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Molecular phylogenetics and evolutionary analysis of body shape in the genus *Cyrtonus* (Coleoptera, Chrysomelidae)

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Abstract

The leaf beetle genus *Cyrtonus* Latreille, 1829 (Chrysomelidae, Chrysomelinae) is a species-rich genus subendemic to the Iberian Peninsula, with only a few species in the neighbouring France and Morocco. All the species are wingless and preferentially inhabiting mountainous areas. The taxonomic knowledge of this group is extremely poor and its systematics almost inexistent. Here, we analyse and characterize with a morphometric analysis one promising systematic trait, the adult body profile, distinguishing between elongated and rounded shapes. Although the monophyly of the genus is not contentious, we test for it using mitochondrial rnl sequences and the chrysomeline homologous sequences available in GenBank. In addition, four genetic markers, two mitochondrial and two nuclear are used to produce a phylogenetic hypothesis for half the species within the genus and to analyse the evolution of shape, summarized as two continuous variables, length and width, and their ratio. These traits covary significantly with the phylogeny, showing a strong phylogenetic association: elongated species appear to constitute a clade within a paraphyletic assemblage of rounded species. In addition, the mitochondrial DNA tree is used to test for constant rate of evolution in this marker and is calibrated using both biogeographical evidence and the standard insect mitochondrial average substitution rate. This molecular clock hypothesis is used to date the age of speciation events on the phylogeny, reconstructing the origin of the genus in the Middle Miocene, with a relatively constant speciation rate until the end of the Pliocene and an apparent increase in this rate in the Pleistocene, possibly associated with the effect of dramatic climatic changes in this period. Finally, the high systematic value of shape profile in *Cyrtonus* is discussed, arguing the absence of evidence relating it to adaptation.

Key words: Morphometry – systematics – phylogenetic inertia – continuous traits

Introduction

The genus *Cyrtonus* Latreille, 1829 is one of the Western Palaearctic genera of Chrysomelidae beetles with worse taxonomic and systematic knowledge (Warchalowski 2003). There are 44 currently accepted taxa in this genus, two of them subspecies of the same taxon; but no proper taxonomic revision of the group has ever been carried out, with most taxa barely known from nothing else than their original descriptions. All species are apterous, with fused elytra. The monophyly of *Cyrtonus* is not contentious, being diagnosed by an important taxonomic character: the pronotum with posterior angles extended and toothed backwards in both sides. These beetles are difficult to find in the wild and poorly represented in most collections (Cobos 1954). This lack of information does not reflect their high biogeographical, evolutionary and systematic interest among leaf beetles. *Cyrtonus* is almost exclusively distributed in mountainous areas of the Iberian Peninsula, with only three species in France and two in the Rif mountains in Morocco (Fairmaire 1850, 1883; Weise 1916; Cobos 1954). The diversification of this orophilic group in a very complex area from a palaeogeographic point of view, with marked contrasts in relief, and considered a faunal refugium during the last severe glaciations, renders *Cyrtonus* a group prone to the study of geographical speciation. Their alleged low-dispersal capability because of apterism may favour isolation processes and speciation.

Very little is known also about the ecology of these insects. They are of nocturnal habits, their larval development occurs in winter or spring and they undergo summer diapause as adults (cycles of *Cyrtonus rotundatus* and *Cyrtonus montanus*; Mulsant and Wachanru 1849; Weise 1906), and can be generally found under stones or under their host plants

(Jolivet 1951). Their trophic relationships are generally not known except for six species, and in every case they seem to be associated to Asteraceae in the genera *Artemisia*, *Dittrichia*, *Helichrysum*, *Hieracium*, *Hyoseris*, *Lappa*, *Leontodon* and *Santolina* (Mulsant and Wachanru 1849; Weise 1906; Jolivet 1951, 1966; Petitpierre 1984; Petitpierre and Garnería 2003; Petitpierre 2004).

The systematics of *Cyrtonus* is even poorer than what is known from a purely taxonomic perspective. There are few hypotheses of relationships among species. Early taxonomic monographs of *Cyrtonus* recognized the existence of elongated and proportionally broader species (Fairmaire 1850, 1883) (Fig. 1), but only Cobos (1954), a century later, proposed that these shapes could in fact reflect natural groupings. The latter author based his opinion not just on the beetle shapes, but also on the type of aedeagus and the geographical distributions of each morphological group (Cobos 1954). The elongated species, distributed predominantly from the Rif in Morocco to France, through the western half of the Iberian Peninsula, also presented straight and only apically curved aedeagi; the species of broader profile, mainly distributed in southeastern Iberian Peninsula, were in addition characterized by regularly curved aedeagi (Cobos 1954). However, this particular systematic character, beetle profile, has not been formulated explicitly, which requires its formalization previously to including it in further systematic analyses.

In this study, the authors aimed at studying the systematics of *Cyrtonus*, making it the subject of a phylogenetic study focusing on species relationships based on molecular markers and contrasting hypotheses about morphological characters. This preliminary analysis will hopefully provide hints towards the refinement of the systematics of the group and assist in

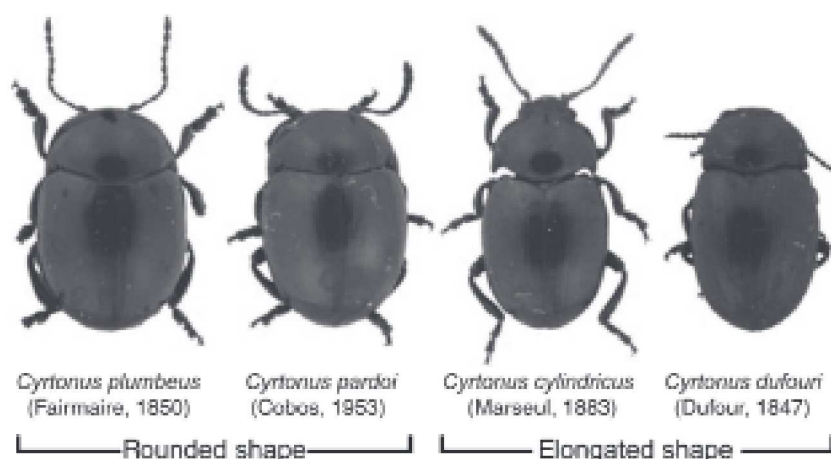


Fig. 1. Examples of habitus shapes in *Cyrtonus*, distinguishing between rounded and elongated profiles

generating hypotheses to test in future studies about speciation in the genus. In particular, it was intended to (1) confirm analytically the existence of a differential shape trait in *Cyrtonus* separating rounded and elongated forms; (2) use molecular markers to reconstruct a phylogenetic hypothesis for the genus for which no previous hypothesis exists [showing its monophyly using a mitochondrial DNA (mtDNA) marker]; (3) add a temporal scale to the evolutionary history of *Cyrtonus*; and (4) analyse in the light of this phylogeny the evolution of shape in *Cyrtonus* and comment on its adaptive and/or systematic value.

Materials and Methods

Sampling

Most species of *Cyrtonus* are very rare, difficult to find in part because of their nocturnal and cryptic habits and in several cases only known from the typical series used for their description. For this study, the authors managed to analyse 32 specimens of 18 species of 42 valid specific taxa, including representatives of the various chromosomal and morphological groups previously identified, which should allow a good approximation to the phylogeny of the genus (Table 1). The polarity of phylogenies was established with the outgroup method using other chrysomelinae genera for which homologous sequences for the four markers were available: *Timarcha balearica* Gory (*Timarchini*), from the University Campus (Mallorca, Spain), and *Calligrapha californica coreopsivora* Brown (*Chrysomelini*), from Almonte (Lanark Co., ON, Canada).

Morphometric analyses

A first step in the analysis of the phylogenetic significance of habitus shape in *Cyrtonus* consisted in confirming analytically the existence of two well-differentiated shapes of suspected systematic relevance. Two homologous simple linear measures, total length (l) along longitudinal axis from apex of pronotum to apex of elytra and width (w) between posterior angles of pronotum, were measured for 348 specimens of both sexes for all species included in the phylogenetic analyses, except *Cyrtonus gibbicollis* (Table 2). Measures were taken using a scaled micrometer in binocular scopes, Olympus VMZ (Olympus Optical España, Barcelona) and a Zeiss Stemi 2000-C (Carl Zeiss S.A., Madrid) on dry-mounted specimens glued on cards and placed perpendicularly to the objective. Subjectivity of measures (e.g. relative position of insect and observer) was compensated by two observers and, when possible, on several specimens, and using averages.

