

Phylogeny of Iberian *Zabrus* (Coleoptera: Carabidae: Zabринi) based on mitochondrial DNA sequence

JOSÉ-FERMÍN SÁNCHEZ-GEA, JOSÉ GALIÁN and JOSÉ SERRANO*

Departamento de Zoología, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, Apto. 4021, 30071 Murcia, Spain; e-mails: jfermin@um.es; jgalian@um.es; jserrano@um.es

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Abstract. The genus *Zabrus* Clairville, 1806 is a Holomediterranean taxon that radiated into about a hundred species most likely during the Cenozoic. There are four endemic subgenera on the Iberian Peninsula, which include 28 species, *Epomidozabrus* (3 spp.), *Euryzabrus* (1 sp.), *Platyzabrus* (2 spp.) and *Iberozabrus* (22 spp.). A mitochondrial fragment comprising part of the cytochrome-oxidase-I, tRNA^{Leu}, and part of the cytochrome-oxidase-II genes was sequenced of most of these species. Taxa of other subgenera of *Zabrus* (*Macarozabrus*, *Polysitus*, *Zabrus*, and *Pelor*), six species of *Amara* (the putative sister taxon of *Zabrus*), and representatives of tribes related to Zabrinini were also sequenced. The results show that the genus *Zabrus* is a monophyletic taxon well separated from *Amara*. The four subgenera endemic to the Iberian Peninsula also make up a monophyletic clade, which stresses the association between the geographic distribution and the monophyly of many supraspecific taxa within this genus. The species-rich subgenus *Iberozabrus* seems to be the sister taxon of the clade made up of the three other subgenera endemic to the Iberian Peninsula. The *Iberozabrus* species-groups proposed by Andújar & Serrano in 2001, based on morphological and geographical grounds, are only partly corroborated by the molecular phylogeny. The lack of congruence between these data sets is mainly between those species-groups with large numbers of species and more complex geographic patterns. Some cases of incongruence are possibly due to homoplastic external characters appeared by convergent evolution.

INTRODUCTION

The genus *Zabrus* Clairville, 1806 is included in the subfamily Pterostichinae and tribe Zabrinini, and comprises about 105 species mostly distributed in the Mediterranean Basin (Andújar & Serrano, 2001). Ganglbauer (1915) established the current taxonomy of the genus and divided it into subgenera based on the external characters of the adult and geographic distribution of the taxa. Freude (1986) questioned the distinctness of *Zabrus* from *Amara* because some of its species occasionally show one of the diagnostic traits of *Zabrus*. However, a karyotypic analysis (Galián et al., 1991), the localisation of rDNA loci (Sánchez-Gea et al., 2000), and the structure of the female genitalia (Ortuño et al., 2003) support the monophyly of the genus *Zabrus*.

Most of the subgenera of *Zabrus* are endemic to particular areas and are probably monophyletic groups characterised by one or more apomorphies (Andújar & Serrano, 2000). On the Iberian Peninsula there are four endemic subgenera, namely *Euryzabrus* (1 species), *Platyzabrus* (2 species), *Epomidozabrus* (3 species) and *Iberozabrus* (22 species), plus two of the three species of the nominal subgenus (Andújar & Serrano, 2001). Andújar & Serrano (2001) investigated the phylogenetic relationships of these subgenera using 24 morphological imaginal characters. Most of those characters were phylogenetically uninformative because they are either autapomorphies or homoplasies. In spite of these limitations, it was shown that the subgenera endemic to the Iberian Peninsula make up a monophyletic group separated from the

subgenus *Zabrus*. Likewise each of the subgenera *Euryzabrus*, *Platyzabrus* and *Epomidozabrus* make up monophyletic groups but these are interspersed within the species of the large subgenus *Iberozabrus*. Moreover, the clades of the species within *Iberozabrus* do not agree with the species-groups postulated on the basis of the morphological and geographic characters currently used in identification keys. These results indicate that *Iberozabrus* has a complex evolutionary history, perhaps starting in the Oligocene. As noted by Oosterbroek & Arntzen (1992), the Mediterranean Basin has undergone drastic changes in sea level, alpine uprisings and climatic oscillations during the last 30–40 MY, when the Tethys Sea started to close. These factors probably exerted major influences on the whole Tethys biota, particularly on the Iberian Peninsula, which became a centre of origin of new taxa, as exemplified by *Iberozabrus*. Most Iberian *Zabrus* are flightless and have a poor dispersal ability, which explains the high number of species in mountainous areas. Other species are found in the lowlands or live in sandy coastal habitats (Andújar & Serrano, 2001). Thus, present day distribution and habitat preferences suggest that the genus has undergone a complex sequence of speciation events in the Iberian Peninsula worthy of investigation, especially in the large subgenus *Iberozabrus*. The aim of this study is to investigate the phylogeny of Iberian taxa of *Zabrus* by analysing their mitochondrial DNA, and compare our results with those of Andújar & Serrano (2001). Sequence data of this molecule has proved useful for phylogenetic studies of many

* Corresponding author.

organisms (review in Avise, 2000), including beetles of the family Carabidae (Howland & Hewitt, 1995; Galián et al., 1999; Emerson et al., 1999, 2000; Martínez et al., in press) to which the genus *Zabrus* belongs.

