

# Assessing the phylogenetic usefulness of a previously neglected morphological structure through elliptic Fourier analyses: a case study in *Bruchus* seed-beetles (Coleoptera: Chrysomelidae: Bruchinae)

G A E L J . K E R G O A T<sup>1</sup> .and N A D I R A L V A R E Z<sup>2</sup>

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**Abstract.** We address the systematics of *Bruchus* seed-beetles through the use of a geometric morphometric outline approach, namely elliptic Fourier analysis. We found that a previously neglected genitalic structure, the ventral plate, provides new evidence in the discussion of taxonomic issues raised by recent molecular studies. Three methods of hierarchical clustering allow investigation of the phylogenetic relationships of the key species that cause the paraphyly of two species groups in recent molecular studies. The resulting reconstructions reveal the phylogenetic usefulness of the structure of interest in recovering consistent relationships of *Bruchus*. Our analyses support the monophyly of the species group whose paraphyletic status was weakly supported by statistical tests in molecular analyses. Our results agree with those molecular and morphological studies that indicate, with relatively strong support, the paraphyletic status of the other species group. We highlight the need to reappraise the use of neglected or presumably uninformative (in traditional morphometrics) morphological characters with geometric morphometrics methods. In addition, we assess the utility of the combination of morphometric descriptors with other sources of phylogenetic information by analysing together an extant molecular dataset and matrix representations based on the results of the elliptic Fourier analyses (to our knowledge our study is the first to investigate such a combination of datasets within a Bayesian framework). Combining morphometric descriptors with other information can improve phylogenetic reconstructions, as suggested by the results of the corresponding analyses we performed using a published molecular dataset.

## Introduction

The Bruchinae are commonly known as seed-beetles because the larval development of all but a few species occurs in seeds. Bruchines exhibit a high degree of dietary specialization: (i) most species are strictly oligophagous or monophagous (Johnson, 1981), and (ii) about 84% of the known host-plant species belong to the family Leguminosae

(Borowiec, 1987). Numerous species are pests of considerable economic importance, as they develop into the seeds of edible leguminous plants (Lukjanovitch & Ter-Minassian, 1957; Southgate, 1979; Delobel & Tran, 1993). This is particularly true of several species belonging to the genus *Bruchus* Linnaeus, which infest lentils (*B. lentis*), field peas (*B. pisorum*) and broad beans (*B. rufimanus*) (Lukjanovitch & Ter-Minassian, 1957; Delobel & Tran, 1993).

Because numerous species of seed-beetles were first described in the genus *Bruchus* (Southgate, 1979; Borowiec, 1987), its status is of particular interest when studying the systematics and taxonomy of seed-beetles. This genus is morphologically homogeneous and is well defined by a

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combination of characters (Lukjanovitch & Ter-Minassian, 1957; Borowiec, 1987): (i) the presence of a lateral pronotal tooth (only absent or indistinguishable in *B. laticollis* and *B. loti*); (ii) highly modified intermediate tibiae in males; and (iii) a unique male genitalic structure with a flat keel-less elongated basal hood. However, only 30 of the 217 species listed as *Bruchus* in the most recent compilation to date, ‘the Catalog of Bruchidae’ (Udayagiri & Wadhi, 1989), appear to be valid. To understand this, one must look at the complexity of the history of seed-beetle taxonomy and systematics (see in particular the detailed review by Borowiec, 1987). In the 19th century, most bruchine species were assigned to the genus *Bruchus*. Only with the development of studies in bruchine systematics in the 20th century were numerous *Bruchus* species transferred to newly defined genera, largely as the result of the work of researchers such as Bridwell, Kingsolver and Johnson in the New World, and Borowiec and Decelle in the Old World (Southgate, 1979; Kergoat & Silvain, 2004). Nonetheless, clarification is required because: (i) numerous researchers have described new species in the genus *Bruchus* without accounting for recent changes in bruchine systematics (Southgate, 1979; Borowiec, 1987); and (ii) the status of many tropical species has not been addressed (Kergoat & Silvain, 2004; Delobel & Delobel, 2006). Table 1 lists 36 valid species that belong to the currently circumscribed genus *Bruchus* (Lukjanovitch & Ter-Minassian, 1957; new species and revisions by Ter-Minassian, 1968; Decelle, 1975, 1979; Borowiec, 1988; Wendt, 1993; Zampetti, 1993; Anton, 1999, 2001). The genus is divided, on the basis of combinations of several morphological characters, into seven species groups (see also Table 1 for details; Borowiec, 1988). Although a preliminary study (Kergoat *et al.*, 2004) has recovered the monophyly of several *Bruchus* species groups, more recent studies (Kergoat, 2004; Kergoat *et al.*, 2007), with increased taxonomic sampling, have indicated that at least two groups (*brachialis* and *rufipes*) are potentially paraphyletic. This latter finding suggests that additional data are needed to address this issue.

In most arthropod groups, genitalic structures evolve rapidly and are thus highly variable, even when considering closely related species (Eberhard, 1985, 2004; Hosken & Stockley, 2004). They tend to show low levels of intraspecific variation, thus constituting a very informative source of diagnostic characters at the species level (but see also Huber & Pérez, 2001; Huber *et al.*, 2005). This is particularly the case for seed-beetles (Kingsolver, 1970; A. Delobel, personal communication), for which numerous closely related taxa can be distinguished only by the male genitalia (see e.g. Alvarez *et al.*, 2004). In *Bruchus*, one putatively diagnostic genitalic character has not previously been considered in taxonomic studies: the eighth abdominal sternite of the male genitalia (Fig. 1). In most bruchine genera this structure, the function of which is not well understood, is represented only by two barely noticeable sclerites (sternal lobes, vestigial sclerites). By contrast, in *Bruchus* species this structure is well developed (with a characteristic boomerang shape), and usually is strongly sclerotized. Many authors have called