The variable w is used to measure how narrow or wide are the beetles with independence of beetle size (l). To eliminate the size-dependent proportion of w , the correlation between these variables was estimated by linear regression and squared Pearson's r and its significance with Statistica 99 (StatSoft Inc. 1995). A positive correlation between both variables means that w is in effect directly related

to size, and further tests were computed with the corresponding residual values (w_2), independent of beetle length. Analyses were carried out using decimal and log-transformed measures and samples were divided into two categories for each sex, elongated and rounded, and statistical differences in w_2 between different classes were estimated with a t -test using Statistica 99 (StatSoft Inc. 1995).

A discriminant analysis was run in Statistica 99 (StatSoft Inc. 1995) to classify ambiguous cases of group ascription. In this case, the shape was used as grouping variable (two groups: rounded and elongated), and l and w were implemented as independent variables and introduced simultaneously in the model. Grouping of ambiguous cases was predicted according to the model and from posterior probabilities. Discriminant analyses were also used to classify predicted ancestral shapes (see the last section of methods).

Genetic markers

Genomic DNA was isolated from abdominal soft tissue (excluding the digestive tract) using a standard phenol:chloroform and ethanol precipitation protocol (Juan et al. 1993). DNA was used as PCR template to amplify four genetic markers, including mitochondrial and nuclear genes. These were (1) partial mitochondrial sequences of the large rRNA subunit (*rrnl*), (2) a mtDNA fragment spanning partial cytochrome oxidase subunits 1 (*cox1*) and 2 (*cox2*) and the intervening tRNA for Leucine (*tRNA^{Leu}*), (3) complete sequence of the second internal transcribed spacer (*its2*) of the nuclear ribosomal gene cluster and (4) a partial sequence of the large rRNA subunit (LSU) of the nuclear ribosomal genes. Primer combinations for each marker are indicated in Table 3. PCR conditions used denaturation at 94°C for 4 min and 35 cycles of 30 s at 94°C, 1 min at 50°C and 2 min at 72°C, with a final extra elongation step of 10 min at 72°C. PCR products were purified using either GeneClean III kit (Bio 101, La Jolla, CA, USA) or QIAquick Gel Extraction kit (QIAGEN, Crawley, West Sussex, UK) and cycle sequenced with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). Sequences were obtained with the genetic analyser ABI PRISM 310 (Applied Biosystems). Basic sequence statistics were calculated with Megn 3.1 (Kumar et al. 2004). Sequences have been deposited at EMBL Nucleotide Sequence Database under accession nos AM403334–AM403459.

Phylogenetic analyses

The monophyly of *Cyrtonus* was tested using partial *rrnl* sequences and 263 homologous sequences available in GenBank for 14 genera of Chrysomelinae (see Appendix S1). Sequences were aligned with a fast phenetic Clustal 1.8 algorithm (Thompson et al. 1994) and analysed with parsimony in PAUP 4.0b10 (Swofford 2003) using a neighbour-joining tree to guide sequence addition. The number of trees was very high and the tree searches were aborted when 50 000 most parsimonious trees were found. Node support was assessed by 100 pseudo-replicates of bootstrap analysis (Felsenstein 1985), each with 5 random sequence addition replicates and the number of TBR tree rearrangements limited to 10 000 000.

Table 1. Species and specimens of *Cyrtonus* studied, their source and morphological and chromosomal characteristics

<i>Cyrtonus</i> species	Code	Source	Shape ¹	Chromosome no. ²
<i>C. arcasi</i> (Fairmaire, 1884)	1	Albacete: Nerpio, Calar de la Osera (SP)	R	40
	2	Granada: Puerto del Pinar (SP)	R	40
	3	Granada: Sierra de Guillimona (SP)	R	40
	4	Granada: Sierra de Guillimona (SP)	R	40
<i>C. contractus</i> (Fairmaire, 1882)		Granada: Sierra Nevada, Trevenque (SP)	R	28
<i>C. cupreiventris</i> (Pérez Arenas, 1872)	1	Zaragoza: Moncayo (SP)	E	28
	2	Zaragoza: Moncayo (SP)	E	28
<i>C. cylindricus</i> (Marseul, 1883)		Granada: Sierra de Guillimona (SP)	E	28
<i>C. dufouri</i> (Dufour, 1847)		Aquitaine: Le Bastit, 46-LOT (FR)	E	28
<i>C. ehlersi</i> (Fairmaire, 1884)		Murcia: Sierra Espuña 1200 m (SP)	R	-
<i>C. elegans</i> (Germar, 1813)		Faro: Cabo São Vicente (PO)	R	28
<i>C. sumolpus</i> (Fairmaire, 1850)		Sierra de Javalambre, Ternel (SP)	R	-
<i>C. fairmairei</i> (Rosenhauer, 1856)	1	Málaga: Puerto de los Pílonos (SP)	R	40
	2	Málaga: Puerto de los Pílonos (SP)	R	40
	3	Málaga: Puerto de los Pílonos (SP)	R	40
	4	Málaga: Puerto de los Pílonos (SP)	R	40
<i>C. gibbicollis</i> (Fairmaire, 1866)	1	Western Rif: Angera, Tleta-Thagramd (MO)	E	-
	2	Western Rif: Angera, Tleta-Thagramd (MO)	E	-
<i>C. majoricensis</i> (Breit, 1908)		Mallorca: Es Teix (SP)	R ³	28
<i>C. parisi</i> (Cobos, 1953)	1	Albacete: Calar de la Sima (SP)	R	46
	2	Albacete: Nerpio, Loma de las Yeguas (SP)	R	46
	3	Albacete: Sierra de Villafuerte (SP)	R	46
	4	Albacete: Sierra de Villafuerte (SP)	R	46
<i>C. plumbus</i> (Fairmaire, 1850)	1	Almería: Nacimiento (SP)	R	28
	2	Alicante: Alcoi, Pto. de Benifallim (SP)	R	28
<i>C. puncticeps</i> (Fairmaire, 1882)		Teruel: Frías de Albarracín (SP)	R	40
<i>C. rotundatus</i> (Herrich-Schaeffer, 1838)	1	Alicante: Sierra Aitana, Pto. de Tudons (SP)	R	28
	2	Provence: Ile Ratonneau (FR)	R	28
<i>C. ruficornis</i> (Fairmaire, 1850)		Soria: Puerto de Oncala (SP)	E	28
<i>C. strictus</i> (Fairmaire, 1883)		Ciudad Real: Cabaneros, Alcobá de los Montes (SP)	E	-
<i>Cyrtonus</i> sp. ⁴	1	Málaga: Sierra de Almijara, Pto. del Collado (SP)	R	28
	2	Málaga: Sierra de Almijara, Pto. del Collado (SP)	R	28

FR, France; MO, Morocco; PO, Portugal; SP, Spain.

¹Shape as indicated in Cobos (1954), distinguishing between elongated (E) and rounded (R) species.

²Chromosome numbers taken from the literature (Petitpierre 1978, 1984; Petitpierre et al. 1993; Petitpierre and Garmerin 2005).

³*Cyrtonus majoricensis* was included among rounded species by Cobos (1954), but the actual shape as confirmed in this study is closer to elongated.

⁴This species is currently under formal description by Jose M. Vela and Gloria Bastazo (Málaga, Spain) and will be named *Cyrtonus cobosi* (pers. comm.).

All markers tested for the ingroup phylogeny presented length variation, which complicated the recognition of homologies in the areas affected by length polymorphisms. Primary homologies were therefore established on the basis of a cladistic approach, direct optimization, which resolves ambiguous alignments maximizing global character congruence (Wheeler 1996). Direct optimization as implemented in *roy* 3.0.11 (Wheeler et al. 2002) produces in a single step the most parsimonious tree(s) and the alignment(s) consistent with the tree topologies (implied alignment; Wheeler 2003). The most parsimonious trees and associated alignments were obtained for two data sets: mitochondrial (including partial sequences of *cox1*, *trnLeu*, *cox2* and *rnl*) and nuclear (including partial sequences of *LSU* and *its2*). Each data set was partitioned prior to analyses in a series of homologous fragments identified as an alternating pattern of conserved and length-variable segments from a preliminary alignment using *Clustal* 1.8 (Thompson et al. 1994). The *trnLeu* and *rnl* were partitioned into 3 fragments each, *LSU* into 7 and *its2* into 18 fragments.

Direct optimization analyses used a step-matrix assigning equal costs to each substitution and indel, and consisted in 10 tree building replicates with 10 random sequence addition replicates each, followed by tree rearrangements under the tree bisection-reconnection (TBR) and subtree pruning-regrafting (SPR) algorithms. The trees obtained were submitted to tree fusion (Goloboff 1999) with further TBR and SPR exploration of tree space. The set of most parsimonious trees obtained in these analyses were used as starting trees in a step of tree ratcheting (Nixon 1999) for further

refinement in finding optimal solutions in tree and alignment reconstruction. The specific analytical conditions are indicated in Appendix S1.