MATERIAL AND METHODS

Material

Sixty-three individuals of the genus *Zabrus* were sequenced. The individuals belong to the four subgenera endemic to the Iberian Peninsula, *Epomidozabrus*, *Euryzabrus*, *Platyzabrus* and *Iberozabrus*, and to the subgenera *Zabrus*, *Macarozabrus*, *Polysitus* and *Pelor*. Six species of the putative sister taxa of *Zabrus*, the genera *Amara* and *Curtonotus* were also sequenced, and species from other tribes of the subfamily Pterostichinae, which includes the tribe Zabrinini, were included as out-groups. Sampling localities are given in Appendix 1. In some cases more than one individual was sequenced to check the influence of intra-specific variation on the results. Body parts of the beetles not used in this study were preserved in 100% ethanol, and deposited in the collection of the Department of Zoology and Physical Anthropology, University of Murcia, Spain.

DNA extraction

One or two legs of each individual were used. Total DNA was extracted using a standard lysis buffer (200µL to 500µL of 1M Tris-HCl pH = 7.4, 0.5M EDTA pH = 8, 10% SDS pH = 7.2 and 10µL 10 mg/ml proteinase K) or Wilson extraction buffer (200µL to 500µL of 100mM Tris-HCl pH = 7.4, 10mM EDTA pH = 8, 100mM NaCl and 10µL 10 mg/ml proteinase K). Phenol (Sambrook et al., 1989) and Chelex (Walsh et al., 1991) extraction protocols were assayed.

DNA amplification

PCR reactions were performed in 12.5 to 25 µL volume using *Taq* DNA Polymerase (Pharmacia Biotech, Little Chalfont, UK) or puRe *Taq* Ready-To-Go PCR Beads (Amersham Biosciences, Little Chalfont, UK). Two different sets of primers, commonly known as Dick (5'-CCTACAGGAATTAAGTTTTAGATGATTAGC-3') and Pat (3'-ATTATACCGTCTAATCACGTAA CCT-5'), or Dick and Marilyn (3'-GTTACTAT(A/G)ACTTGA ATACT-5') were used. These primers amplify a fragment of mitochondrial DNA that includes the end of cytochrome oxidase I (CO-I), tRNA^{Leu}, and the beginning of cytochrome oxidase II (CO-II), as well as the intergenic spacers (Simon et al., 1994).

Cycle parameters were 45 s denaturalisation at 94°C, 1 min of annealing at 45–52°C, and 1 min extension at 72°C for 35 cycles. No purification method was used and DNA amplifications were simply precipitated with 2/3 vol. of 5M ammonium acetate and 1 vol. of isopropanol.

DNA sequencing

PCR products were sequenced with the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California) using Ampli *Taq* DNA Polymerase FS (Applied Biosystems, Tres Cantos, Madrid, Spain) in an ABI PRISM Tm 377 DNA Sequencer (Applied Biosystems, Tres Cantos, Madrid, Spain). When needed sequencing was performed in both directions using the same primers for amplification.

Alignment and correction of sequences

Sequences were aligned using ClustalW v. 1.7 (Thomson et al., 1994). Incongruent and missing data in sequences were manually corrected using the Chromas program v. 2.22 (Technelysium Pty Ltd 2002). Aligned sequences were checked for a post-correction of gaps. Some bases were disregarded at the

beginning and the end of the sequence. The final sequence data set consisted of 876 base pairs: 1-535 for CO-I, 544-611 for tRNA^{Leu} and 618-876 for the CO-II. GenBank accession numbers for these sequences are AY551824-AY551904.

Phylogenetic analysis

The sequence was analysed using the maximum parsimony (MP), maximumlikelihood (ML) and two different distance methods: neighbour joining (NJ) and minimum evolution (ME). Two different programs were used for these analyses: PAUP v. 4.0b10 (Swofford et al., 1996) and MEGA v. 2.0 (Kumar et al., 2001). PAUP was used to perform MP and ML analyses with unweighted and weighted characters. MEGA was used to perform NJ and ME analyses with unweighted characters. MacClade v. 3.0 program (Maddison & Maddison, 1992) was used to weight the bases of the sequenced mitochondrial fragment.

For distance analyses NJ and ME, pairwise sequence differences were compared using the Kimura 2-parameter method using two different tests of phylogeny: bootstrap (BT) and interior branch (IBT) test, with 10,000 replications in each analysis.

MP analysis was done using heuristic searches and a BT of 10,000 replications (TBR branch swapping, MULPARS option in effect) with simple stepwise addition of taxa, to find the most parsimonious tree. A 50% majority-rule consensus tree was constructed if more than one parsimonious tree was obtained.

To choose the best possible model to do the ML analysis, Model Test version 3.06 program was used (Posada & Crandall, 1998). This program tests 56 different models using two criteria: Akaike information criterion (AIC) and hierarchical likelihood ratio tests (hLRTs). After implementing the data matrix with this model, a ML analysis was done using heuristic searches and a BT of 10,000 replications (TBR branch swapping, MULPARS option in effect) with simple stepwise addition of taxa. A 50% majority-rule consensus tree was constructed when more than one tree was obtained.

RESULTS

423 bases out of the 876 in the sequence data set were variable and 303 were parsimony informative. Within the tribe Zabrinini there were 356 variable sites of which 274 were parsimony informative. For species of the genus *Zabrus* there were 321 variable sites of which 239 were parsimony informative. All the species of the genus *Zabrus* are included in a single clade (Fig. 1) supported by values ranging from 72 to 100 depending on the kind of analysis used.