**Table 1.** *Bruchus* species list and taxon sampling.

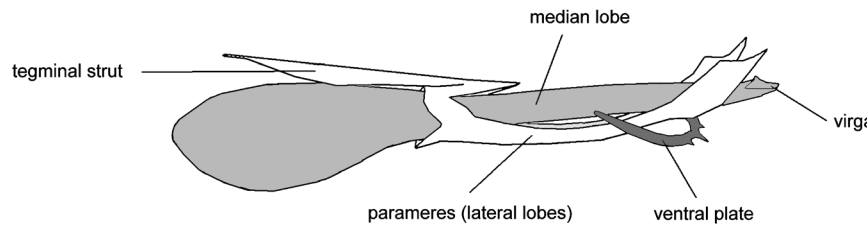
Species	Species groups	Sampling localities <sup>a</sup>
<i>B. affinis</i> Frölich, 1799	<i>affinis</i>	Hte. Corse (Fr.)
<i>B. mirabilicollis</i> Luk. & Ter., 1968	<i>affinis</i>	[Not sampled]
<i>B. mulkaki</i> Luk. & Ter., 1957	<i>affinis</i>	[Not sampled]
<i>B. viciae</i> Olivier, 1795	<i>affinis</i>	Basilicata (It.)
<i>B. altaicus</i> Fahraeus, 1839	<i>atomarius</i>	Talysh (Az.)
<i>B. atomarius</i> (Linnaeus, 1761)	<i>atomarius</i>	Htes. Alpes (Fr.); Rhône (Fr.)
<i>B. dentipes</i> (Baudi, 1886)	<i>atomarius</i>	Vaucluse (Fr.)
<i>B. rufimanus</i> Boheman, 1833	<i>atomarius</i>	Vaucluse (Fr.); Basilicata (It.)
<i>B. anatolicus</i> Anton, 1999	<i>brachialis</i> <sup>b</sup>	[Not sampled]
<i>B. brachialis</i> Fahraeus, 1839	<i>brachialis</i>	Hte. Corse (Fr.); Voiotia (Gr.)
<i>B. brisouti</i> Kraatz, 1868	<i>brachialis</i>	(Mo.)
<i>B. canariensis</i> Decelle, 1975	<i>brachialis</i>	[Not sampled <sup>c</sup> ]
<i>B. hamatus</i> Miller, 1881	<i>brachialis</i>	(Tu.)
<i>B. hierroensis</i> Decelle, 1979	<i>brachialis</i>	[Not sampled]
<i>B. ibericus</i> Anton, 1999	<i>brachialis</i> <sup>b</sup>	[Not sampled]
<i>B. laticollis</i> Boheman, 1833	<i>brachialis</i>	Vaucluse (Fr.)
<i>B. perezi</i> Kraatz, 1868	<i>brachialis</i>	(Tu.)
<i>B. signaticornis</i> Gyllenhal, 1833	<i>brachialis</i>	Hérault (Fr.)
<i>B. ulicis</i> Mulsant & Rey, 1858	<i>brachialis</i>	Basilicata (It.)
<i>B. venustus</i> Fahraeus, 1839	<i>brachialis</i>	Rhône (Fr.)
<i>B. loti</i> Paykull, 1800	<i>loti</i>	Oise (Fr.)
<i>B. lugubris</i> Fahraeus, 1839	<i>loti</i>	[Not sampled]
<i>B. pavlovskii</i> Luk. & Ter., 1954	<i>loti</i>	[Not sampled]
<i>B. emarginatus</i> Allard, 1868	<i>pisorum</i>	Vaucluse (Fr.); Lakonia (Gr.)
<i>B. ervi</i> Frölich, 1799	<i>pisorum</i>	Adiyaman (Tu.)
<i>B. lentis</i> Frölich, 1799	<i>pisorum</i>	Hts-de-Seine (Fr.)
<i>B. pisorum</i> (Linnaeus, 1758)	<i>pisorum</i>	Basilicata (It.)
<i>B. tetragonus</i> (Baudi, 1886)	<i>pisorum</i>	[Not sampled]
<i>B. griseomaculatus</i> Gyllenhal, 1833	<i>rufipes</i>	Essonne (Fr.)
<i>B. libanensis</i> Zampetti, 1993	<i>rufipes</i> <sup>b</sup>	(Tu.)
<i>B. luteicornis</i> Illiger, 1794	<i>rufipes</i>	Vaucluse (Fr.); Basilicata (It.)
<i>B. occidentalis</i> Luk. & Ter., 1957	<i>rufipes</i>	Hte. Corse (Fr.)
<i>B. rufipes</i> Herbst, 1783	<i>rufipes</i>	Hte. Corse (Fr.)
<i>B. sibiricus</i> Germar, 1824	<i>rufipes</i>	Aksu (Ka.)
<i>B. tristiculus</i> Fahraeus, 1839	<i>tristis</i>	Vaucluse (Fr.); Alentejo (Po.)
<i>B. tristis</i> Boheman, 1833	<i>tristis</i>	Vaucluse (Fr.)

<sup>a</sup>Names of countries are abbreviated as follows: Azerbaijan (Az.); France (Fr.); Greece (Gr.); Italy (It.); Kazakhstan (Ka.); Morocco (Mo.); Portugal (Po.); Turkey (Tu.).

<sup>b</sup>These species were described after the study of Borowiec (1988) and assigned to Borowiec taxonomic groups by Anton (2001).

<sup>c</sup>An individual of this species was sequenced (but not dissected) in the study of Kergoat *et al.* (2007).

this structure ‘urosternite’ (e.g. Anton, 2001; Schott, 2003; Kergoat, 2004; Kergoat *et al.*, 2004), but we advocate the more precise term ‘ventral plate’, because ‘urosternite’ commonly is synonymous with ‘ventral abdominal sternite’ (A. Delobel, personal communication). Among the various male genitalic structures in *Bruchus*, the ventral plate is of particular interest as it shows both marked interspecific variation and no intraspecific variation (Anton, 2001), but



**Fig. 1.** Schematic representation of *Bruchus* male genital structures (note the position of the ventral plate).

the potential phylogenetic usefulness of this structure has been suggested only recently and until now has been untested (Kergoat *et al.*, 2004).

Here the ventral plate structure provides a new source of data (over the previously used qualitative morphological characters and molecular data) to address issues concerning *Bruchus* systematics. A geometric morphometric outline approach, elliptic Fourier analysis (EFA; Kuhl & Giardina, 1982; Rohlf & Archie, 1984), was preferred to geometric morphometric landmark approaches because the ventral plates lack useful landmarks. EFA has proved to be a powerful tool with which to analyse complex shape variations at various taxonomic levels both in plants and in animals (e.g. Bertin *et al.*, 2002; Garnier *et al.*, 2005; Polihronakis, 2006), especially if landmarks are unavailable (Monti *et al.*, 2001; Tatsuta *et al.*, 2004). In EFA, the two-dimensional outlines of the structure of interest are decomposed into a sum of harmonically related ellipses (Kuhl & Giardina, 1982). For each harmonic, four elliptic Fourier descriptors (EFDs) are generated. These are further analysed by standard statistical methods (which generally involve multivariate analyses). We specifically use EFA to question the phylogenetic relationships of the three key-species (namely *B. griseomaculatus*, *B. laticollis* and *B. loti*) that account for the paraphyly of the *brachialis*-group and *rufipes*-group in recent molecular studies. We investigate the placement of these species using hierarchical clustering methods to estimate corroboration with previous studies. Finally, we assess the potential interest of the combination of EFA with other sources of phylogenetic information by analysing together an extant molecular dataset (from the study of Kergoat *et al.*, 2007) and matrix representations based on the results of the EFA. We follow the numerous authors who have assessed the phylogenetic utility of morphological characters using combined analyses (e.g. see Spalik & Downie, 2001; Collin, 2003).

## Materials and methods

### Collection of data

Twenty-seven of the 36 valid species of the genus *Bruchus* were sampled (Table 1). Identifications of specimens were conducted by two recognized bruchine taxonomists (A. Delobel and K.-W. Anton). Whenever possible, multiple specimens from distinct localities were examined. However,