The recovered implied alignments were used as homology hypotheses for other analyses, including parsimony and maximum likelihood (ML) tree reconstructions in *PAUP* 4.0b10 (Swofford 2003), bootstrap analysis for assessing node confidence (Felsenstein 1985), and congruence analysis between mitochondrial and nuclear data sets. Parsimony heuristic searches consisted in 100 replicates of random addition of taxa, with TBR branch swapping and saving multiple trees; gaps were specified as a fifth character state. The ML tree reconstruction method required the previous specification of the substitution model best fitting the variability observed in the data. This was accomplished using *ModelTest* 3.7 (Posada and Crandall 1998) to evaluate the fit of various models to the data as ran in *PAUP*, implementing two selection criteria: a hierarchical likelihood ratio test (LRT) and the Akaike information criterion. The resulting model was implemented in a ML heuristic search in *PAUP* using a neighbour-joining tree as starting topology for the analyses. Bootstrap and congruence analyses were run in *PAUP*. Parsimony bootstrap consisted in 1000 data pseudoreplicates and was obtained both including gaps as a fifth character state and removing all gapped positions, whereas likelihood bootstrap was based on 100 data pseudoreplicates. The congruence analysis used the partition homogeneity test with 1000 pseudoreplicates, each with 50 starting points using random addition of sequences, removing all invariable sites (Cunningham 1997) and saving a single tree per pseudoreplicate.

Table 2. Samples used for statistical analysis of beetle shape (n , no. of specimens; l , beetle length; w , width between posterior angles of pronotum; SD, standard deviation)

Cyrtonus species	Males				Females			
	n	$l \pm SD$	$w \pm SD$	$l/w \pm SD$	n	$l \pm SD$	$w \pm SD$	$l/w \pm SD$
Elongated shape								
<i>C. cupreovirens</i>	14	6.37 \pm 0.574	3.34 \pm 0.227	1.90 \pm 0.090	16	7.09 \pm 0.429	3.58 \pm 0.208	1.98 \pm 0.088
<i>C. cylindricus</i>	10	5.88 \pm 0.385	2.97 \pm 0.157	1.98 \pm 0.157	18	6.71 \pm 0.215	3.25 \pm 0.157	2.07 \pm 0.123
<i>C. dufouri</i>	11	5.63 \pm 0.354	2.96 \pm 0.156	1.91 \pm 0.121	5	6.84 \pm 0.288	3.38 \pm 0.239	2.03 \pm 0.065
<i>C. ruficornis</i>	6	5.65 \pm 0.176	2.93 \pm 0.052	1.93 \pm 0.062	2	6.20 \pm 0.141	3.20 \pm 0.141	1.94 \pm 0.041
<i>C. strictus</i>	4	6.08 \pm 0.230	2.80 \pm 0.141	2.17 \pm 0.386	2	6.13 \pm 0.295	2.85 \pm 0.141	2.15 \pm 0.210
Rounded shape								
<i>C. arcus</i>	15	6.35 \pm 0.382	3.70 \pm 0.277	1.72 \pm 0.058	15	7.22 \pm 0.375	4.05 \pm 0.370	1.79 \pm 0.114
<i>C. contractus</i>	2	6.05 \pm 0.071	3.35 \pm 0.071	1.81 \pm 0.017	—	—	—	—
<i>C. ehlersi</i>	3	6.17 \pm 0.321	3.63 \pm 0.231	1.70 \pm 0.113	—	—	—	—
<i>C. elegans</i>	21	7.89 \pm 0.282	4.68 \pm 0.217	1.69 \pm 0.058	15	8.57 \pm 0.274	5.02 \pm 0.170	1.71 \pm 0.075
<i>C. eumolpus</i>	3	6.37 \pm 0.231	3.50 \pm 0.265	1.82 \pm 0.076	1	6.60	3.70	1.78
<i>C. fairmairei</i>	12	6.95 \pm 0.505	4.00 \pm 0.215	1.74 \pm 0.102	24	8.00 \pm 0.379	4.40 \pm 0.308	1.82 \pm 0.085
<i>C. majoricensis</i> ¹	19	5.47 \pm 0.277	2.74 \pm 0.130	2.00 \pm 0.094	18	6.11 \pm 0.175	3.07 \pm 0.091	1.99 \pm 0.055
<i>C. pardoi</i>	25	6.42 \pm 0.315	3.72 \pm 0.239	1.73 \pm 0.085	27	7.59 \pm 0.443	4.15 \pm 0.250	1.83 \pm 0.081
<i>C. plumbeus</i>	16	7.71 \pm 0.305	4.18 \pm 0.189	1.84 \pm 0.093	19	8.57 \pm 0.419	4.74 \pm 0.215	1.81 \pm 0.066
<i>C. puncticeps</i>	11	5.43 \pm 0.237	3.14 \pm 0.136	1.73 \pm 0.051	1	5.80	3.20	1.81
<i>C. rotundatus</i>	7	6.24 \pm 0.231	3.58 \pm 0.191	1.75 \pm 0.114	4	7.28 \pm 0.222	3.90 \pm 0.141	1.87 \pm 0.083
<i>Cyrtonus</i> sp.	2	5.75 \pm 0.354	3.40 \pm 0.141	1.69 \pm 0.034	—	—	—	—

¹This species was included in the 'rounded' group of *Cyrtonus* by Cubos (1954). However, it is conspicuously elongated in shape. We treat the two alternative placements in the analyses.

Table 3. Markers and PCR primer combinations used in this study

Marker	Primer name	Primer sequence	Reference
Rml	LR-N-13398	5'-CGC CTG TTT ATC AAA AAC AT-3'	Simon et al. (1994)
	LR-J-12887	5'-CTC CGG TTT GAA CTC AGA TCA-3'	Simon et al. (1994)
Cox1-trnaLeu-cox2	C1-J-2792a	5'-ATA CCT CGA CGT TAT TCA GA-3'	Bogdanowicz et al. (1993)
	C2-N-3661mod	5'-GCT CCA CAA ATT TCT GAG CA-3'	Emerson and Wallis (1995)
its2	ITS3	5'-GCA TCG ATG AAG AAC GCA GC-3'	White et al. (1990)
	ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'	White et al. (1990)
LSU	28sMM	5'-GAA GTT ACG GAT CTA RTT TG-3'	Hillis and Dixon (1991)
	28sEE	5'-CCG CTA AGG AGT GTG TAA-3'	Hillis and Dixon (1991)

The molecular clock hypothesis on the mtDNA data was also tested using an LRT, enforcing and without enforcing equal rates of evolution throughout lineages in the ML analyses in PAUP under appropriate evolutionary models (as selected with ModelTest). A linearized tree (see results, below) was used to calibrate the molecular clock using a putative vicariant event separating *C. gibbicollis* from the Rif and Tanger in Morocco from their sibling Iberian species in SE Spain, using the opening of the Gibraltar strait in the Miocene (5.3 Mya; e.g. Maldonado 1985) as biogeographical event leading to the vicariance. This calibration was compared with a standard insect mitochondrial clock, 0.0115 substitutions/site/lineage (Brower 1994). A 94% confidence threshold for the latter age estimates was obtained by bootstrapping 100 times the data with SeqBoot (Phylip package version 3.65; Felsenstein 2004) and recalculating branch lengths and node depths on the fixed clock-constrained ML topology in PAUP (Baldwin and Sanderson 1998).

Tests of monophyly were implemented in the context of ML searches using LRT to compare the null unconstrained hypothesis with an alternative hypothesis constraining a node of interest.

Trait evolution analyses

Comparative morphometric data analysis across species in the phylogeny was carried out based on the generalized least squares model described by Pagel (1997, 1999) and implemented in his software Continuous (version 1.0d13). Only averages of male measures for each species were used in the tests to minimize the amount of missing data,

and two parallel sets of analyses were run, one for l and w simultaneously, and one for the ratio of these variables (l/w). LRT were used in each case to select among competing models and parameters explaining the evolution of the corresponding traits. Three relevant tree scaling parameters informing about trait evolution were estimated via likelihood: (1) κ , which tests the tempo, (2) λ , which tests the mode and (3) δ , which tests for phylogenetic associations of trait evolution. Additionally, these parameters were implemented to compare two competing models of evolution (A and B, respectively, in Continuous): (1) random, describing the evolution of traits without trends in change, and (2) directional, accounting for traits with a prevailing direction of change. In the case of the simultaneous analysis of l and w , evidence of trait covariation on the phylogeny was tested. This was carried out by calculating the evolutionary regression coefficient and its significance (Pagel 1993, 1997). Finally, Continuous was used to infer the ancestral trait values for interior nodes in the phylogeny (parameter α , Continuous manual). These ancestral states were used to deduce the shape of the ancestral species and their class assignment was deduced using discriminant analysis as described earlier. The tree topology and associated branch lengths used for all the analyses were obtained using ML in PAUP from a reduced matrix of mtDNA sequences including a single representative of each species, and a heuristic search with 10 replicates of random addition of taxa with TBR, implementing the evolutionary model selected by ModelTest.