The sequence analysis shows that species of the genus *Zabrus* have always one or two extra adenines in the intergenic spacer between tRNA^{Leu} and the cytochrome-oxidase-II, except for those of the subgenus *Iberozabrus*, which have three extra adenines.

Distance analysis

The NJ and ME analyses done by the MEGA program using IBT produced similar condensed trees. Three different clades of *Zabrus* species were obtained at the level of subgenera (Fig. 1). Clade 1 included Eastern Mediterranean taxa (Bulgaria, Greece and Turkey), which are members of the subgenus *Pelor*. Clade 2 included species of subgenera *Zabrus*, *Macarozabrus* and *Polysitus*. Clade 3 included all species endemic to the Iberian Peninsula. This last clade includes two subclades, clade 3b, comprising the subgenera *Euryzabrus*, *Epomidozabrus*,

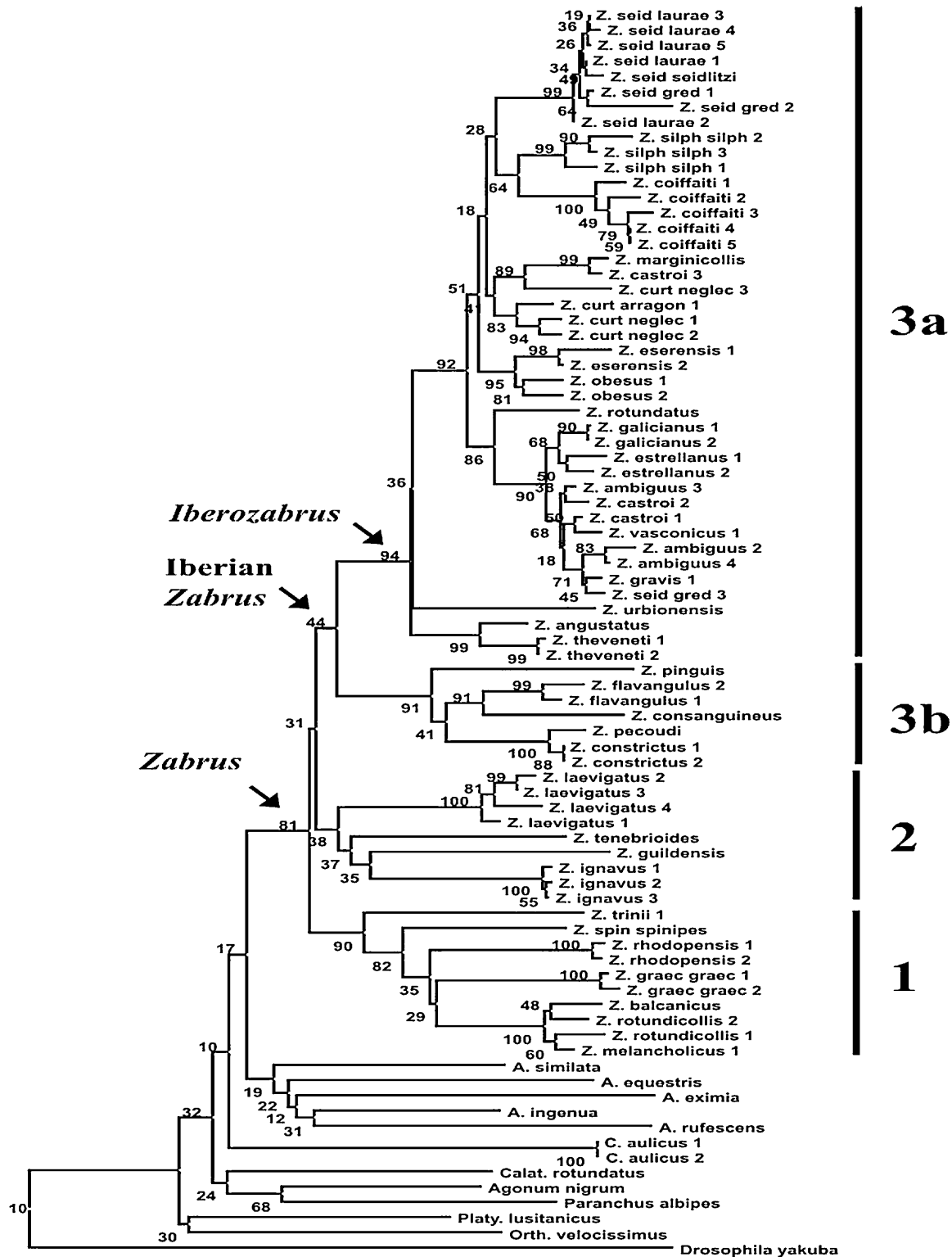


Fig. 1. Minimum evolution interior branch test tree for species of *Zabrus* and related taxa, based on a mitochondrial DNA fragment of cytochrome-oxidase-I, tRNA^{Leu} and cytochrome-oxidase-II. Numbers refer to interior branch test values based on 10,000 replications. Clade 1 = *Zabrus* subgenus *Pelor*; clade 2 = *Zabrus* subgenera *Macarozabrus*, *Zabrus*, and *Polysitus*; clade 3a = *Zabrus* subgenus *Iberozabrus*; clade 3b = *Zabrus* subgenera *Euryzabrus*, *Epomidozabrus* and *Platyabrus*.

Platyabrus and one species of the subgenus *Iberozabrus*, and clade 3a the other taxa of *Iberozabrus*.