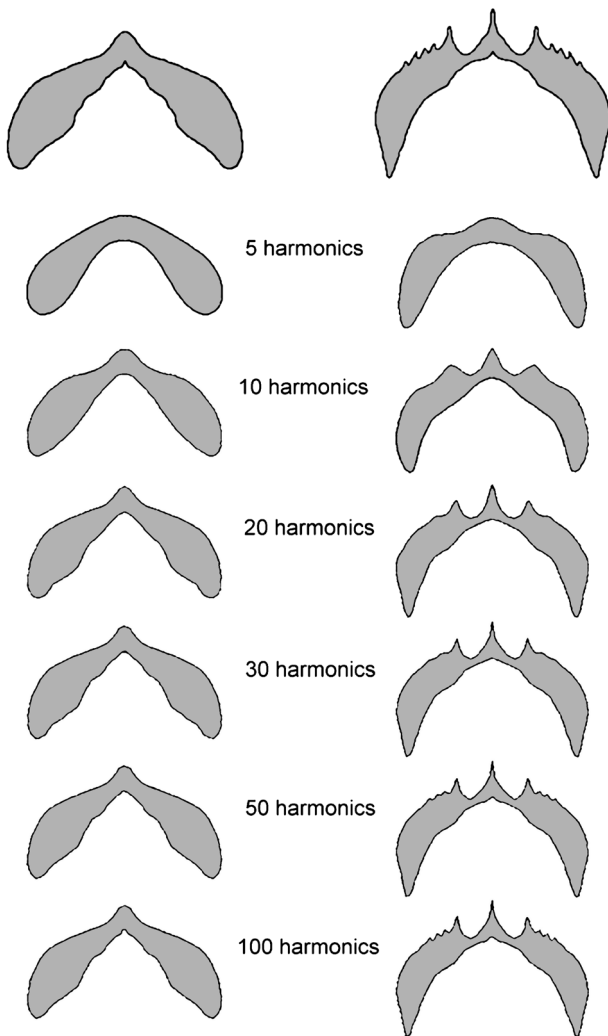
for some rare species, only a few specimens were available and/or precise information on sampling localities was missing. Male genitalia were dissected and cleared in 5% KOH. Special attention was given to the ventral plates, which were mounted on microscope slides and kept as vouchers in the Centre de Biologie et de Gestion des Populations (CBGP, Montferrier-sur-Lez, France). A Leica microscope coupled with a Canon EOS 350D digital camera was used to digitize the ventral plates. Original image files were processed into full colour bitmap (\*.bmp) format and converted to grey-scale images using Adobe PHOTOSHOP CS2 (Adobe Systems Incorporated, Mountain View, California, U.S.A.).

The program package SHAPE (Iwata & Ukai, 2002) was used to analyse the image files. This package is composed of four distinct programs that are able to convert outlines to chaincode (CHAINCODER program), calculate normalized EFDs (CHC2NEF program), perform principal-component analysis (PCA) of EFDs (PRINCOMP program), and visualize shape variations in relation to principal-component scores (PRINPRINT program). We used a standard procedure based on the ellipse of the first harmonic to normalize the orientation, size, and starting points of the EFDs (Kuhl & Giardina, 1982). As a result of this normalization procedure, three of the four coefficients of the first harmonic were degenerated (Rohlf & Archie, 1984; Ferson *et al.*, 1985). For our dataset, the default setting of 20 harmonics was insufficient to approximate some complex contour shapes, and at least 50 harmonics were necessary to resolve details such as spiny protrusions (see Fig. 2). We used this number as a trade-off that allows a good approximation of contour shapes without the undesired estimation of too many parameters for the statistical analyses (Monti *et al.*, 2001).

Means of the EFDs in each species provided the principal-component analysis used by the PRINCOMP program, which is based on the analysis of the variance-covariance matrix of the coefficients. The use of 50 harmonics resulted in the estimation of 197 principal-component scores (this number corresponds to four times the number of harmonics minus the number of degenerated coefficients), which were saved in tab-delimited text format for further statistical analysis.

### Statistical analyses

A square matrix comprising species pairwise Euclidian distances was computed based on the values of the principal



**Fig. 2.** Inverse Fourier reconstruction of the ventral plate of two *Bruchus* species (*B. affinis* on the left and *B. sibiricus* on the right) with increasing numbers of harmonics (from 5 to 100).

components accounting for at least 90% of the total variance ('dist' function, R package; R Development Core Team, 2006). This threshold minimized the number of parameters in the analysis (Monti *et al.* 2001). The distance matrix was then used for hierarchical cluster analyses (HCA) of the 27 analysed species (functions 'hclust' and 's.class', ADE4 package for R; Thioulouse *et al.*, 1996). HCA were carried out using widespread agglomerative methods (Johnson, 1967), in which the  $n$  taxa are progressively reunited into clusters of growing size. At each step, the two most similar clusters are merged until all taxa are clustered into a single cluster of size  $n$ . The three distinct clustering methods used, namely single linkage clustering (SLC), average linkage clustering (ALC), and complete linkage clustering (CLC), differ in the estimation of the similarity between clusters. In SLC, the closest pair of observations between two clusters is used to estimate their

similarity. By contrast, CLC uses the farthest pair of observations, and ALC uses the average similarity of observations to estimate the similarity of two clusters.

Inter-cluster inertia (i.e. the proportion of inter-cluster variance compared with the global variance) was calculated for the three methods of HCA, in order to choose the optimal number of clusters in each analysis. For cluster values ranging from 2 to 10, the variations of inter-cluster inertia were plotted graphically and the rate of change of the inertia function (with respect to the number of groups considered) was determined by computing its second derivative (i.e. in order to make salient the first break in the slope of the distribution of the inertia). A visual representation of the different clusters resulting from the PCA analysis was then obtained using the results of the PCA based on the two most-explanatory axes (PC1 and PC2). In addition, a dendrogram was reconstructed for each HCA method. In the absence of outgroups, the roots of the dendrograms were placed halfway between the two most distinct taxa (mid-point rooting; Farris, 1972).

#### Combined analyses

Following Cannon & Manos (2001), we performed combined analyses using a molecular dataset from Kergoat *et al.* (2007) and matrix representations based on EFA results. To convert the HCA dendrograms into matrix representations, the method of matrix representation with parsimony analysis (MRP; Baum, 1992; Ragan, 1992) was used. In MRP, each node is represented by a binary matrix element, with each terminal taxon in the corresponding cluster being coded either as 1 or 0 (Bininda-Emonds & Bryant, 1998). The program RAINBOW version 1.3 (Chen *et al.*, 2004) was used to compute a single MRP matrix based on the three dendrograms from each HCA analysis (i.e. ALC, CLC and SLC). This matrix (of 90 characters) was concatenated further with a molecular dataset of four genes encompassing 25 *Bruchus* species and four outgroups (see Kergoat *et al.*, 2007 for details). The resulting combined dataset consists of 28 *Bruchus* species (plus four outgroups) and 3035 characters.

To address the heterogeneous nature of our dataset better, we used a partitioned Bayesian inference (BI) approach (Nylander *et al.*, 2004). This method enables the use of different stochastic evolutionary models to analyse datasets consisting of different data types simultaneously, thus increasing the fit of the evolutionary models with the data (Ronquist & Huelsenbeck, 2003). The corresponding analyses were carried out using MRBAYES version 3.12 (Ronquist & Huelsenbeck, 2003) with the following settings: two independent runs of 2 000 000 generations with four Markov chains, random starting trees, default priors, trees sampled every 100 generations, and a burn-in period of 100 000 generations. For clarity, only the best partitioning strategy, as described in Kergoat *et al.* (2007), was retained in the BI analyses (i.e. a ten-partition strategy); therefore 11 partitions were used (ten partitions for the molecular plus

one partition to account for the additional MRP matrix). A supplementary analysis (with the same settings) was performed to identify whether convergence had been reached (Huelsenbeck *et al.*, 2002). To enable a more meaningful discussion, the contribution of the morphological dataset to the combined analysis, we performed the same analyses on a reduced dataset that included only the taxa sampled by Kergoat *et al.* (2007). For all analyses the support of nodes was assessed using clade posterior probability (CPP) estimates.