A complementary approach to the tests in Continuous, was carried out using the module CoMET (Lee et al. 2005) of Mesquite 1.06 (Maddison and Maddison 2005). CoMET determines the best

fitting evolutionary model for a set of observed continuous trait data, selecting based on ML among nine different evolutionary models. These models result from the combination of three model types with three model classes (Oakley et al. 2005). The model types change the relation of branch lengths with phenotype divergence: 'distance model', with branch lengths proportional to phenotype change, 'equal model', with branch lengths constant representing a constant change in phenotype between nodes, and 'free model', with branch lengths to be estimated via a likelihood function. The model classes modify the mode of phenotype change: 'pure phylogenetic', where changes occur in every branch of the phylogeny, 'non-phylogenetic', where changes do not occur in internal branches, and 'punctuated', where changes affect only one daughter lineage. The tree used to implement CoMET searches is the same ML tree as for Continuous analyses.

An attempt to reconstruct ancestral states for continuous characters was carried out with Mesquite 1.06 (Maddison and Maddison 2005) using the linear cost assumption, in which the parsimony optimization is carried out, assuming that the cost of change from state x to y is $(x-y)$.

Results

Morphological character correlations

A regression analysis of width (w) against length (l) across species in *Cyrtomus* produced always highly significant ($p < 0.000001$) regression coefficients for all data, all males, all females, rounded and elongated males and females, and treating *Cyrtomus majoricensis* either as a rounded or an elongated species. Classifying *C. majoricensis* as elongated generally improved the fit of elongated forms ($r^2 = 0.482$ to 0.568 in males, and 0.491 to 0.620 in females), slightly reducing that of rounded forms ($r^2 = 0.848$ to 0.835 in males, and 0.880 to 0.761 in females).

Testing differences in the mean values of width residuals (w_r) with t -tests proved highly significant in all contrasts of males versus females, males versus females of each morphological group, of morphological groups for each gender and for all samples (Table 4; Fig. 2). The inclusion of *C. majoricensis* within elongate-shaped species increased the t -value of the respective tests, suggesting a trend towards increasing the significance (Table 4). Moreover, w_r showed no significant associations with beetle size (neither in decimal or logarithmic representation), suggesting no allometric effects over shape.

The discriminant analysis of l and w for rounded and elongated forms (excluding *C. majoricensis* samples) correctly predicted 91.96% of observations for model estimation [Wilks' lambda = 0.45761, approx. $F(2,308) = 182.53$; $p < 0.000001$]. As expected from the results of previous

analyses, all *C. majoricensis* samples were recognized in the discriminant analysis as belonging to the group of elongated forms, with posterior probabilities in the range $0.6604412 < p < 0.995159$.

Phylogenetic trees

The parsimony analysis of partial chrysome line rml sequences (541 characters for analysis; 214 parsimony informative) produced a very high number of equally parsimonious trees of tree length 2211 (CI = 0.249; RI = 0.865). The strict consensus rooted in *Timarcha* showed a monophyletic *Cyrtomus* within a clade including *Ambrostoma*, *Chrysolina* and *Oreina*, as expected from morphology; bootstrap support for the clade was moderately high, 78% (not shown). Following results are based on the choice of two divergent outgroups, *Timarcha* and *Calligrapha*, for which homologous sequences are available for all markers, and provide a clear separation with *Cyrtomus* without affecting in-group estimates of divergence.

Summary statistics of the sequences included in this study are reported in Table 5. These values are within the expectations derived for other insects, including a high $A + T$ bias for the mitochondrial genes (over 70%) or balanced nucleotide compositions for the nuclear ribosomal genes. The most variable and informative marker is *cox1-trnA-Leu-cox2* and the most conserved is LSU. In the case of nuclear ribosomal genes, sites affected by length variation are very informative.

The parsimony analysis of mtDNA markers combined resulted in one island of 24 trees (1494 steps, CI = 0.507, RI = 0.716). The consensus tree is shown in Fig. 3a. The mtDNA markers produced a generally well-resolved and supported phylogeny, except for a polytomy from which four multi-species clades stem. A parsimony analysis of the nuclear markers combined found a single island of 24 trees (535 steps, CI = 0.873, RI = 0.938; Fig. 3b). The nuclear parsimony tree was well resolved but the support was generally low. An incongruence length difference test for congruence between mitochondrial and nuclear markers was highly significant ($p = 0.001$ with gaps as a fifth character state; $p = 0.008$ with gapped positions excluded), hence the authors opted for not combining them in a simultaneous analysis. Topological incongruence between hypotheses included the position of (1) *Cyrtomus puncticeps*, which was basal to a clade containing *Cyrtomus arcasi*, *Cyrtomus contractus*, *Cyrtomus fairmairei* and *Cyrtomus pardoi* in the mtDNA tree and to the remaining

Table 4. Results of t -tests for differences in width residuals (w_r) for different groupings of *Cyrtomus* (r , rounded; e , elongated)

Group 1	Group 2	Mean w_{r1}	Mean w_{r2}	t -value	df	p -value
Males $r + e$	Females $r + e$	0.0909	-0.0985	6.6702	346	< 0.000001
Males r^1	Females r^1	0.1622	-0.0131	6.0526	258	< 0.000001
Males r^2	Females r^2	0.2123	0.0211	6.5290	221	< 0.000001
Males e^1	Females e^1	-0.1245	-0.3450	4.8446	86	0.000006
Males e^2	Females e^2	-0.1309	-0.3064	5.0273	123	0.000002
Males r^1	Males e^1	0.1622	-0.1245	7.0029	179	< 0.000001
Males r^2	Males e^2	0.2123	-0.1309	10.4111	179	< 0.000001
Females r^1	Females e^1	-0.0131	-0.3450	8.6200	165	< 0.000001
Females r^2	Females e^2	0.0211	-0.3064	9.7755	165	< 0.000001
r^1	e^1	0.0786	-0.2322	10.2266	346	< 0.000001
r^2	e^2	0.1214	-0.2166	13.1839	346	< 0.000001

¹*C. majoricensis* considered a rounded species.

²*C. majoricensis* considered an elongated species.

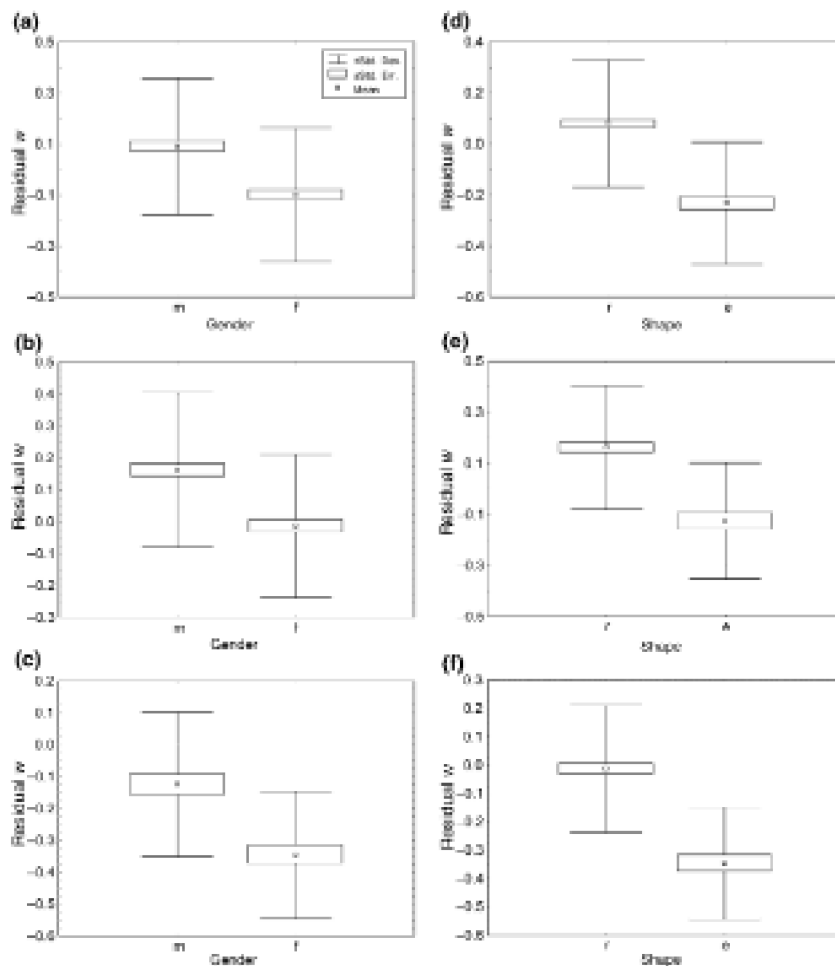


Fig. 2. Box and whisker plots contrasting width residual (w) values in *Cyrtomus* in males versus females (a), rounded males versus females (b), elongated males versus females (c), rounded versus elongated species (d), rounded versus elongated males (e) and rounded versus elongated females (f). All comparisons show highly significant differences. Legend – m: males, f: females, r: rounded shape, e: elongated shape