The NJ and ME analyses done by the MEGA program using BT also produced similar consensus trees. These

consensus trees were less resolved than those obtained using IBT (consensus values were lower) and some clades were resolved as polytomies. Seven different clades were obtained at the level of subgenera (not

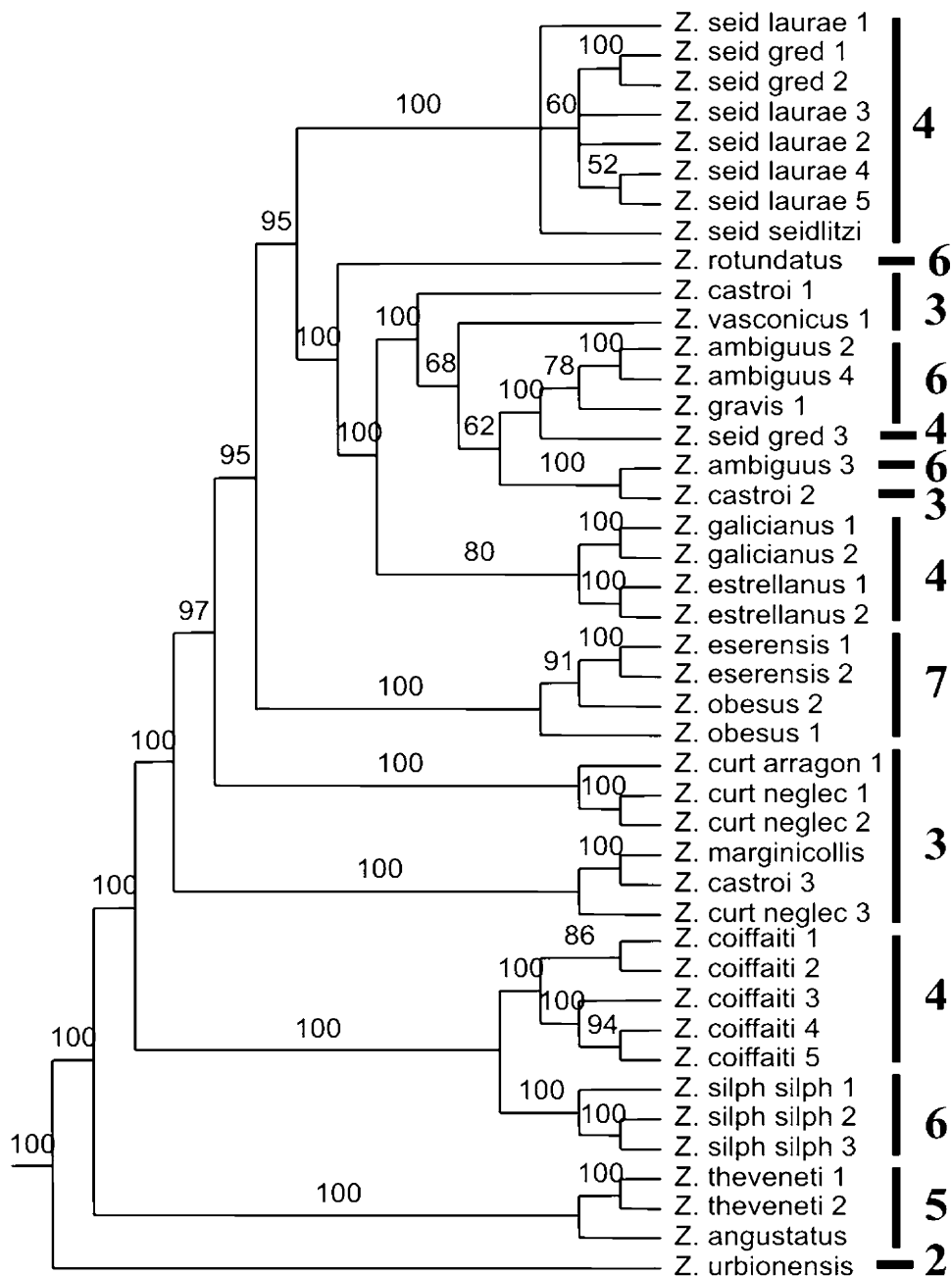


Fig. 2. Fragment of the maximum parsimony bootstrap consensus tree showing only the species of the subgenus *Iberozabrus*. Unweighted characters based on the same mitochondrial fragment as in Fig. 1. Numbers to the left of each branch refer to the majority rule bootstrap values based on 10,000 replications. Numbers to the right of the reconstructed phylogeny show the species-groups to which the species belongs. The seven species-groups are indicated in Appendix 2.

shown). Clade 1 includes all of the East Mediterranean species, as obtained using IBT. Clades 2 to 5 correspond to clade 2 obtained by IBT. Clade 2 includes only one species of the nominal subgenus *Zabrus*, *Z. tenebrioides*. Clade 3 includes the species from the Canary Islands, *Z. (Macarozabrus) laevigatus*. Clade 4 includes the Moroccan species of the subgenus *Polysitus*. Clade 5 includes another species of the subgenus *Zabrus*, *Z. ignavus*. Finally, clades 6 and 7 obtained using BT correspond to clade 3 obtained by IBT, and in both cases are unresolved. Clade 6 includes species of the subgenera *Euryzabrus*, *Epomidozabrus*, *Platyzabrus* and *Z. (Iberozabrus)*

consanguineus. Clade 7 includes the rest of the species of the subgenus *Iberozabrus*, all endemic to the Iberian Peninsula. The subclades of the subgenus *Iberozabrus* only partly conform with the species-groups postulated for this subgenus on the basis of morphology and geographic distribution (Andújar & Serrano, 2001).

