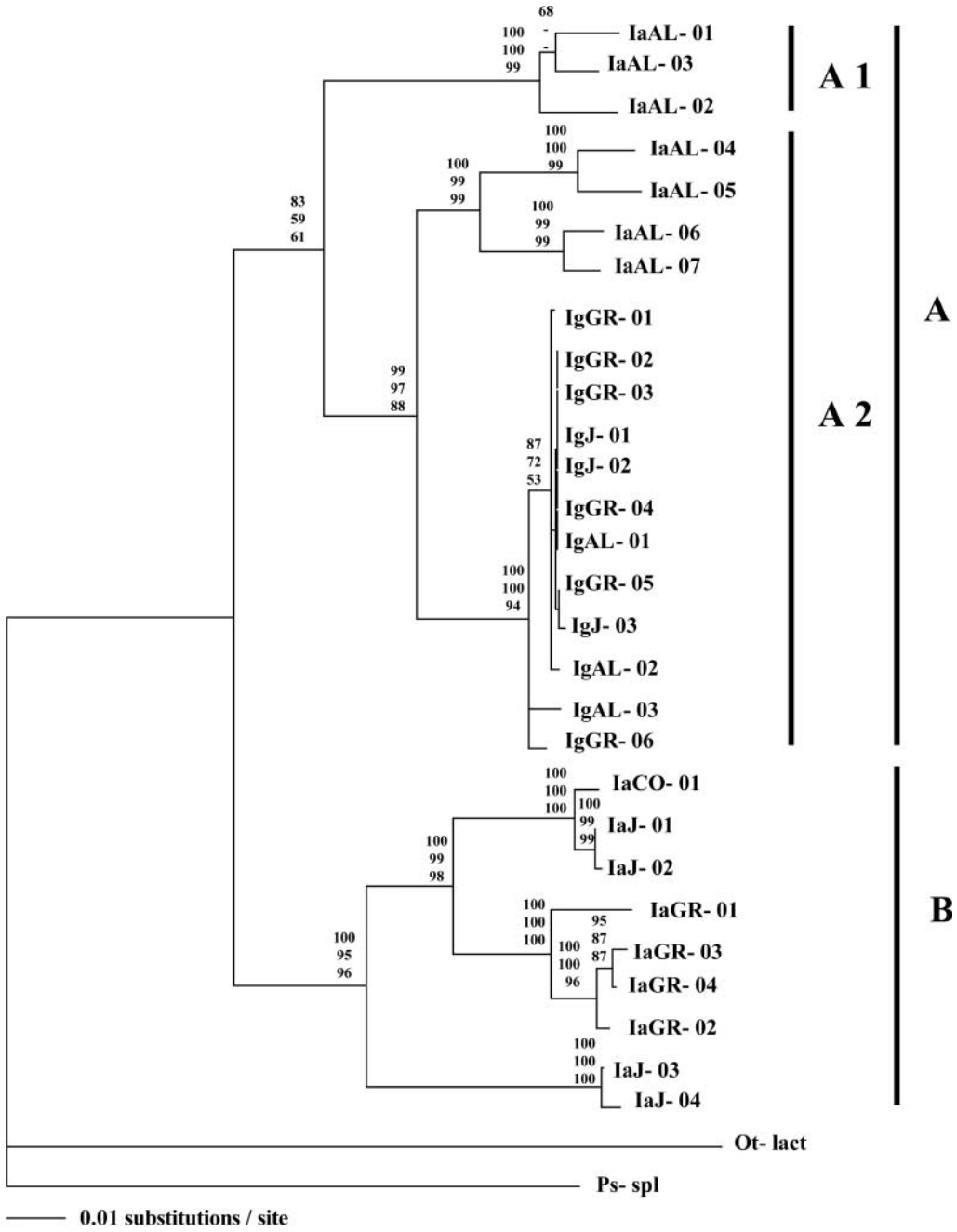

Figure 2. Neighbour-joining tree constructed with pairwise distances calculated following the Hasegawa-Kishino-Yang (HKY85) model. Numbers on branches are NJ, MP and ML bootstrap support (>50%), respectively.

DISCUSSION

The present study provides a robust phylogenetic hypothesis for examining the relationship between *Iberus alonensis* and *I. gualtieranus*. The phylogenies obtained with COI and 16S rRNA are in agreement when they are considered as independent sequences and when they are treated together. Both fragments are useful for inferring phylogenies at the intraspecific level in *Iberus*, as has been found in other terrestrial molluscs (Pfenninger, Eppenstein & Magnin, 2003; Gittenberger *et al*., 2004).