Table 5. Sequence summary statistics (ti, transitions; tv, transversions)

Marker	Length	Aligned length	Informative sites ¹	A	C	G	ti/tv	Overall divergence ²	Intraspecific range ²	Interspecific range ²
rnl	509–510	514	91 (86)	39.6	15.5	9.0	0.7	0.065 ± 0.006	0–0.026	0.007–0.103
cox1-cox2	800–803	806	269 (261)	35.1	16.6	11.7	1.3	0.130 ± 0.006	0.005–0.035	0.009–0.182
its2	427–499	626	188 (44)	24.4	23.0	24.7	1.4	0.048 ± 0.006	0–0.006	0–0.089
LSU	708–721	750	36 (6)	23.4	23.6	31.7	0.4	0.003 ± 0.001	0–0	0–0.014

¹Values are given considering gaps a fifth character state and excluding gaps (within brackets).

²Values for uncorrected p-distances.

species in the nuclear one, of (2) *Cyrtomus elegans* sister to *Cyrtomus* sp. and *Cyrtomus eumolpus* sister to *Cyrtomus plumbeus* in the mtDNA tree, and the reverse relationships in the nuclear one and of (3) *C. majoricensis* sister to *Cyrtomus ehlersi* and *C. rotundatus* in the mtDNA tree and to the elongated *Cyrtomus* in the nuclear one (Fig. 3).

The evolutionary model best fitting mtDNA data was a GTR [$f(A) = 0.40$, $f(C) = 0.14$, $f(G) = 0.08$; $f(A-C) = 2.02$, $f(A-G) = 12.66$, $f(A-T) = 2.46$, $f(C-G) = 3.43$, $f(C-T) = 18.04$], with a proportion of invariant sites ($I = 0.45$) and variable sites with rate heterogeneity (shape of Γ distribution: $\alpha = 0.67$). A single ML tree of $-\ln$ likelihood = 8394.5192 was recovered, topologically compatible with the parsimony consensus tree (Fig. 4a). A GTR + I + Γ model also fitted the nuclear data (as selected based on the AIC criterion) [$f(A) = 0.24$, $f(C) = 0.23$, $f(G) = 0.28$; $f(A-C) = 1.86$, $f(A-G) = 3.80$, $f(A-T) = 3.73$, $f(C-G) = 0.86$, $f(C-T) = 6.20$; $I = 0.72$; $\alpha = 0.65$]. Four ML trees of $-\ln$ likelihood = 3093.3537 were

recovered, differing in the position of *C. gibbicollis* 2 related to other members in its clade. The ML tree topology differed from the parsimony consensus for several taxa, but the only difference taking into account the supported clades was *C. puncticeps* basal to the *C. arcasi*, *C. contractus*, *C. fairmairei* and *C. pardoi* clade in the ML tree and to the other species in the parsimony consensus (Fig. 4b).

In the mitochondrial trees, the species of elongated shape appeared in a clade, whereas in the nuclear genes, the elongated species were paraphyletic with *C. majoricensis* (identified as rounded by Cobos 1954) in the parsimony tree, and monophyletic (without support) and also closely related to *C. majoricensis* in the ML reconstruction. Interestingly, in spite of its inclusion in the broad-shape group by Cobos (1954), *C. majoricensis* is rather conspicuously elongated (as confirmed statistically, see above section) and, in fact, constraining this species to be monophyletic with the other elongated species produced non-significant LRT results for mtDNA

Fig. 3. Parsimony trees based on *rnl* + *cox1-trnaLeu-cox2* (a) and *its2* + LSU (b). Numbers above branches are bootstrap values higher than 50% for the data sets including gaps as a fifth character state, and numbers below branches (in brackets) are the corresponding bootstrap values excluding gapped sites. Thicker branches in the phylogeny correspond to species of elongated shape

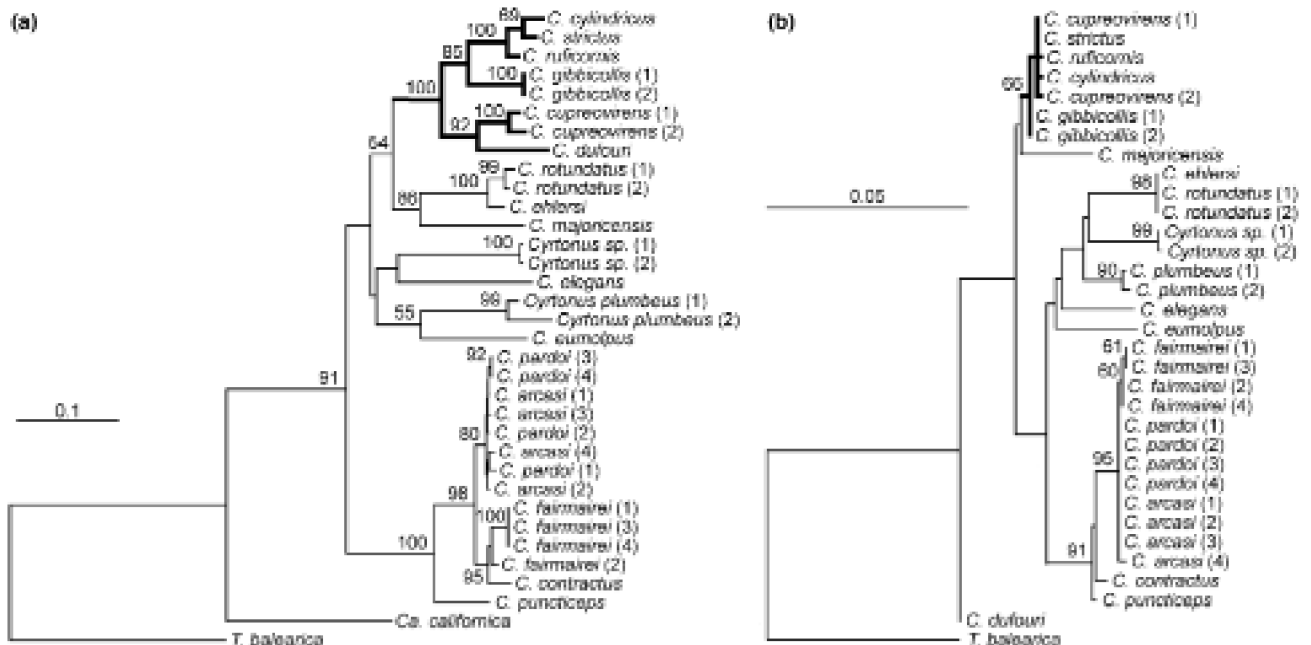
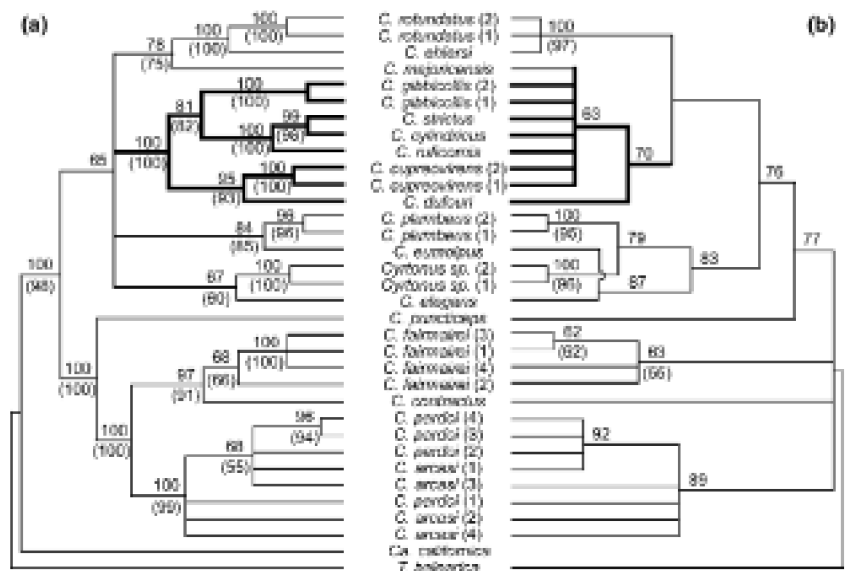


Fig. 4. Maximum likelihood trees based on mtDNA (a) and nuclear ribosomal (b) data. Numbers above branches are bootstrap supports higher than 50%. Thicker branches in the phylogeny correspond to species of elongated shape

$[-\ln \text{ likelihood} = 8400.51272; \delta(32 \text{ df}) = 11.98704; p = 0.9994]$ and nuclear $[-\ln \text{ likelihood} = 3093.3723; \delta(31 \text{ df}) = 0.03718; p = 0.9999]$ markers.