## Results

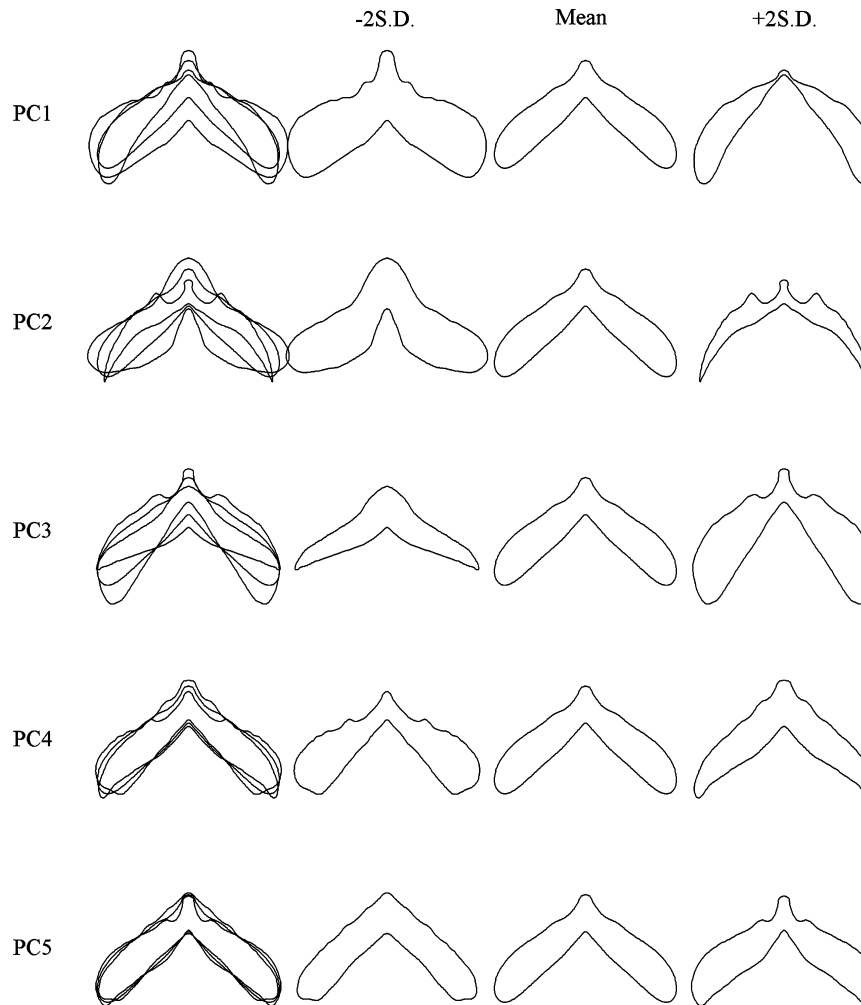
### Shape variations

90.5% of the total shape variation was captured by the first three principal components (PC1, PC2 and PC3; see Table 2). We thus retained the first three principal compo-

nents in all subsequent analyses. To visualize the effect of each principal component on shape variations, EFDs were estimated inversely using both mean and extreme values ( $\pm 2\sigma$  for each principal component), following Ohsawa *et al.* (1998). The resulting outlines (Fig. 3) show the effects of each principal component on variations of ventral plate shapes. For instance, a high PC2 value will produce a characteristic shape with narrow parts and two marked spines (like those found in *B. loti* and all but one member of the *rufipes* group).

### Cluster compositions

For all HCA, four clusters provided the best clustering strategy. The first negative value in the second derivative of the inertia function was reached with four clusters in ALC and CLC analyses. In the SLC analysis the first negative value corresponded to one single cluster, this value was not



**Fig. 3.** Effect of each principal component on shape variation (mean and extreme values are figured). On the left are illustrated the superimposed outlines corresponding to the mean and extreme values of the principal components.

**Table 2.** Contribution of principal components to shape variation.

	Eigenvalue	Proportion (%)	Cumulative (%)
PC1	$1.856 \times 10^{-2}$	52.73	52.73
PC2	$8.333 \times 10^{-3}$	23.67	76.40
PC3	$4.969 \times 10^{-3}$	14.11	90.52
PC4	$1.328 \times 10^{-3}$	3.77	94.29
PC5	$7.626 \times 10^{-4}$	2.16	96.46
PC6	$4.695 \times 10^{-4}$	1.33	97.79
PC7	$1.759 \times 10^{-4}$	0.49	98.29
PC8	$1.332 \times 10^{-4}$	0.37	98.67
PC9	$1.074 \times 10^{-4}$	0.30	98.97
PC10	$8.444 \times 10^{-5}$	0.23	99.21

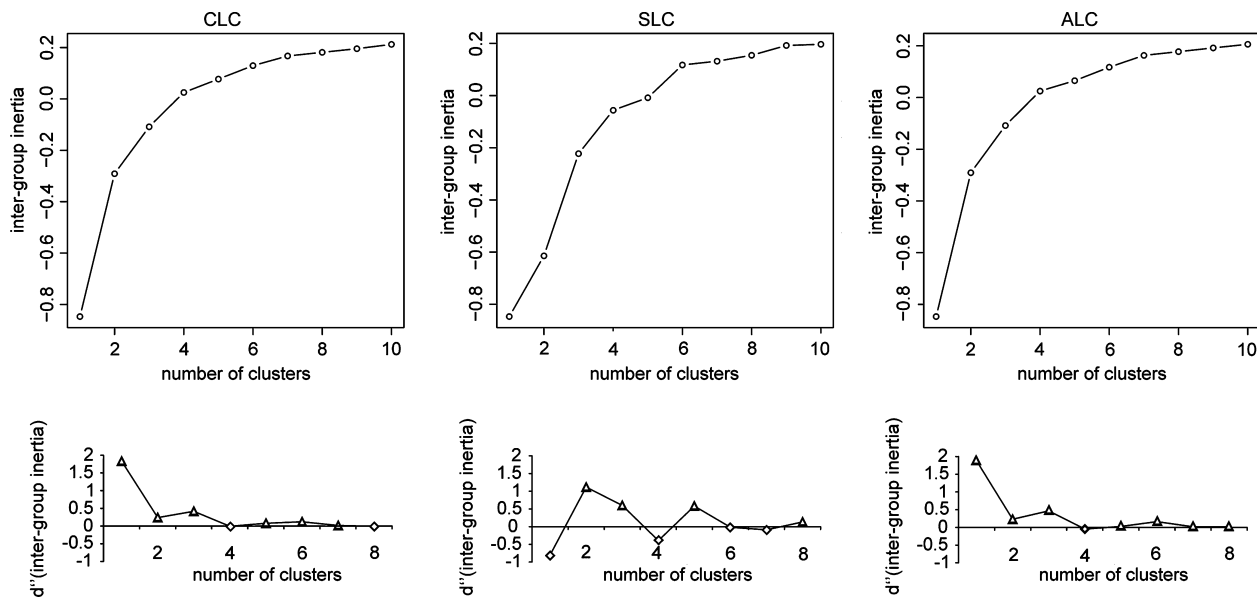
meaningful for our purpose, and in this particular case the second negative value had to be considered (see Fig. 4). Two distinct results are recovered by the HCA (see Fig. 5), as both ALC and CLC methods produce similar cluster compositions. In the ALC and CLC analyses, with the exception of *B. griseomaculatus* and *B. libanensis*, all species of the *rufipes* group are found in one cluster (referred to as cluster D) in addition to the sole sampled representative of the *loti* group, *B. loti*. A further cluster (B) includes the members of groups *affinis*, *atomarius* and *tristis* (plus *B. griseomaculatus*, *B. lentis* and *B. libanensis*), and the two remaining clusters (A and C) gather all members of the *brachialis* group (*B. laticollis* is found in cluster C) plus the remaining members of the *pisorum* group. In the SLC analysis, the five species found in cluster D are split into two distinct clusters (#1 and #3) comprising two and three

species, respectively. A further cluster (#2), which is identical in composition to cluster C, gathers almost all the members of the *brachialis* group (plus *B. pisorum* from the *pisorum* group). The large cluster #4 includes the 15 remaining species, which belong to six taxonomic groups (*affinis*, *atomarius*, *brachialis*, *pisorum*, *rufipes* and *tristis*).