All phylogenetic analyses performed recovered two distinct lineages within *I. alonensis*. One of them joins the *I. alonensis* specimens from Almería (east and south), together with the samples of *I. gualtieranus*. The other clade brings together the specimens collected from Granada and Jaén-Córdoba regions. *Iberus alonensis* is a paraphyletic group because of the inclusion of *I. gualtieranus* within the Almería clade. Genetic distances between *I. alonensis* and *I. gualtieranus* populations from the same province are greater than those shown between all three populations of *I. gualtieranus*. These results are not consistent with the hypothesis suggesting that the 'form' *gualtieranus* has arisen repeatedly as a result of an iterative process of adaptation to similar environmental conditions from the geographically closest populations of *I. alonensis* (López-Alcántara *et al*., 1983, 1985; Alonso *et al*., 1985).

All phylogenetic analyses reveal that *I. gualtieranus* is a monophyletic group. The presence of a keeled, flattened shell with a prominent ornamentation should be considered as a

synapomorphy supporting this clade. This is just the opposite condition to that described for *Arianta arbustorum* (Gittenberger *et al*., 2004) where depressed shells are plesiomorphic and globular shells are derived. Teshima *et al*. (2003) found that depressed shells in *Ainohelix editha* (Bradibaenidae) evolved independently in the two populations analysed, showing also a different condition to that found in *I. gualtieranus*.

Iberus gualtieranus populations are recovered as a terminal node within the *I. alonensis* group from Almería showing that all the current populations of *I. gualtieranus* from Jaén, Granada and Almería were derived from the Almería population of *I. alonensis*. In consequence, the populations of *I. alonensis* living in Jaén and Granada are different lineages from those of *I. gualtieranus* living in the closest areas. The genetic distance between *I. gualtieranus* and the closest *I. alonensis* populations ranges from 4.9% to 6.2%. Laboratory cross experiments carried out over several generations and maintained upon the same soil conditions, indicate that shell morphology is genetically inherited (Muñoz, unpublished results). Genetic data, together with laboratory experiments, suggest that *I. gualtieranus* is not an ecotype of *I. alonensis*, but instead constitutes a phylogenetic lineage. Several intermediate forms are known to occur in nature, in the contact zones between these two vicariant taxa (Kobelt, 1904; García San Nicolás, 1957; López-Alcántara *et al*., 1985). These intermediate forms have been given different names, *I. intermedius* Boettger, 1913 and *I. laurenti* (Bourguignat, 1870), but they are no longer considered to be taxonomically valid (Serradell, 1912; García San Nicolás, 1957; Cobos, 1979).

The topology of the *I. gualtieranus* clade shows a general polytomy, indicating that the phylogeny within this group is unresolved. The geographically different *I. gualtieranus* populations are not grouped in different clades. This indicates that geographical isolation has not resulted in genetic diversification of the three populations. Moreover, identical haplotypes are shared between specimens from the three localities: one specimen from Almería, two from Jaén, and another three from Granada have the same mtDNA haplotype for the two sequenced fragments. Currently, there is no evidence of gene flow among these three populations (Alonso *et al*., 1985) and *I. gualtieranus* subfossil shells are not known in the intermediate zones. The only explanation for the presence of the same mtDNA haplotype in all three *I. gualtieranus* populations, and the very low genetic distances among them, is that two of these populations have originated by introductions carried out by translocation from the only autochthonous population of *I. gualtieranus*.

The phylogenetic relationships indicate that *I. gualtieranus* originated from the Almería population of *I. alonensis*. On the basis of biogeographical considerations, the population of Sierra de Gádor should be considered the only native population of *I. gualtieranus*. It is placed inside the geographical range of *I. alonensis* from Almería, and has the largest geographical range of all three populations of *I. gualtieranus* (Cobos, 1979; Alonso *et al*., 1985). This is also the only locality where subfossil forms have been found (Alonso *et al*., 1985), which further supports the more ancient origin of the population of Sierra de Gádor.