Molecular clock

The ML analysis of mtDNA data constraining a regular rate of evolution produced trees with $-\ln \text{ likelihood} = 8412.4829$ (six trees only differing in the relative branching of *C. arcasi* and *C. pardoi* sequences, the rest, topology and branch lengths, identical). The difference with the unconstrained analysis was non-significant according to an LRT ($df = 32; p = 0.29$). One clock-constrained tree is shown in Fig. 5. Two different calibrations, one based on a biogeographical event and one based on an average estimate of the evolutionary rate of insect

mitochondrial genomes were calculated, showing a remarkable concordance, which deviates slightly only towards the root of the tree (Fig. 5). The *Cyrtomus* clade appear to have an age around 12–17 Mya old (Middle Miocene), with a phase of early diversification between 7 and 15 Mya. Several speciation events, including *Cyrtomus cylindricus*-*Cyrtomus ruficornis*-*Cyrtomus strictus*, *C. rotundatus*-*C. ehlersi*, *C. arcasi*-*C. pardoi* and *C. contractus*-*C. fairmairei*, were very recent, in the last 2 Mya (Pleistocene).

Trait evolution

The kind of comparative results described here are entirely dependent on the underlying phylogenetic hypothesis, hence

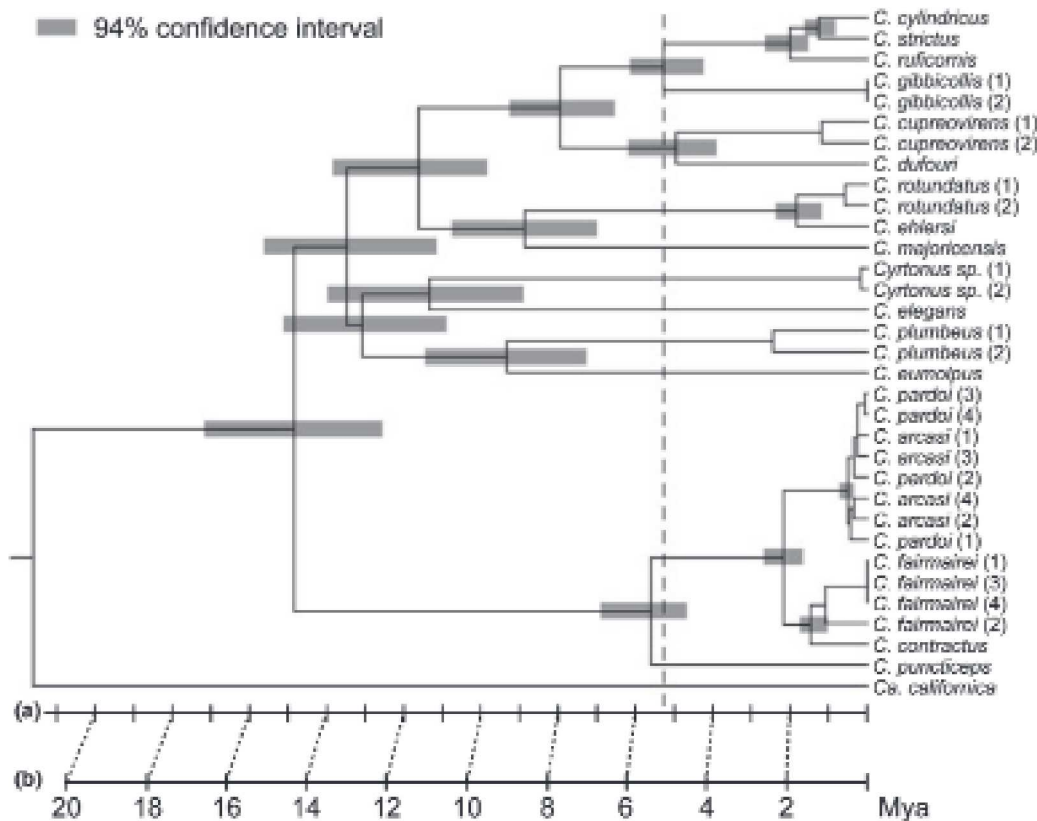


Fig. 5. Clock constrained maximum likelihood tree based on mtDNA for *Cyrtomus*. The tree is calibrated using a biogeographical event 5.3 Mya (dashed vertical line) resulting in the alleged vicariance of North African *Cyrtomus gibbicollis* and southern Iberian *Cyrtomus* relatives (a), and adjusting the standard mtDNA average clock of 2.3% substitutions/Mya (b). Grey bars are the 94% confidence intervals for the age estimates of branching events relating two or more species

the reader is cautioned about their interpretation (Pagel 1997). The ML tree topology for the set of comparative analysis was obtained implementing a GTR + I + Γ [$f(A) = 0.39$, $f(C) = 0.14$, $f(G) = 0.09$; $f(A-C) = 3.34$, $f(A-G) = 16.85$, $f(A-T) = 3.96$, $f(C-G) = 4.26$, $f(C-T) = 29.17$; $I = 0.52$; $\alpha = 0.83$] (Fig. 6a).

Regarding tree-scaling parameters for (l, w) and both random and directional models of trait evolution, κ was significantly different from 0 ($p < 0.01$) and not significantly different from 1.0, δ was not significantly different from 1.0 and λ was significantly different from 0 ($p < 0.01$) and not significantly different from 1. The same scaling parameters for l/w resulted: κ not significantly different from 0 and marginally significantly different from 1.0 ($p < 0.10$), δ significantly different from 1.0 ($p < 0.01$), and λ significantly different from 0 ($p < 0.01$) and not significantly different from 1.0.

The random model was not significantly different from the directional model for (l, w) ($p = 0.09$, using in both models the parameters estimated for the random model; $p = 0.18$, using the respective estimated parameters for each model). The null random model was rejected against the directional model for l/w ($p = 0.01$, using the parameter values of the random model; $p = 0.01$, using the optimal parameter values for each model). The ML estimates of the parameters for each set of data under the preferred trait evolutionary model are shown in Table 6.

The morphological traits l and w appeared correlated (0.91306) and covarying on the phylogeny (evolutionary

regression coefficient = 0.60178, $p < 0.00001$). Fig. 6b shows this correlation with the evolutionary tree plotted on the morphospace defined by these variables. The clade of elongated species plus *C. majoricensis* appeared clustering in a particular region of the morphospace.

Although the generalized least squares model used for the above analyses does not rely on estimated trait values for internal nodes (Pagel 1997), these ancestral states can be reconstructed. Table 7 shows the ancestral reconstructions of l and w for selected nodes in the phylogeny (Fig. 6a), as well as their predicted shape class using discriminant analysis. The graphical representation of ancestral nodes in morphospace is shown in Fig. 6c.

The comparison of models of continuous evolution with CoMET applied to each morphological variable and the l/w ratio identified as the best model fitting the data a free model of punctuated average class in every case.

Discussion

Beetle shape and systematics

Here, for the first time, analytical evidence is presented for the existence of two morphologically distinguishable groups in *Cyrtomus*, differing by their relative widths irrespective of total beetle length – a group of rounded and a group of elongated species. This characteristic can be simplified as the ratio of beetle length and width and constitutes a non-overlapping trait