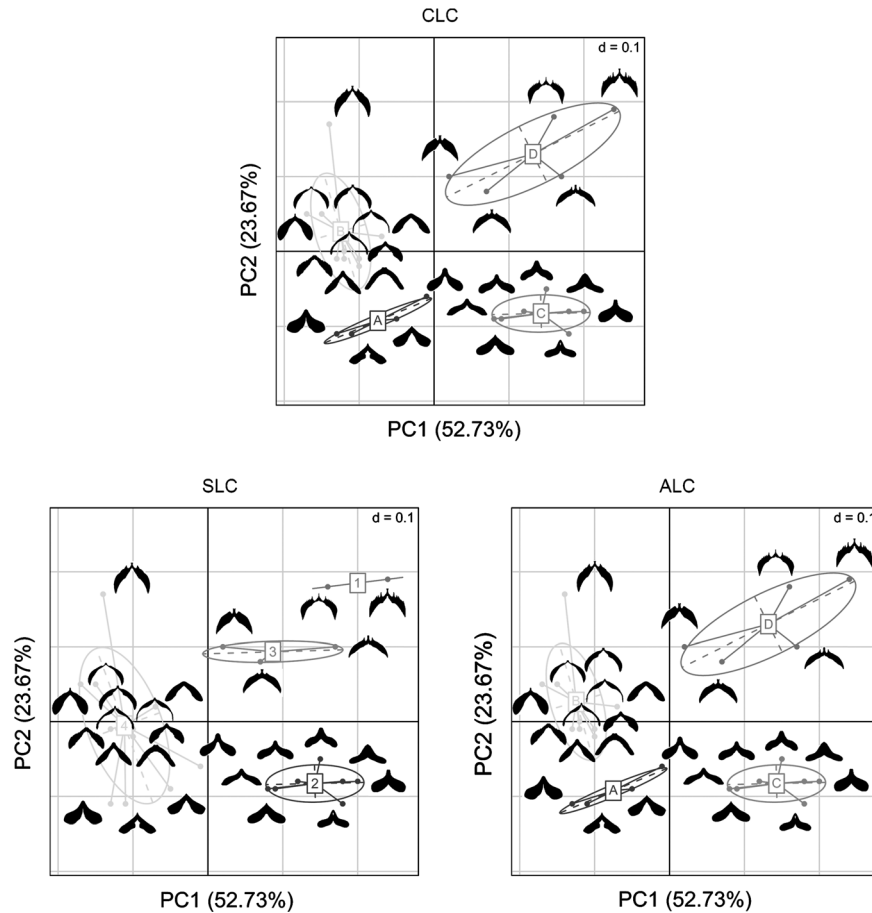
#### HCA dendrograms

Additional information is provided by an examination of the HCA dendrograms (Fig. 6). Interestingly, ALC and CLC analyses result in dendrograms that are very similar, differing only in the position of a single species (*B. griseomaculatus*). In the CLC analysis, *B. griseomaculatus* is grouped with *B. libanensis*, whereas in the ALC analysis it diverged recently from its sister group (which includes species from three other species groups).

Overall, the ALC and CLC dendrograms differ from the SLC dendrogram by minor differences in the branching order of members of two groups (*brachialis* and *pisorum*), and because they cluster together four species of the *rufipes* group (plus *B. loti*) whereas the SLC analysis splits them into two distinct clusters. It is important to note that *B. loti* is nested in the *rufipes* group in all analyses. Regarding *B. laticollis*, this species is found consistently in a cluster composed of other members of the *brachialis* group, plus a member of the *pisorum* group. With reference to species of groups *affinis*, *atomarius* and *tristis*, the three analyses yield a similar topology in which the *atomarius* group constitutes a single cluster, whereas members of groups *affinis* and *tristis* are found in distinct clusters.



**Fig. 4.** The top graphs show the variation of inter-group inertia with increasing numbers of clusters (from 1 to 10) for each HCA method. The bottom graphs show the variation of the second derivative of the inertia function (with respect to the number of groups considered): triangles indicate positive values whereas diamonds indicate negative values.



**Fig. 5.** Cluster representation for each HCA analysis illustrated on the PCA pattern based on the two most explanatory axes (CLC, top; SLC, bottom left; ALC, bottom right). The ventral plate outlines of each species are also shown.

### Combined analyses

The two independent BI runs converged on a similar topology (Fig. 7), which is well supported by the CPP values ( $\text{CPP} \geq 90\%$  for 20 of the 28 nodes). This topology is mostly congruent with the trees resulting from the analyses of the molecular data alone (see Fig. 8); however, it presents noticeable discrepancies with the HCA dendrograms. The groups *affinis*, *atomarius* and *tristis* are recovered as monophyletic with high support (CPP of 100%), whereas the groups *brachialis*, *pisorum* and *rufipes* are recovered as paraphyletic. Interestingly, the three taxa for which we have only morphometric descriptors (*B. ervi*, *B. perezi* and *B. ulicis*) are associated with members of their own taxonomic groups. *Bruchus ervi* belongs to a clade that includes the other members of the *pisorum* group (plus a member of the *brachialis* group), whereas *B. perezi* and *B. ulicis* belong to a clade exclusively comprising members of the *brachialis* group.

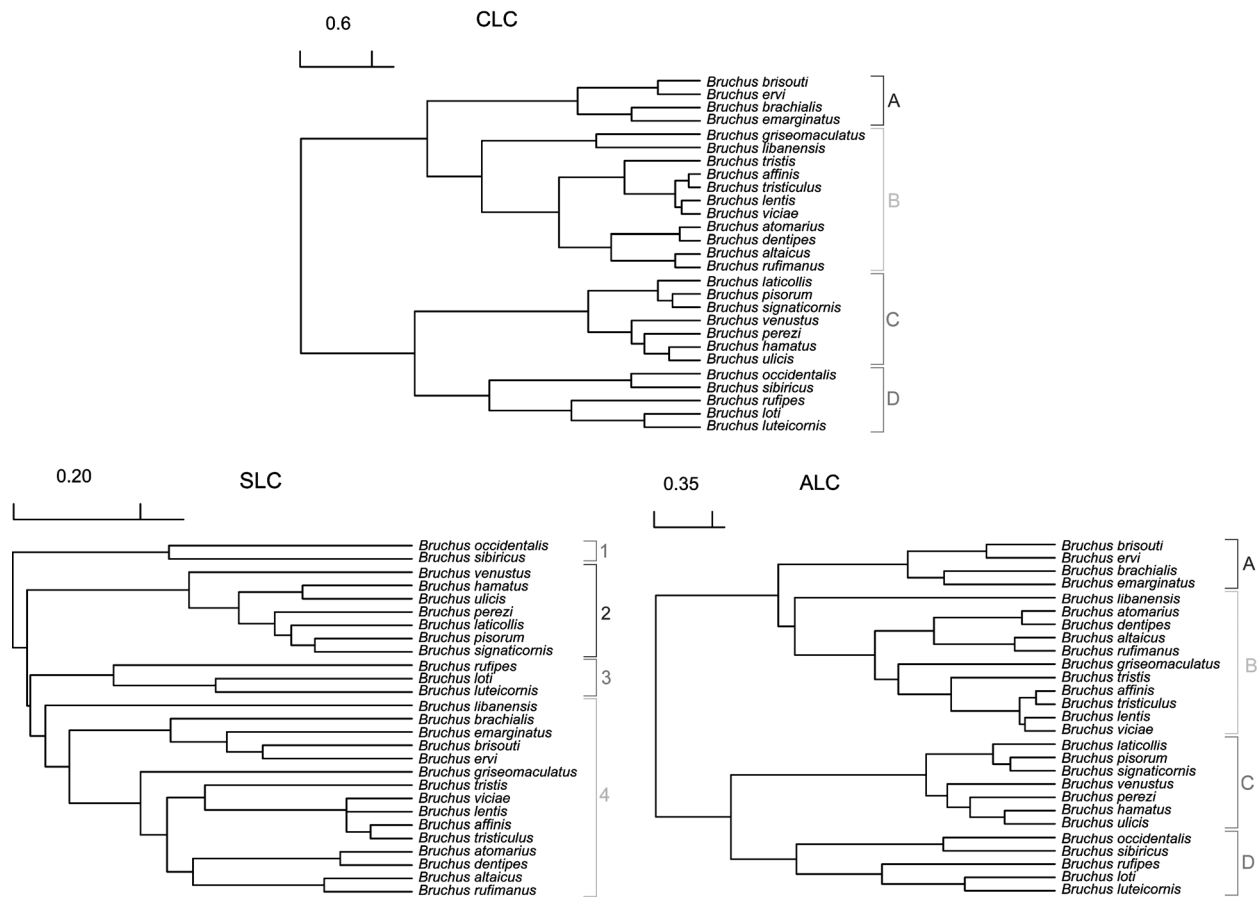
Analyses of the reduced dataset (which only includes those taxa sampled in Kergoat *et al.*, 2007) yield a highly supported topology (not figured). In comparison with the