NJ and ME analyses produced different arrangement of the clades when using IBT. The NJ analyses indicated that the Iberian *Zabrus* are more closely related to Eastern Mediterranean taxa than to *Zabrus* + *Macarozabrus* + *Polysitus*, and ME analyses indicated a closer relationship of Iberian taxa to *Zabrus* + *Macarozabrus* + *Polysitus*

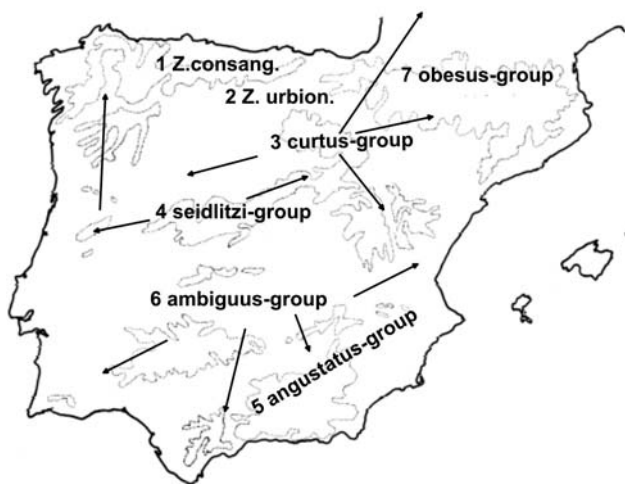


Fig. 3. Map of the Iberian Peninsula showing the location of the *Iberozabrus* species-groups indicated in Appendix 2.

than to Eastern Mediterranean taxa. However, these relationships are weak as they are only supported by low values.

Maximum likelihood

Model Test chose TVM + G + I as the best model based on AIC and hLRTs criteria with unweighted characters. When bases were assigned weights 2 : 5 : 1 Model Test chose TVM + G + I under hLRTs criterion, and GTR + G + I under AIC criterion. The heuristic search analysis performed by PAUP using these models found 1 or 2 trees. The trees had a score of 8,256.72155 with unweighted characters, and a score of 17,845.95246 (AIC) or 17,493.10825 (hLRTs) with weighted characters. The trees had a C.I. of 0.3987 and a R.I. of 0.6888 with unweighted characters, and a C.I. of 0.4497 and a R.I. of 0.6907 with weighted characters.

At the level of subgenera five different clades were obtained in the majority rule consensus tree. These clades matched those obtained using distance methods (NJ and ME) (Fig. 1), with the exception of clade 2 where one species of the nominal subgenus *Zabrus*, *Z. tenebrioides*, is excluded and is in a clade of its own. The differential weighting of the three codon positions produced no effect at this level.

At the level of species included in each subgenera several subclades are obtained. Some of them that belong to the subgenus *Iberozabrus* (Fig. 2) partially conform to species-groups derived from morphological and geographic characters, although the rearrangement of these subclades differs from those obtained using distance methods.

Parsimony analysis

The heuristic search analysis done by PAUP found 9,768 most parsimonious trees when the three bases of each codon were assigned weights of 2 : 5 : 1. The trees had a length of 2,906 steps, a C.I. of 0.4604 and a R.I. of 0.7037. The heuristic search analysis done by PAUP found 40,600 most parsimonious trees when the three bases were assigned equal weights. These trees had a

length of 1,496 steps, a C.I. of 0.4051 and a R.I. of 0.6970.

In both cases, at the level of subgenera, there were five clades in the majority rule consensus tree. These clades also matched those obtained with distance methods (Fig. 1), with the exception of clade 2. This clade was split into two clades by this analysis, which differ depending on the weighting of the characters. With unweighted characters the first clade is formed by species from Morocco and the Canary Islands (subgenus *Polysitus* and *Macarozabrus*, respectively) and the second by the two species of the subgenus *Zabrus*. With weighted characters species of the subgenus *Polysitus* were assigned to the second clade. Clades within the subgenus *Iberozabrus* are similar (but not coincident) to those ones obtained by distance and maximum likelihood methods.

DISCUSSION

The monophyly of the genus *Zabrus*

All the trees indicate that *Zabrus* species make up a monophyletic group with high BT and IBT values, whose sister taxon is still uncertain, although all the analyses indicate either *Amara* or *Curtonotus* (currently regarded as a subgenus of *Amara*) as the sister group (Fig. 1). This corroborates the conclusions of a karyotype analysis (Galián et al., 1991; Sánchez-Gea et al., 2000), and those based on morphology and geographic distribution of taxa (Andújar & Serrano, 2001), and the structure of the female genitalia (Ortuño et al., 2003). *Amara* and *Curtonotus* are Holarctic taxa, whereas *Zabrus* is a Mediterranean genus. This suggests that *Zabrus* originated from *Amara*-like ancestors after the separation of the Eurasiatic and North American plates, which started at the beginning of the Cenozoic Era, about 65 MY ago.