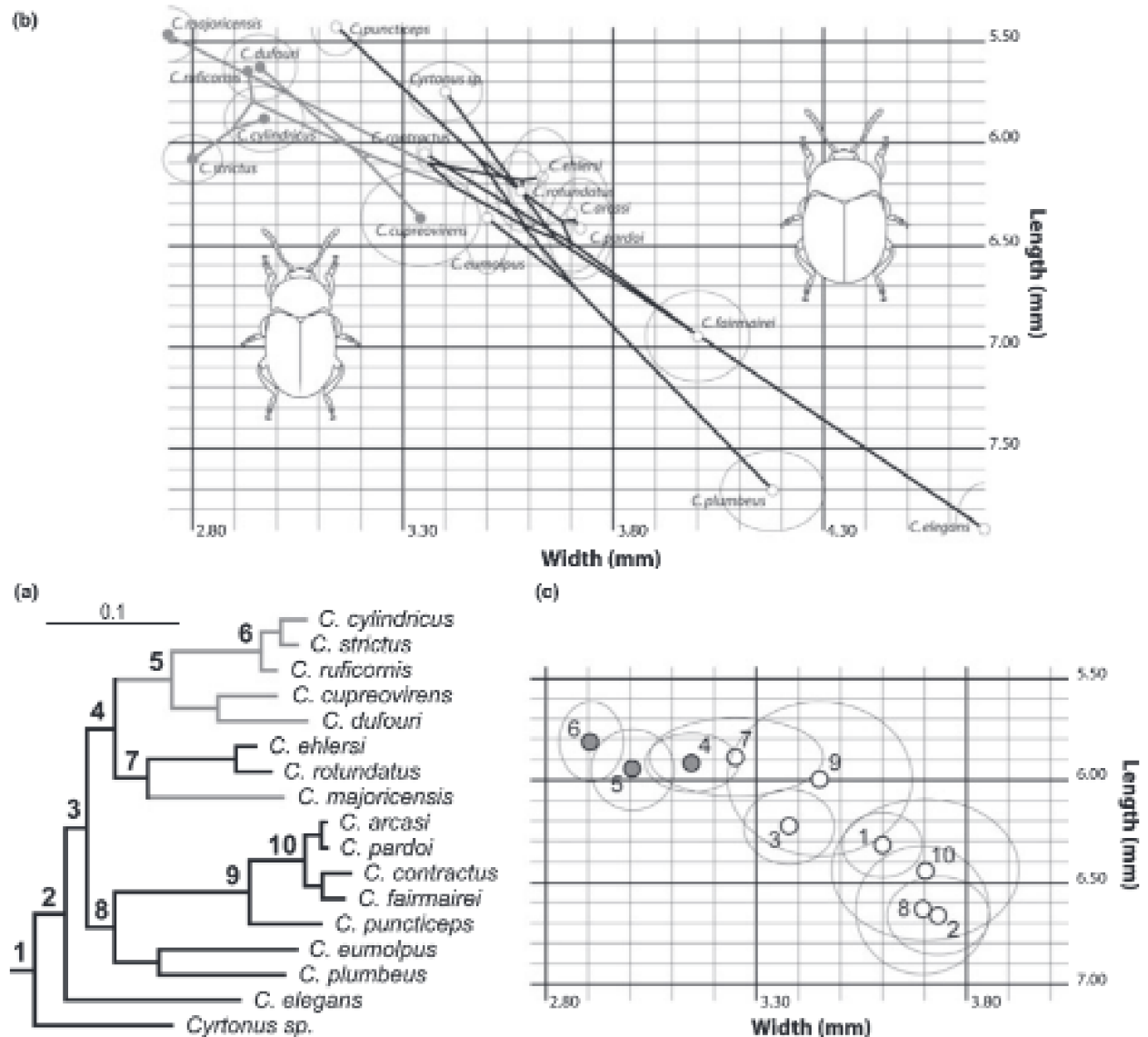


Fig. 6. (a) Maximum likelihood mtDNA phylogeny of *Cyrtomus* species with available morphometric data; elongate-shaped lineages are identified with grey-shaded branches. (b) Plot of the phylogeny on the morphospace defined by the variables length and width (standard deviations shown as an ellipse around the species average values); elongated species, with a common phylogenetic origin, appear clearly separated of rounded species using these two traits. (c) Parsimony reconstruction using the linear cost assumption of ancestral trait values of internal nodes identified on the phylogeny (see methods for details)

Table 6. Maximum likelihood estimated scaling parameter values for trait evolution in *Cyrtomus* (*l*, length; *w*, width)

	κ (tempo)	δ (phylogenetic inertia)	λ (mode)
$(l, w)^1$	0.86907	1.60653	0.91290
l/w^2	0.31603	3.32562	0.79379

¹Random evolutionary model.

²Directional evolutionary model.

with two categories, with their threshold around 1.87 for males and 1.90 for females. Interestingly, these characteristic shapes mapped onto independent molecular phylogenies reveal that they are exclusive of particular clades. This finding confirms the intuitive thinking of Cobos (1954), who speculated on the existence of natural groupings in *Cyrtomus* defined by their shape.

The elongated shape appears in a group of species forming a supported clade in the parsimony nuclear tree (a solution compatible with the unresolved ML tree for these markers), and a paraphyletic lineage in the mtDNA trees because of the endemic species of Mallorca, *C. majoricensis*, clustering in the sister clade to the other elongated species (although the monophyly of these species is nevertheless supported statistically).

The trait reflects phylogeny, hence it is a good predictor of natural groupings, which can be used for a much needed refinement of the systematics of this poorly known leaf beetle genus (Cobos 1954; Bastazo and Vela 1985).

Phylogeny of *Cyrtomus*

According to the molecular clock calibrated using two different strategies, the origin of *Cyrtomus* and the separation of the two main lineages mentioned above can be dated around

Table 7. Reconstructed ancestral trait values in the phylogeny of *Cyrtomus* (Fig. 6a) and their predicted shape class (*l*, length; *w*, width; *r*, rounded; *e*, elongated; *p*, posterior probability)

Node	<i>l</i>	var(<i>l</i>)	SE(<i>l</i>)	<i>w</i>	var(<i>w</i>)	SE(<i>w</i>)	<i>l/w</i>	Shape ¹	p-value
1	6.3269	0.0871	0.2952	3.6090	0.0379	0.1946	1.75	r	0.9024
2	6.6708	0.1273	0.3567	3.7335	0.0617	0.2484	1.79	r	0.8988
3	6.2245	0.1154	0.3397	3.3871	0.0486	0.2205	1.84	r	0.5926
4	5.9157	0.0717	0.2678	3.1555	0.0513	0.2265	1.87	e	0.6892
5	5.9408	0.1353	0.3678	3.0508	0.0412	0.2029	1.95	e	0.8750
6	5.8071	0.1321	0.3634	2.9052	0.0242	0.1555	2.00	e	0.9483
7	5.8941	0.1277	0.3574	3.2455	0.1792	0.4233	1.82	r	0.5478
8	6.6296	0.3412	0.5841	3.6910	0.1050	0.3240	1.80	r	0.8712
9	5.9972	0.4933	0.7024	3.4552	0.2012	0.4485	1.74	r	0.8717
10	6.4456	0.4147	0.6440	3.7044	0.2070	0.4549	1.74	r	0.9393

¹Predicted based on discriminant analysis.

12–17 Mya, in the Middle Miocene. During this period, the main part of the Iberian Peninsula was separated from the southeastern portion, corresponding to a large Betic Riflean island between the European and North African plates (Andeweg 2002). The current range of *Cyrtomus* includes these two regions of the Iberian Peninsula and the present phylogenetic knowledge of the genus does not allow to precise a geographical origin. The proportionally higher species richness in the Betic area related to surface (over 50% of the species) may suggest an origin in this region, but until further phylogenetic evidence is gathered, the authors prefer to leave this question unanswered.

The main feature that characterizes the phylogeny of *Cyrtomus* is the separation from the base of two different clades, one consisting of *C. arcasi*, *C. contractus*, *C. fairmairei*, *C. pardoi* and *C. puncticeps*, and the other of the remaining species. Both clades include rounded species and only the latter contains elongated species. But there is a distinctive characteristic between clades: a homogeneous karyotype of $2n = 28$ chromosomes in the larger clade, and $2n = 40$, $2n = 46$ or $2n = 28$ (the latter with clear structural differences with the other $2n = 28$ karyotypes; Petitpierre and Garnería 2003) in the clade of *C. puncticeps* and relatives (Petitpierre et al. 1993; Petitpierre and Garnería 2003). Although the karyotype of 28 chromosomes has previously been suggested as the ancestral number for the genus (Petitpierre and Garnería 2003), the molecular phylogeny with the sampling available does not support this reconstruction of the ancestral state.

The elongated forms appeared 10–14 Mya during the Late Miocene, derived from the rounded morphology. This finding contrasts with the opinion of Cobos (1954), who based on the species distributions considered the elongated forms primitive and the rounded ones a monophyletic assemblage of more recent origin. The island endemic *C. majoricensis* is of ancient origin, perhaps from the common ancestor of elongated forms. But its inferred age (8–10 Mya) is considerably younger than the last land connection of the Balearics and the Betic–Riflean promontory, some 15–18 Mya (Andeweg 2002), indicating that a trans-marine dispersal would explain the presence of the genus in the islands. The disjunct chorology of *C. rotundatus* in eastern Spain and southeastern France has a recent origin according to their high genetic similarity.