Morphological studies are consistent with this consideration. López-Alcántara *et al*. (1985) and Alonso *et al*. (1985) consider that the shell of the specimens of *I. gualtieranus* from Sierra de Gádor is the best adapted to the dry, karstic environment. The high variability of shell morphology in the *I. gualtieranus* population of Sierra Elvira is considered by López-Alcántara *et al*. (1985) and Alonso *et al*. (1985) to be the result of disruptive selection at the beginning of a process of expansion (Alonso *et al*., 1985). The *I. gualtieranus* population from Sierra de Jaén is the most homogeneous in shell morphology, which is supposed to be a stage of an incipient typogenesis (Alonso *et al*., 1985). These authors regarded these differences in shell adaptations as evidence of three independent processes of ecotype adaptation. However, according to Teshima *et al*. (2003) the evolution of keeled-flat shells does not simply occur as an adaptation to limestone substrates and all theories about the evolution of this shell form are basically speculative. Nevertheless, the ideas of López-Alcántara *et al*. (1985) and Alonso *et al*. (1985) are consistent with the hypothesis that the last two populations are the result of recent introductions of *I. gualtieranus*. Furthermore, both populations are close to human settlements, which may have favoured the deliberate anthropogenic introduction of snails with a high gastronomic value.

As a result of this work, we conclude that *I. gualtieranus* is an independent lineage and should be considered an Evolutionarily Significant Unit (ESU) for management plans. It is closely related to *I. alonensis* with which it can hybridize, producing fertile hybrids. Intermediate shells are known to occur in the contact zones (Boettger, 1913). Besides, fertile hybrids have been obtained under laboratory conditions in our laboratory. On the basis of their great differences in shell morphology, together with the lack of reproductive isolation between keeled and globular snails, we suggest that these two taxa should be designated as subspecies: *Iberus gualtieranus gualtieranus* and *I. gualtieranus alonensis*. Finally, the phylogenetic tree shows that there are at least three main lineages with the rounded '*alonensis*' shell form in Andalusia. The possibility of the existence of more taxa in the *alonensis–gualtieranus* complex (including all the bigger forms of this complex) is currently being investigated.

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REFERENCES

ALONSO, M.R., LOPEZ-ALCÁNTARA, A., RIVAS, P. & IBAÑEZ, M. 1985. A biogeographic study of *Iberus gualtierianus* (L.) (Pulmonata: Helicidae). *Soosiana*, **13**: 1–10.

APARICIO, M.T. 1983a. *Estudio morfológico y citotaxonómico de algunos helícidos de la fauna española, en especial de la región central*. PhD thesis, Universidad Complutense, Madrid.

APARICIO, M.T. 1983b. The chromosomes of eight species of the subfamily Helicinae (Gastropoda, Pulmonata, Helicidae) from Spain. *Malacological Review*, **16**: 71–78.

APARICIO, M.T. & RAMOS, M.A. 1988. A comparative study of the morphology of the pulmonate snail *Pseudotachea litturata* (Pfeiffer) and other species of *Pseudotachea*, *Iberus* and *Cepaea*. *Journal of Molluscan Studies*, **54**: 287–294.

ARRÉBOLA, J.R. 1995. *Caracoles terrestres (Gastropoda, Stylommatophora) de Andalucía con especial referencia a las provincias de Sevilla y Cádiz*. PhD thesis, Universidad de Sevilla.

ARRÉBOLA, J.R. 2002. *Manuales de conservación de la naturaleza, 1. Caracoles terrestres de Andalucía*. Consejería de Medio Ambiente.

ARRÉBOLA, J.R. & ÁLVAREZ, R. 2001. La explotación de los caracoles terrestres: aspectos ecológicos y socio-culturales. *Temas de Antropología Aragonesa*, **11**: 139–172.

AVISE, J.C. 1994. *Molecular markers, natural history, and evolution*. Chapman & Hall, New York.

AVISE, J.C. 2000. *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge, MA.

BOETTGER, C.R. 1913. Aus der Schausammlung. Die Verändeer lichkeit der Échale von *Iberus gualterianus* L. *Bericht der Senckenbergischen Naturforschenden Gesellschaft*, **44**: 183–197.

BOURGUIGNAT, J.R. 1870. Mollusques nouveaux, litigieux ou peu connus. *Revue et Magasin de Zoologie*, **15–22**.

COBOS, A. 1979. Sobre algunos *Iberus* Montfort de la provincia de Almería (Gastrop. Pulmon.). *Boletín de la Sociedad de Historia Natural de Baleares*, **23**: 35–46.