The monophyly of the subgenera of *Zabrus* endemic to the Iberian Peninsula

In all analyses the four subgenera endemic to the Iberian Peninsula, *Epomidozabrus*, *Euryzabrus*, *Platyabrus* and *Iberozabrus* make up a monophyletic group (Fig. 1), which is separated from the species of the subgenera from the Canary Islands (*Macarozabrus*: *Z. laevigatus*), Morocco (*Polysitus*: *Z. guildensis*), the Eastern Mediterranean region (*Pelor*: various species) and the widespread subgenus *Zabrus* (*Z. ignavus* and *Z. tenebrioides*).

This is associated with the poor dispersal power of the genus, as only species of the subgenus *Zabrus* have fully developed wings and are able to fly. With the exception of this subgenus, the other subgenera are restricted to particular areas of the Mediterranean-Turanian region. In terms of morphology and geography the large subgenus *Pelor* lacks consistency, and so Ganglbauer (1915), and more recently Freude (1986, 1988, 1989, 1990) attempted to split this subgenus into four species-groups with better congruency. Andújar & Serrano (2000) indicated that *Pelor* is probably polyphyletic and should be divided into monophyletic units, and proposed the subgenera *Italozabrus* and *Himalayozabrus*. Nonetheless, the few species of *Pelor* included in our analysis are probably close

relatives (they were collected in Bulgaria, Greece and Turkey) and thus it is not surprising that all of them are grouped in the same clade.

The Iberian subgenera with few species, *Epomidozabrus*, *Euryzabrus* and *Platyzabrus* seem to share a common ancestor (Fig. 1), and their sister group is the larger subgenus *Iberozabrus* (22 species). High BT and IBT values support both clades. *Epomidozabrus*, *Euryzabrus* and *Platyzabrus* are not interspersed within the species of *Iberozabrus*, as indicated by the phylogenetic analysis based on morphological characters (Andújar & Serrano, 2001). The presence of three extra adenines at the end of the tRNA^{Leu} sequence of species of the subgenus *Iberozabrus* supports the monophyletic nature of this subgenus. As these three subgenera are restricted to the oldest massifs on the Peninsula (mountains of Galicia, the Cantabrian Chain and the Sistema Central Chain, respectively), they may represent the first evolutionary radiation of the genus *Zabrus* in Iberia.

The limits and internal relationships of *Iberozabrus*

Ganglbauer (1915) and Freude (1986) placed some of the *Iberozabrus* species in the large subgenus *Pelor* group I (*Z. consanguineus*, *Z. theveneti*, *Z. angustatus*, *Z. gravis*, *Z. silphoides*, *Z. rotundatus*). Jeanne (1968) included these species in *Iberozabrus*, a decision corroborated by our study and that using karyotypic data (Galián et al., 1991). These conclusions again emphasize the congruence between the monophyly of subgenera of *Zabrus* and their geographic distribution.

Distance, maximum likelihood and maximum parsimony analyses indicate (Fig. 2) that *Iberozabrus* includes clades that coincide in part with the seven species-groups proposed by Andújar & Serrano (2001) on the basis of morphology and geographic distribution. These groups are listed in Appendix 2 and their geographic distributions are shown in Fig. 3.

The species-group 1 is made up exclusively of *Zabrus* (*Iberozabrus*) *consanguineus*. It is the most distinct species in the subgenus and is related to the subgenus *Epomidozabrus* by the three phylogenetic analyses (Fig. 1). This relationship is congruent with their morphology and geographic distribution as *Z. consanguineus* is the only species of the genus that occurs in the Cantabrian Mountains, which are adjacent to the Galician Mountains occupied by *Epomidozabrus*. All the data suggest that this species belongs to a lineage related to *Epomidozabrus*, which should be placed outside *Iberozabrus*, perhaps in a new subgenus as suggested by Andújar & Serrano (2001).

The species-groups supported by this study are *Z. urbinensis* (species-group 2), *Z. angustatus* and *Z. theveneti* (species-group 5, Betic Mountains), and *Z. obesus* and *Z. eserensis* (species-group 7, Pyrenees). However, species-groups 3 (*curtus*-group), 4 (*seidlitzii*-group) and 6 (*ambiguous*-group) are not well supported (Fig. 2).

The species of the *curtus*-group make up two unrelated terminal clades, one formed by *Z. curtus* and *Z. marginicollis*, and the other by *Z. castroi* and *Z. vasconicus* together with some members of the *ambiguous*-group. This group has a pronotum with an emarginate posterior basis

and outward protruding posterior angles, and a geographic distribution mainly centred in the Sistema Ibérico and the Basque Mountains (Andújar & Serrano, 2001). As the shape of the pronotum is an adaptive character it is possible that this group is polyphyletic based on a homoplastic character. Likewise, the *curtus*-group shows a heterogeneous geographic pattern (Fig. 3) as it occupies a broad altitudinal range from the coast (*Z. inflatus*, not sequenced, inhabits the French Landas) to the montane belt (the preferred altitude) and the high mountains. In the north it reaches the Fontainebleau forest close to Paris, and in the south the southern border of the Sistema Ibérico. Further studies are needed to test the monophyly of the group and reconstruct its complex evolution.