mean CPP value of 90.6% obtained by the best partitioning strategy of the study by Kergoat *et al.* (2007), the combined analyses of the reduced dataset yield a tree with a mean CPP value of 95.8%. This finding indicates that the morphological dataset contributes significantly to the robustness of the phylogenetic reconstruction in the combined analyses.

## Discussion

### Elliptic Fourier analyses

Despite some unexpected, perhaps artefactual, results (with respect to the composition of extant taxonomic groups), the EFA indicate that the ventral plate is a phylogenetically informative structure. Most members of a given taxonomic group are generally found in the same cluster, and the members of the *atomarius* group always form a single cluster. Collectively, these results demonstrate that the general phylogenetic pattern recovered by the EFA is far from random, and that relevant information



**Fig. 6.** Inferred dendrograms for each HCA method (CLC, top; SLC, bottom left; ALC, bottom right). Note that, in the absence of outgroups, the roots of the dendrograms are placed halfway between the two most distinct taxa. The cluster composition is shown to the right of each dendrogram.

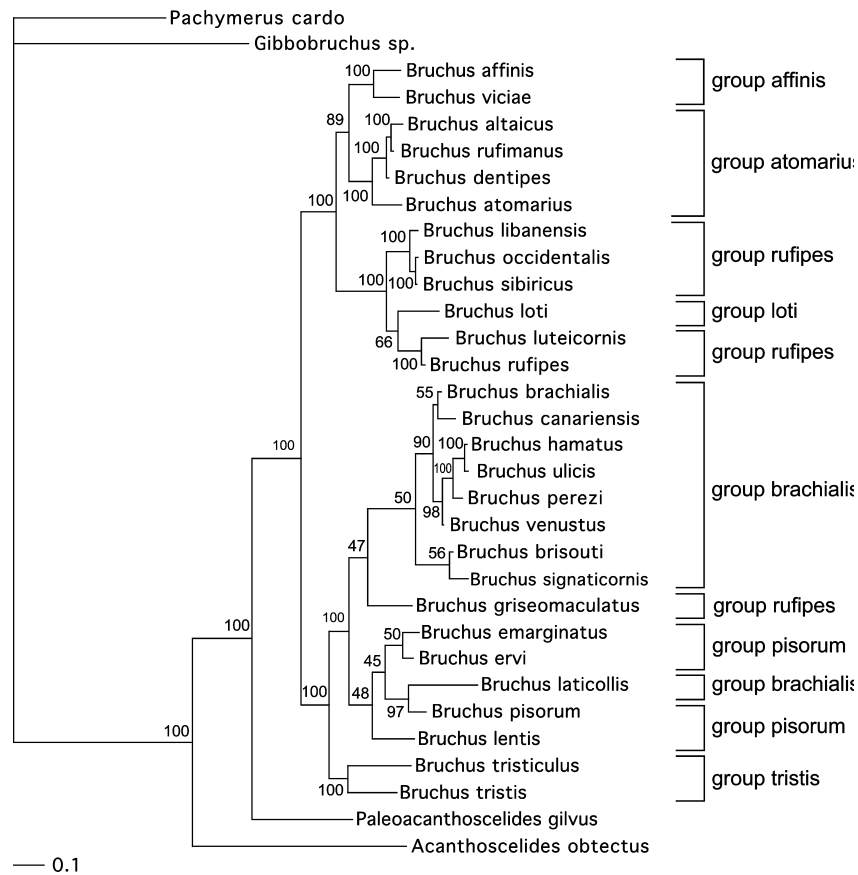
can be recovered from the analysis of the ventral plate. In addition, the HCA dendrograms provide insights into the position of three species (namely *B. ervi*, *B. perezi* and *B. ulicis*) for which, to our knowledge, no molecular data are available.

However, limits in the resolution power of this approach are detectable when putative sister taxa are not paired in the cluster analysis (e.g. the four species from groups *affinis* and *tristis*). The repeated inclusions of members of the *pisorum* group among members of the *brachialis* group are not supported by previous molecular and morphological analyses. These results may be accounted for by the phylogenetic content of the ventral plate being uninformative in resolving phylogenetic relationships. The fact that closely related taxa are not all assigned correctly could result also from a failure to extract all the phylogenetic content from the structure of interest. These hypotheses are supported partially by the fact that some of these species have a very short branch in all HCA reconstructions. By contrast, artefactual reconstructions may be explained by reconstructed long branches, as in the case of another species whose place-

ment was unexpected, namely *B. libanensis*. Both these potential limitations, in conjunction with the apparent low level of congruency found between the HCA dendrograms and recent molecular phylogenies (Fig. 8; Kergoat *et al.*, 2007), cause us to consider some EFA results with caution.

#### Taxonomic considerations

In accordance with recent studies (Kergoat, 2004; Kergoat *et al.*, 2007) and the views of K.-W. Anton (personal communication), the EFA support the exclusion of *B. griseomaculatus* from the *rufipes* group because this species does not group with species belonging to the *rufipes* group. This proposal is supported by the noticeable differences found in a re-examination of several genitalic structures, such as the shape of parameres and the presence/absence of a characteristic sclerite in the distal part of the internal sac (L. Borowiec, personal communication). The ventral plate of this species also lacks the two characteristic spines associated with high PC2 values. EFA do not support



**Fig. 7.** Phylogenetic hypothesis resulting from the BI analyses of the combined datasets (11 partitions were used). Numbers at nodes indicate the CPP values. Taxonomic groups are given to the right of the tree.

the inclusion of *B. griseomaculatus* in the *brachialis* group, providing further support for the suggested transfer of *B. griseomaculatus* to a group of its own (*griseomaculatus* group) (Kergoat *et al.*, 2007).