DAVIS, G.M., WILKE, T., SPOLSKY, C., QIU, C.-P., QIU, D.-C., XIA, N.-Y., ZHANG, Y. & ROSENBERG, G. 1998. Cytochrome oxidase I-based phylogenetic relationships among the Pomatiopsidae, Hydrobiidae, Rissoidae and Truncatellidae (Gastropoda: Caenogastropoda: Rissoacea). *Malacologia*, **40**: 251–266.

DAVISON, A. 2000. An East-West distribution of divergent mitochondrial haplotypes in British populations of the land snail, *Cepaea nemoralis* (Pulmonata). *Biological Journal of the Linnean Society*, **70**: 697–706.

DAVISON, A. 2002. Land snail as a model to understand the role of history and selection in the origins of biodiversity. *Population Ecology*, **44**: 129–136.

DOURIS, V., CAMERON, R.A.D., RODAKIS, G.C. & LECANIDOU, R. 1998. Mitochondrial phylogeography of the land snail *Albinaria* in Crete: long-term geological and short-term vicariance effects. *Evolution*, **52**: 116–125.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution*, **17**: 368–376.

FÉRUSSAC, D. 1821. *Tableaux systématiques des animaux mollusques, en classes et familles naturelles*. Paris.

FITCH, W.M. 1971. Toward defining the course of evolution: minimal change for a specific tree topology. *Systematic Zoology*, **20**: 406–416.

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**: 294–299.

FRATI, F., DELL'AMPIO, E., CASASANTA, S., CARAPELLI, A. & FANCIULLI, P.P. 2000. Large amounts of genetic divergence among Italian species of the genus *Orchesella* (Insecta, Collembola) and the relationships of two new species. *Molecular Phylogenetics and Evolution*, **17**: 456–461.

GARCÍA SAN NICOLÁS, E. 1957. Estudios sobre la biología, la antomía y la sistemática del género *Iberus* Montfort, 1810. *Boletín de la Real Sociedad Española de Historia Natural*, **55**: 199–390.

GITTENBERGER, E., PIEL, W.H. & GROENENBERG, D.S.J. 2004. The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Pulmonata, Helicidae). *Molecular Phylogenetics and Evolution*, **30**: 64–73.

GÓMEZ-MOLINER, B.J., MORENO, D., ROLÁN, E., ARAUJO, R. & ÁLVAREZ, R.M. 2001. *Protección de moluscos en el catálogo nacional de especies amenazadas*. Reseñas Malacológicas XI. Sociedad Española de Malacología.

GRANDE, C., TEMPLADO, J., CERVERA, J.L. & ZARDOYA, R. 2004. Molecular phylogeny of Euthyneura (Mollusca: Gastropoda). *Molecular Biology and Evolution*, **21**: 303–313.

GUILLER, A., COUTELLEC-VRETO, M., MADEC, L. & DEUNFF, J. 2001. Evolutionary history of the land snail *Helix aspersa* in the Western Mediterranean: preliminary results inferred from mitochondrial DNA sequences. *Molecular Ecology*, **10**: 81–87.

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

HIDALGO, J.G. 1875–84. *Catálogo iconográfico y descriptivo de los moluscos terrestres de España, Portugal y las Baleares*. S. Martínez, Madrid.

HILLIS, D.H., MABLE, B.K., LARSON, A., DAVIS, S.K. & ZIMMER, E.A. 1996. Nucleic acids IV: sequencing and cloning. In: *Molecular Systematics* (A. D. Sinauer, D. H. Hillis, C. Moritz & B. K. Mable, eds), 320–381. Sinauer Associates, Sunderland, Massachusetts.

KOBELT, W. 1904. *Iberus* Montfort und *Otala* Schum. *Malakozoologischen Gesellschaft*: **88**.

LINNAEUS, C. 1758. *Systema Naturae*. Edn 10. Facsimile (1956) by the British Museum, Natural History, London.

LÓPEZ-ALCÁNTARA, A., RIVAS, P., ALONSO, M.R. & IBAÑEZ, M. 1983. Origen de *Iberus gualtierianus*. Modelo evolutivo. *Haliotis*, **13**: 145–154.

LÓPEZ-ALCÁNTARA, A., RIVAS, P., ALONSO, M.R. & IBAÑEZ, M. 1985. Variabilidad de *Iberus gualtierianus* (Linneus, 1758) (Pulmonata, Helicidae). *Iberus*, **5**: 83–112.