The species of the *seidlitzii*-group make up three terminal clades, one includes the *Z. seidlitzii* species complex, another *Z. estrellanus* and *Z. galicianus*, and a third *Z. coiffaiti* and *Z. silphoides silphoides*, a species of the *ambiguous*-group. This indicates that this group differentiated in two areas, the oriental and central Sistema Central, and the western Sistema Central (Portugal, Serra da Estrela) plus the mountains of Galicia. In the east the range occupied by the subspecies of *Z. seidlitzii* (the sierras of Guadarrama and Gredos) partially overlaps that of *Z. coiffaiti* and its molecular sister taxa *Z. silphoides* (Sierra de Gredos). If the relatedness of *Z. coiffaiti* and *Z. silphoides* is corroborated by other characters then the morphological characters used to place these species in different species-groups (lateral margin of elytron widened or narrow near humeral region, pronotum sides straight or rounded, posterior angles almost square or obtusely rounded) are not entirely reliable. To the north-east *Z. cameranus* and *Z. gibbulus* (not sequenced) are also putative members of this species-group (Andújar & Serrano, 2001). In the west area *Z. estrellanus* (Serra da Estrela) is closely related to *Z. galicianus* (mountains of Galicia). These two areas are faunistically related (Novoa et al., 1996).

The *ambiguous*-group was defined on the basis of a pronotum with rounded sides and a geographic distribution centred in the southern half of the Iberian Peninsula, in contrast to the northern distribution of most of the other species-groups of *Iberozabrus*. It includes *Z. ambiguus*, a species with a large altitudinal range from the Southern Meseta to the high sierras (e.g., Sierra Nevada). This species shows incipient morphological differentiation, which has resulted in the description of taxa currently regarded as synonyms (Andújar & Serrano, 2001). *Z. rotundatus* is morphologically a sibling species of *Z. ambiguus* restricted to the western Betic sierras (Sierra de Ronda), but the phylogenetic trees indicate that these taxa are distantly related (Fig. 2). *Z. gravis* is a lowland species related to *Z. ambiguus* based on the characters investigated. Finally, *Z. silphoides* should be removed from this species-group, because all of the consensus trees place this species close to *Z. coiffaiti* (a member of the *seidlitzii*-group). That the geographic area of *Z. silphoides* (Northern Meseta) and *Z. coiffaiti* (Sierra de Gredos) meet at the upper limit of the montane belt on the north

side of the Sierra de Gredos, makes this relationship more plausible.

Our analysis of a mitochondrial fragment suggests more complex relationships within the subgenus *Iberozabrus* than the morphology and geographic distribution. As noted by Andújar & Serrano (2001) the phylogenetic value of morphological characters is low, mostly because they are adaptive and subject to convergence and homoplasy. The reconstruction of the phylogeny of this taxon is also hindered by changes that occurred on the Iberian Peninsula during the Miocene due to alpine tectonics and climatic oscillations. The parallel orientation of Iberian massifs, which are connected by the Sistema Iberico Mountains in a North-South direction (Fig. 3), has probably favoured repeated periods of isolation and speciation followed by dispersal and colonisation. This evolutionary scenario makes it more difficult to reconstruct the phylogeny of *Iberozabrus* than that of the genus *Calathus* on the Macaronesian Archipelagos (Emerson et al., 1999, 2000). The Macaronesian islands are strong barrier to dispersal, which explains why each island has its own endemic *Calathus* (Machado, 1992). Only Tenerife, one of the oldest islands, has probably experienced more than one colonisation event and a complex intra-island radiation, giving rise to different species-groups of *Calathus* (incidentally, there are two *Zabrus* species on Tenerife and one on Gran Canaria).

In summary, there is a lack of congruence between the different data sets for the subgenus *Iberozabrus*, which might be solved in the future by sequencing additional genes and surveying other morphological, genomic and ecological characters.

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APPENDIX 1. Localities (major mountain massifs or lowland areas) from which *Zabrus* and related taxa sequenced in this study were collected. SP Spain, PT Portugal, MO Morocco, CI Canary Islands, GR Greece, BU Bulgaria, TR Turkey. Sampling dates and precise data of localities can be obtained upon request.