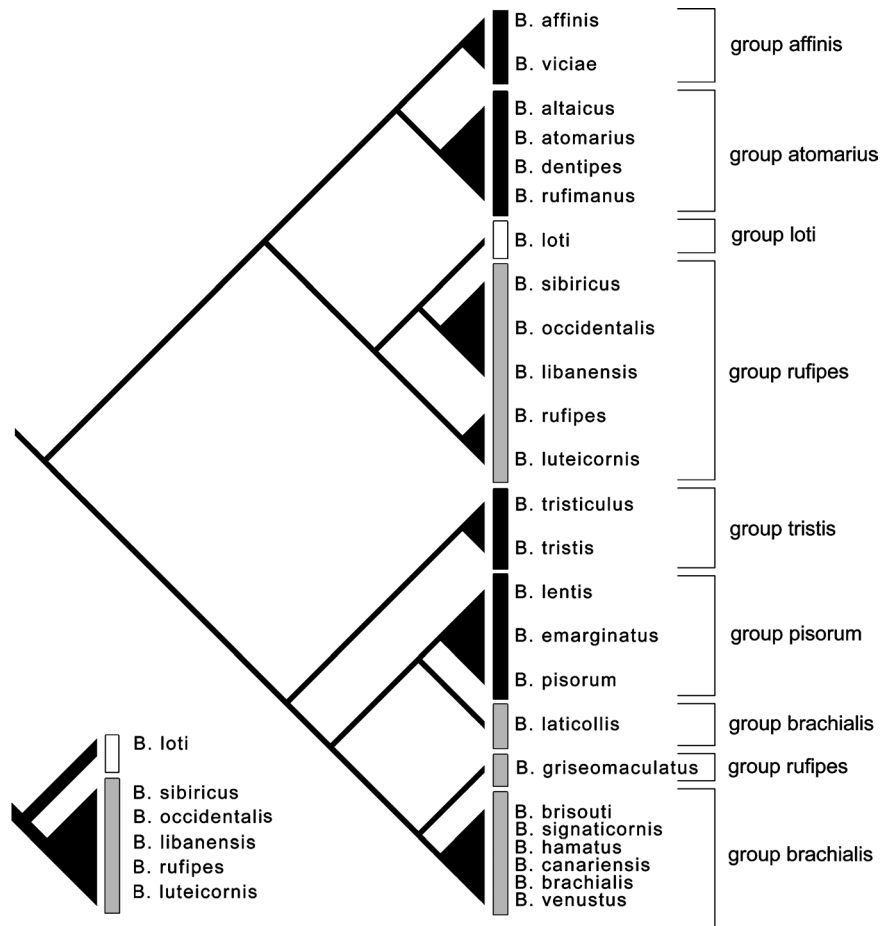
The status of the *rufipes* group is not resolved because there is uncertainty in the placement of *B. loti*. In all HCA dendrograms, *B. loti* is positioned within the *rufipes* group. This placement is supported by the fact that the ventral plate of *B. loti* possesses two spines (associated with a high PC2 value), as found for all members of the *rufipes* group (with the noticeable exception of *B. griseomaculatus*). A similar placement is recovered in the molecular phylogenies obtained under parsimony, whereas under Bayesian inference *B. loti* is found as the sister species of the *rufipes* group (Kergoat, 2004; Kergoat *et al.*, 2007). Traditional morphometrics support a close relationship between the two groups, which are defined by similar combinations of morphological characters (Borowiec, 1988). The traditional assignation of a species to these groups is determined by a sole criterion, the presence/absence of an elytral pattern, a character whose taxonomic usefulness in Coleoptera is limited owing to frequent homoplasy (e.g. Morgan *et al.*, 2000; Robertson *et al.*, 2004). However, given that we have only one representative of the *loti* group in collection (out

of a possible three species), it seems premature to propose the incorporation of the *loti* group within an enlarged *rufipes* group.

Recent molecular studies question the position of *B. laticollis*: this member of the *brachialis* group was found repeatedly in previous studies (but with a low statistical support) as the sister species of members of the *pisorum* group (see Fig. 8), consequently rendering the *brachialis* group paraphyletic. This latter result was unexpected, because the respective members of the two groups are well discriminated by several diagnostic morphological characters (members of the *brachialis* group are well characterized by two synapomorphous characters: the enlarged fore tibiae in male and the presence of two groups of sclerites in the median lobe). Here the EFA provide new evidence that strengthens the hypothesis of a monophyletic *brachialis* group, because *B. laticollis* is systematically found within a cluster of seven species that gathers five other representatives of the *brachialis* group.

#### Combined analyses

With their associated broader sampling, combined analyses provide new insights into *Bruchus* taxonomy. The two



**Fig. 8.** Pruned tree from the study by Kergoat *et al.* (2007). This tree is a simplification of the consensus tree obtained under parsimony from a multi-gene analysis of the phylogenetic relationships of 29 bruchine species (including 25 *Bruchus* species). For discussion purposes, the alternative position of *B. loti* under BI is shown on the bottom left of the tree. The monophyletic or paraphyletic status of a given taxonomic group is indicated either by black (for monophyletic groups) or grey (for paraphyletic groups) sidebars. A white sidebar is used for group *loti*, which was represented only by a sole species (the monophyletic status of this group was therefore not assessable).

members of the *brachialis* group for which no molecular data are available (*B. perezi* and *B. ulicis*) lie within a large clade exclusively constituted by members of the *brachialis* group. Likewise, *B. ervi* is associated with other members of the *pisorum* group. In addition, a significant contribution to the robustness of trees is revealed by the combined analyses of the reduced dataset, which yielded higher CPP values in comparison to the analyses of the molecular dataset alone. Collectively, these results underline not only the phylogenetic utility of the ventral plate as a valuable source of phylogenetic information, but also the interest of our combined approach, which associates morphometric descriptors and molecular data. In relation to the EFA results, the conclusions reached previously on the status of the *rufipes* group are supported by the combined analyses, as *B. griseomaculatus* appears unrelated to other members of the *rufipes* group whereas *B. loti* is positioned within a clade composed of members of the *rufipes* group. Nonetheless, combined analyses still do not support the hypothesis of

a monophyletic *brachialis* group because of the position of *B. laticollis*, which is found in a clade comprising members of the *pisorum* group.

## Conclusion

Despite reservations concerning the reliability of reconstructions obtained through the various HCA methods, the analyses performed on *Bruchus* ventral plates have generated useful results, especially in relation to the issue of the position of the three key species of interest. Our study thus provides additional evidence to support the monophyly of the *brachialis* group and the exclusion of *B. griseomaculatus* from the *rufipes* group. However, sampling of the two other members of group *loti* is essential to reach a decision on the status of the *loti* group. We believe that our study, which focused on a relatively simple structure, clearly underlines the potential interest of geometric morphometric outline

approaches in systematics, because it allows information to be extracted that would not be gathered through traditional morphological- or molecular-based approaches. Our study provides further evidence to support the usefulness of combined analyses based on morphometric descriptors. On a case-to-case basis such approaches may permit the reassessment of neglected or presumed uninformative (in traditional morphometrics) morphological characters, by providing additional data that can be compared with molecular and/or traditional morphological datasets.