LYDEARD, C. & LINDBERG, D.R. (eds)2003. *Molecular systematics and phylogeography of mollusks*. Smithsonian Institution, Washington, DC.

ORTIZ DE ZÁRATE, A. 1991. *Descripción de los moluscos terrestres del valle del Najerilla*. Gobierno de la Rioja, Consejería de Educación, Cultura y Deportes, Logroño.

PALUMBI, S.R., MARTIN, A.P., ROMANO, S., McMILLAN, W.O., STICE, L. & GRABOWSKI, G. 1991. *The simple fool's guide to PCR*. Special Publication, Department of Zoology, University of Hawaii, Honolulu.

PARMAKELIS, A., SPANOS, E., PAPAGIANNKIS, G. & MYLONAS, M. 2003. Mitochondrial DNA phylogeny and morphological diversity in the genus *Mastus* (Beck, 1837): a study in a recent (Holocene) island group (Koufonisi, southeast Crete). *Biological Journal of the Linnean Society*, **78**: 389–399.

PFENNINGER, M. & POSADA, D. 2002. Phylogeographic history of the land snail *Candidula unifasciata* (Poiret, 1801) (Helicellinae, Stylommatophora): fragmentation, corridor migration and secondary contact. *Evolution*, **56**: 1776–1788.

PFENNINGER, M., POSADA, D. & MAGNIN, F. 2003. Evidence for survival of Pleistocene climatic changes in Northern refugia by the land snail *Trochoidea geyeri* (Soós 1926) (Helicellinae, Stylomatophora). *BMC Evolutionary Biology*, **3**: 8.

PFENNINGER, M., EPPENSTEIN, A. & MAGNIN, F. 2003. Evidence for ecological speciation in the sister species *Candidula unifasciata* (Poiret, 1801) and *C. rugosiuscula* (Michaud, 1831) (Helicellinae, Gastropoda). *Biological Journal of the Linnean Society*, **79**: 611–628.

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

PUENTE, A.I. 1994. *Estudio taxonómico y biogeográfico de la superfamilia Helicoidea Rafinesque, 1815 (Gastropoda: Pulmonada: Stylommatophora) de la Península Ibérica e Islas Baleares*. PhD thesis, Universidad del País Vasco.

SAITOU, N. & NEI, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**: 406–425.

SCHANDER, C. & HAGNELL, J. 2003. Death by drowning degrades DNA. *Journal of Molluscan Studies*, **69**: 387–388.

SCHANDER, C.H., HALANYCH, K.M., DAHLGREN, T.H. & SUNDBERG, P. 2002. Test of the monophyly of *Odostomiinae* and *Tubonilliinae* (Gastropoda, Heterobranchia, Pyramidellidae) based on 16S mtDNA sequences. *Zoologica Scripta*, **32**: 234–254.

SERRADELL, B. 1912. *Helix Gualtiero-campesina* Serradell. Especie, ó mejor dicho, forma nueva, intermedia entre el grupo de la *H. Gualtierana* L. y de la *H. campesina* Ezq. *Boletín de la Real Sociedad Española de Historia Natural*, **12**: 377–384.

STEINKE, D., ALBRECHT, C. & PFENNINGER, M. 2004. Molecular phylogeny and character evolution in the Western Palaeartic Helicidae *s.l.* (Gastropoda: Stylommatophora). *Molecular Phylogenetics and Evolution*, **32**: 724–734.

SOKOLOV, E.P. 2000. An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues. *Journal of Molluscan Studies*, **66**: 573–575.

SWOFFORD, D.L. 2002. *PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10*. Sinauer Associates, Sunderland, Massachusetts.

THOMPSON, J.D., GIBSON, T.D., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**: 4876–4882.

TESHIMA, H., DAVISON, A., KUWAHARA, Y., YOKOYAMA, J., CHIBA, S., FUKUDA, T., OGIMURA, H. & KAWATA, M. 2003. The evolution of extreme shell shape variation in the land snail *Ainohelix editha*: a phylogeny and hybrid zone analysis. *Molecular Ecology*, **12**: 1869–1878.

WADE, C.M., MORDAN, P.B. & CLARKE, B. 2001. A phylogeny of the land snails (Gastropoda: Pulmonata). *Proceedings of the Royal Society of London, Series B*, **268**: 413–422.