Conditioned by the current sampling available, speciation in *Cyrtomus* appears as a gradual, constant process with separation of lineages early from its origin, suggesting little lineage extinction, which typically would result in a convex shape

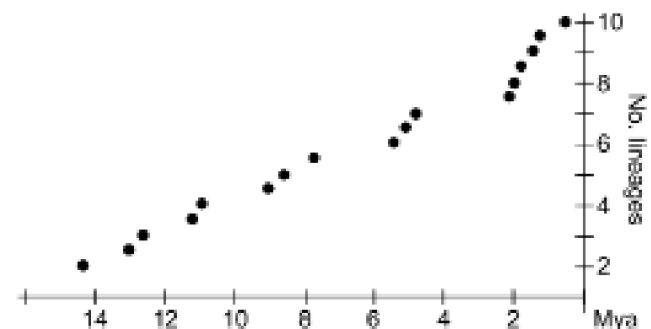


Fig. 7. Lineage-through time plot based on the linearized tree in Fig. 5. The slope of this plot is fairly regular, slightly rising in the last time interval towards the recent, possibly indicating an increase in the rate of speciation events.

(Barraclough and Nee 2001; Fig. 7). Interestingly, during the Pleistocene (<2 Mya), there is a perceptible increase in the branching rate (Fig. 7), which could be interpreted as the result of new speciation events triggered by climatic conditions, again supporting that the background extinction rate is not higher than the speciation rate (Barraclough and Nee 2001; Hewitt 2004). The isolation of populations and the reduction of effective population sizes may facilitate differentiation processes leading to taxonomic segregation in these cases, but these hypotheses remain to be tested. These recent speciation events include the separation of three species such as *C. cylindricus*, *C. ruficornis* and *C. strictus*, currently with clearly allopatric ranges – in the south, centre-northeast and centre of the Iberian Peninsula, respectively, – suggestive of geographically dispersed refugia. Something similar could be said for *C. ehlersi* and *C. rotundatus*, in southeastern and eastern Iberian Peninsula, respectively. More interesting is the case of *C. arcasi* and *C. pardoi*, sympatric in mountainous areas of the Spanish southeast, with incomplete lineage sorting for mtDNA markers, but probably reproductively isolated instantaneously, less than 1 Mya, by their different karyotype and chromosome numbers. *Cyrtomus arcasi* has 40 chromosomes (Petitpierre et al. 1993) and *C. pardoi* has 46 chromosomes (Petitpierre and Garnería 2003), with the latter karyotype likely evolved from the former. The separation of these species based on genetic grounds is not possible, which perhaps reflects their recent speciation, with the chosen markers not capable of resolving it; however, with the current knowledge, an unjustified taxonomic oversplitting cannot be ruled out.

Evolutionary meaning of shapes

Scaling tree parameters used on the continuous character matrix including beetle length and width are compatible with an evolutionary model of gradualism (κ and δ not significantly different from 1.0) and trait change following a constant variance model where phylogeny predicts the covariance patterns among species (λ not significantly different from 1.0). In other words, these characters covary on the phylogeny in a gradual manner according to tree topology, proportionally to branch lengths. This is expected, as neither of these traits shows apparent segregation on the molecular phylogenies.

A different, contrasting picture emerges when the ratio of these two measures is used, as a simplified estimation of the relative beetle width, which is expected from mapping these traits onto a tree related to morphospace. The mode parameter α is not significantly different from 0.0, which reflects punctuational evolution, a trait specific of a lineage; the parameter δ is indeed significantly higher than 1.0, indicative of late trait changes on the phylogeny associated to lineage adaptation; and λ is not different from 1.0, which again indicates that the variation of this trait is correlated with the phylogeny. The evolution of beetle narrow proportions seems to have occurred in a derived lineage in the phylogeny, perhaps responding to adaptation (but see below). A compatible model of evolution was selected with CoMET, recognizing change to occur in a single descendant lineage, but with trait changes not equivalent to tree branch lengths.

With the current poor state of knowledge of the biology and ecology of *Cyrtonus*, it is really difficult to hypothesize on the biological meaning of beetle proportions, if any. It would be possible to relate beetle shape and proportions to environmental parameters and physiological adaptations such as temperature and thermoregulation, for instance. Broader, more compact bodies are expected to thermoregulate better than the narrower counterparts, and could be an adaptation for more extreme environments, such as high altitudes (equivalent to Bergman's rule in warm-blooded animals). However, both shapes can be found in a range of elevations, sometimes syntopically, arguing against an adaptive value for this trait. Since the beetles pupate and probably refugiate in the soil, their shape could perhaps be correlated with soil characteristics, allowing more efficient burrowing; yet again, syntopy of alternative body shapes disagrees with this interpretation. The evidence so far suggests that the evolutionary segregation of this trait is tightly linked to the phylogeny of the group, and is not affected by convergence because of selection and adaptation, which reinforces its systematic importance.

Differences in beetle proportions may represent, however, some sort of reproductive isolation mechanism or allow ecological segregation. Although most species and populations of *Cyrtonus* are allopatric, there are six reported instances of local sympatry for some species pairs (Petitpierre 2004). Remarkably, in five of these six cases of sympatry, the coexisting species are one of each morphological group, which is a highly improbable outcome assuming a random model of species pairing independent of geographical distribution [$p(\text{rounded}) = 0.56$, $p(\text{elongated}) = 0.44$; $p(\text{five species pairs rounded/elongated, one pair of rounded taxa}) = 0.0003$]. Interestingly, the only reported case of rounded species sympatry corresponds to the above mentioned case of *C. arcasi* and *C. pardoi* with *C. plumbeus*, species which differ radically in their

chromosome numbers, with $2n = 40$, $2n = 46$ and $2n = 28$, respectively, which also warrants reproductive isolation (Petitpierre 2004). Some of these sympatric species pairs have been documented in the molecular phylogeny, including *Cyrtonus capreovirens* and *C. puncticeps*; and *C. cylindricus* with *C. arcasi*, *C. pardoi* and/or *C. plumbeus*. In every case, these are divergent taxa separated over 12 Mya, suggesting that their morphological differences did not evolve in sympatry to avoid competition, rather they became sympatric secondarily and their coexistence was possible because of effective reproductive barriers and possibly absence of competition.

Acknowledgements

The authors are indebted to I. Ribera (MNCN, Madrid) for discussion and assistance on morphometric analyses. G. Bastazo and J.M. Vela, J.C. Bourdonné, F. Burle and Ph. Ponel are gratefully acknowledged for providing living samples of some species. J.L. Lencina (University Murcia, Murcia) supplied *Cyrtonus* specimens for genetic and morphometric analyses. Pictures were taken by C. Ruiz (University of Murcia, Murcia). Alessandro Minelli and three anonymous referees are acknowledged for their help to improve the manuscript. JGZ has been funded by the project SPA/1121941 STP (Humboldt Research Fellowship, Germany). JG and FP have been supported by the projects BOS2000-0822 and REN2003-03667, Ministry of Science and Technology, Spain.

Resumen

Filogenia molecular y análisis evolutivo de la forma en el género Cyrtonus (Coleoptera, Chrysomelidae)

Cyrtonus Latreille (Chrysomelidae, Chrysomelinae) es un género muy diverso subendémico de la península Ibérica, con apenas unas pocas especies en las vecinas Francia y Marruecos. Todas las especies son ápteras y preferentemente habitan zonas montañosas. El conocimiento taxonómico sobre este grupo es extremadamente pobre y su sistemática prácticamente inexistente. En este trabajo analizamos y caracterizamos mediante análisis morfométricos un carácter sistemático prometedor, el perfil de los adultos, distinguiendo entre formas alargadas y redondeadas. Aunque la monofilia del género no ofrece discusión, contrastamos esta hipótesis usando secuencias mitocondriales del gen *rnl* y las homologas para crismelinos disponibles en GenBank. Además usamos cuatro marcadores genéticos, dos mitocondriales y dos nucleares, para generar una hipótesis filogenética para la mitad de las especies reconocidas en el género y para estudiar la evolución de la forma, resumida como dos variables continuas, longitud y anchura. Estos atributos covarían significativamente con la filogenia, mostrando una fuerte asociación filogenética: las especies alargadas parecen constituir un clado en un grupo parafilético de especies redondeadas. El árbol de ADN mitocondrial se usó también para investigar la constancia en la tasa de evolución para este marcador y se calibró tanto usando evidencia biogeográfica como la tasa de sustitución promedio del genoma mitocondrial de insectos. La hipótesis del reloj molecular se usó para datar eventos de especiación sobre la filogenia, reconstruyendo el origen del género en el Mioceno medio, con una tasa de especiación relativamente constante hasta finales del Plioceno y un aparente aumento durante el Pleistoceno, posiblemente asociado con el efecto de cambios climáticos dramáticos durante este período. Por último, se propone el elevado valor sistemático de la forma en *Cyrtonus* debido a la ausencia de evidencias relacionándola con adaptaciones.

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Supplementary Material

The following supplementary material is available for this article online:

Appendix S1. Taxa, mitochondrial rnl GenBank accession numbers and references of Chrysomelinae genera. Tree and implied alignment search in POY.

This material is available as part of the online article from <http://www.blackwell-synergy.com>.