Taxon	Number of individuals	Localities
Tribe Zabrini		
Genus <i>Zabrus</i>		
subgenus <i>Epomidozabrus</i> Ganglbauer, 1915		
<i>flavangulus</i> Chevrolat, 1840	2	Serra da Estrela, PT
subgenus <i>Euryzabrus</i> Ganglbauer, 1915		
<i>pinguis</i> Dejean, 1831	1	SW coast of Galicia, SP
subgenus <i>Iberozabrus</i> Ganglbauer, 1915		
<i>ambiguus</i> Rambur, 1838	1	NW Betic Mountains-1, SP
	1	NW Betic Mountains-2, SP
	1	Sierra Nevada, SP
<i>angustatus</i> Rambur, 1838	1	Sierra Nevada, SP
<i>castroi</i> Martínez y Saez, 1873	1	S Sistema Ibérico, SP
	2	E Sistema Central, SP
<i>coiffaiti</i> Jeanne, 1970	5	Sierra de Gredos, SP
<i>consanguineus</i> Chevrolat, 1865	1	W Cantabrian Mountains, SP
<i>curtus arragonensis</i> Heyden, 1883	1	S Sistema Ibérico, SP
<i>curtus neglectus</i> Schaum, 1864	3	N Sistema Ibérico, SP
<i>eserensis</i> C. Bolívar, 1918	2	Central Pyrenees, SP
<i>estrellanus</i> Heyden, 1880	2	Serra da Estrela, PT
<i>galicianus</i> Jeanne, 1970	2	SE Mountains of Galicia, SP
<i>gravis</i> Dejean, 1828	1	Southern Meseta, SP
<i>marginicollis</i> Dejean, 1828	1	Northern Meseta, SP
<i>obesus</i> Audinet-Serville, 1821	2	Central Pyrenees, SP
<i>rotundatus</i> Rambur, 1838	1	W Betic Mountains, SP
<i>seidlitzii gredosanus</i> Jeanne, 1970	2	Sierra de Gredos-1, SP
	1	Sierra de Gredos-2, SP
<i>seidlitzii laurae</i> Toribio, 1989	5	E Sierra de Guadarrama, SP
<i>seidlitzii seidlitzii</i> Schaum, 1864	1	C Sierra de Guadarrama, SP
<i>silphoides silphoides</i> Dejean, 1828	1	Northern Meseta, SP
	2	Sierra de Francia, SP
<i>theveneti</i> Chevrolat, 1874	2	NW Betic Mountains, SP
<i>urbionensis</i> Jeanne, 1970	1	N Sistema Ibérico, SP
<i>vasconicus</i> Uhagón, 1904	2	Basque Mountains, SP
subgenus <i>Macarozabrus</i> Ganglbauer 1915		
<i>laevigatus</i> Zimmermann, 1831	3	Tenerife, CI
subgenus <i>Pelor</i> Bonelli, 1810		
<i>balcanicus balcanicus</i> Heyden, 1883	1	W Rhodopi Mountains, BU
<i>balcanicus rhodopensis</i> Apfelbeck, 1904	2	S Pirin Mountains, BU
<i>spinipes spinipes</i> Fabricius, 1798	1	W Rhodopi Mountains, BU
<i>graecus graecus</i> Dejean, 1828	2	Micenas, GR
<i>melancholicus melancholicus</i> Schaum, 1864	1	Ak Mountains, TR
<i>rotundicollis</i> Ménétrés, 1836	2	Tahtali Mountains, TR
<i>tririi tririi</i> Fischer von Waldheim, 1817	2	Musgüney Mountains, TR
subgenus <i>Platyzabrus</i> Jeanne, 1970		
<i>constrictus</i> Graells, 1858	2	Sierra de Béjar, SP
<i>pecoudi</i> Colas, 1942	1	Sierra de Gredos, SP
subgenus <i>Polysitus</i> Zimmermann 1831		
<i>guildensis</i> Alluaud, 1932	1	Fes, MO
subgenus <i>Zabrus</i> Clairville, 1806		
<i>ignavus ignavus</i> Csiki, 1907	1	NW Betic Mountains, SP
	2	Southern Meseta, SP

<i>tenebrioides tenebrioides</i> Goeze, 1777	1	Gerona, SP
Genus <i>Amara</i>		
subgenus <i>Amara</i> Bonelli, 1810		
<i>similata</i> Gyllenhal, 1810	1	Alicante, SP
subgenus <i>Amathitis</i> Zimmermann, 1832		
<i>rufescens</i> Dejean, 1829	1	Murcia, SP
subgenus <i>Camptocelia</i> Jeanne, 1942		
<i>eximia</i> Dejean, 1828	1	NW Betic Mountains, SP
subgenus <i>Celia</i> Zimmermann, 1832		
<i>ingenua</i> Duftschmid, 1812	1	Southern Meseta, SP
subgenus <i>Percosia</i> Zimmermann, 1832		
<i>equestris</i> Duftschmid, 1812	1	SE Mountains of Galicia, SP
subgenus <i>Leironotus</i> Ganglbauer 1892		
<i>rotundicollis</i> Schaufuss, 1862	1	SE Mountains of Galicia, SP
Genus <i>Curtonotus</i>		
<i>aulicus</i> Panzer, 1796	2	W Rhodopi Mountains, BU
Tribe Sphodrini		
<i>Calathus (Neocalathus) rotundatus rotundatus</i> Jacquelin du Val, 1857	1	SE Mountains of Galicia, SP
<i>Platyderus (Platyderus) lusitanicus lusitanicus</i> Dejean, 1828	1	SE Mountains of Galicia, SP
Tribe Platynini		
<i>Agonum (Agonum) nigrum</i> Dejean, 1828	1	Southern Meseta, SP
<i>Paranchus albipes</i> Fabricius 1792	1	Málaga, SP
Tribe Pterostichini		
<i>Orthomus velocissimus pardoii</i> Mateu, 1957	1	Málaga, SP

APPENDIX 2. Species-groups of *Iberozabrus* postulated by Andújar & Serrano (2001) on the basis of morphological and geographic distribution. * Taxa not sequenced.

Group 1. *Zabrus consanguineus* Chevrolat

Group 2. *Zabrus urbionensis* Jeanne

Group 3. *Z. curtus* Audinet-Serville, *Z. castroi* Martínez, *Z. notabilis* Martínez*, *Z. vasconicus* Uhagón, *Z. marginicollis* Dejean and *Z. inflatus* Dejean*

Group 4. *Z. seidlitzii* Schaum, *Z. estrellanus* Heyden, *Z. galicianus* Jeanne, *Z. coiffaiti* Jeanne, *Z. cameranus* Arribas* and *Z. gibbulus* Jeanne*

Group 5. *Z. theveneti* Chevrolat and *Z. angustatus* Rambur

Group 6. *Z. ambiguus* Rambur, *Z. rotundatus* Rambur, *Z. gravis* Dejean and *Z. silphoides* Dejean

Group 7. *Z. obesus* Audinet-Serville and *Z. eserensis* C. Bolívar

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