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## References

- Alvarez, N., Hossaert-McKey, M., Rasplus, J.-Y. *et al.* (2004) Sibling species of bean bruchids: a morphological and phylogenetic study of *Acanthoscelides obtectus* Say and *Acanthoscelides obvelatus* Bridwell. *Journal of Zoological Systematics and Evolutionary Research*, **43**, 29–37.
- Anton, K.-W. (1999) Two new species of the *Bruchus brachialis* group from the Mediterranean region (Coleoptera: Bruchidae: Bruchinae). *Linzer Biologische Beiträge*, **31**, 655–660.
- Anton, K.-W. (2001) Bemerkungen zur faunistik und taxonomie mitteleuropäischer samenkafer (Coleoptera: Bruchidae). *Folia Entomologica Hungarica*, **62**, 43–49.
- Baum, B.R. (1992) Combining trees as a way of combining data sets for phylogenetic inference, and the desirability of combining gene trees. *Taxon*, **41**, 3–10.
- Bertin, A., David, B., Cezilly, F. & Alibert, P. (2002) Quantification of sexual dimorphism in *Asellus aquaticus* (Crustacea: Isopoda) using outline approaches. *Biological Journal of the Linnean Society*, **77**, 523–533.
- Bininda-Emonds, O.R.P. & Bryant, H.N. (1998) Properties of matrix representation with parsimony analyses. *Systematic Biology*, **47**, 497–508.
- Borowiec, L. (1987) The genera of seed-beetles (Coleoptera, Bruchidae). *Polskie Pismo Entomologiczne*, **57**, 3–207.
- Borowiec, L. (1988) *Bruchidae-Strakowce (Insecta: Coleoptera)*. *Fauna Polski, Tom 11*. PWN, Warszawa, Poland.
- Cannon, C.H. & Manos, P.S. (2001) Combining and comparing morphometric shape descriptors with a molecular phylogeny: the case of fruit type evolution in Bornean *Lithocarpus* (Fagaceae). *Systematic Biology*, **50**, 860–880.
- Chen, D., Eulenstein, O. & Fernandez-Baca, D. (2004) Rainbow: a toolbox for phylogenetic supertree construction and analysis. *Bioinformatics*, **20**, 2872–2873.
- Collin, R. (2003) The utility of morphological characters in gastropod phylogenetics: an example from the Calyptraeidae. *Biological Journal of the Linnean Society*, **78**, 541–593.
- Decelle, J. (1975) Les Bruchidae des îles Canaries. *Bulletin et Annales de la Société Royale Belge d'Entomologie*, **111**, 109–142.
- Decelle, J. (1979) Une nouvelle espèce de *Bruchus* (Coleoptera: Bruchidae) des îles Canaries. *Vieraea*, **8**, 143–146.
- Delobel, B. & Delobel, A. (2006) Dietary specialization in European species groups of seed beetles (Coleoptera: Bruchidae: Bruchinae). *Oecologia*, **149**, 428–443.
- Delobel, A. & Tran, M. (1993) *Les Coléoptères des denrées alimentaires entreposées dans les régions chaudes*. Faune tropicale XXXII. Orstom/CTA, Paris.
- Eberhard, W.G. (1985) *Sexual Selection and Animal Genitalia*. Harvard University Press, Cambridge, MA.
- Eberhard, W.G. (2004) Male–female conflict and genitalia: failure to confirm predictions in insects and spiders. *Biological Reviews*, **79**, 121–186.
- Farris, J.S. (1972) Estimating phylogenetic trees from distance matrices. *American Naturalist*, **106**, 645–668.
- Ferson, F., Rohlf, F.J. & Koehn, R.K. (1985) Measuring shape variation of two-dimensional outlines. *Systematic Zoology*, **34**, 59–68.
- Garnier, S., Magniez-Jannin, F., Rasplus, J.-Y. & Alibert, P. (2005) When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia. *Journal of Evolutionary Biology*, **18**, 269–280.
- Hosken, D.J. & Stockley, P. (2004) Sexual selection and genital evolution. *Trends in Ecology and Evolution*, **19**, 87–93.
- Huber, B.A. & Perez, G.A. (2001) Female genital dimorphism in a spider (Araneae: Pholcidae). *Journal of Zoology, London*, **255**, 301–304.
- Huber, B.A., Rheims, C.A. & Brescovit, A.D. (2005) Speciation without changes in genital shape: a case study on Brazilian pholcid spiders (Araneae: Pholcidae). *Zoologischer Anzeiger*, **243**, 273–279.
- Huelsensbeck, J.P., Larget, B., Miller, R.E. & Ronquist, F. (2002) Potential applications and pitfalls of Bayesian inference of phylogeny. *Systematic Biology*, **51**, 673–688.
- Iwata, H. & Ukai, Y. (2002) SHAPE: a computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. *Journal of Heredity*, **93**, 384–385.
- Johnson, S.C. (1967) Hierarchical clustering schemes. *Psychometrika*, **2**, 241–254.
- Johnson, C.D. (1981) Seed beetle host specificity and the systematics of the Leguminosae. *Advances in Legume Systematics* (ed. by R. M. Polhill and P. H. Raven), pp. 995–1027. The Royal Botanic Gardens, Kew.
- Kergoat, G.J. (2004) *Le genre Bruchidius (Coleoptera, Bruchidae): un modèle pour l'étude des relations évolutives entre les insectes et les plantes*. PhD Thesis, University of Paris VI.
- Kergoat, G.J. & Silvain, J.-F. (2004) Le genre *Bruchidius* (Coleoptera: Bruchidae) est-il monophyletique? Apports des méthodes de parcimonie, maximum de vraisemblance et inférence bayésienne. *Biosystema*, **22**, 113–125.
- Kergoat, G.J., Delobel, A. & Silvain, J.-F. (2004) Phylogeny and host-specificity of European seed beetles (Coleoptera, Bruchidae), new insights from molecular and ecological data. *Molecular Phylogenetics and Evolution*, **32**, 855–865.
- Kergoat, G.J., Silvain, J.-F., Delobel, A., Tuda, M. & Anton, K.-W. (2007) Defining the limits of taxonomic conservatism in host-plant use for phytophagous insects: molecular systematics and evolution of host-plant associations in the seed-beetle genus *Bruchus* Linnaeus (Coleoptera: Chrysomelidae: Bruchinae). *Molecular Phylogenetics and Evolution*, **43**, 251–269.

- Kingsolver, J.M. (1970) A study of male genitalia in Bruchidae (Coleoptera). *Proceedings of the Entomological Society of Washington*, **72**, 370–386.
- Kuhl, F.P. & Giardina, G.R. (1982) Elliptic Fourier features of a closed contour. *Computer Graphics and Image Processing*, **18**, 236–258.
- Lukjanovitch, F.K. & Ter-Minassian, M.E. (1957) Zhuki-zernovski (Bruchidae). *Fauna SSSR, Zhestkokrylye*, **24**, 1–209.
- Monti, L., Baylac, M. & Lalanne-Cassou, B. (2001) Elliptic Fourier analysis of the form of genitalia in two *Spodoptera* species and their hybrids (Lepidoptera: Noctuidae). *Biological Journal of the Linnean Society*, **72**, 391–400.
- Morgan, M., Knisley, C.B. & Vogler, A.P. (2000) New taxonomic status of the endangered tiger beetle *Cicindela limbata albissima* (Coleoptera: Cicindelidae): evidence from mtDNA. *Annals of the Entomological Society of America*, **93**, 1108–1115.
- Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P. & Nieves-Aldrey, J.L. (2004) Bayesian phylogenetic analysis of combined data. *Systematic Biology*, **53**, 47–67.
- Ohsawa, R., Tsutumi, T., Uehara, H., Namai, H. & Ninomiya, S. (1998) Quantitative evaluation of common buckwheat (*Fagopyrum esculentum* Moench) kernel shape by elliptic Fourier descriptor. *Euphytica*, **101**, 175–183.
- Polihronakis, M. (2006) Morphometric analysis of intraspecific shape variation in male and female genitalia of *Phyllophaga hirticula* (Coleoptera: Scarabaeidae: Melolonthinae). *Annals of the Entomological Society of America*, **99**, 144–150.
- Ragan, M.A. (1992) Phylogenetic inference based on matrix representation of trees. *Molecular Phylogenetics and Evolution*, **1**, 53–58.
- R Development Core Team (2006) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. [WWW document]. URL <http://www.R-project.org>. [accessed 21 October 2006].
- Robertson, J.A., McHugh, J.V. & Whiting, M.F. (2004) A molecular phylogenetic analysis of the pleasing fungus beetles (Coleoptera: Erotylidae): evolution of colour patterns, gregariousness and mycophagy. *Systematic Entomology*, **29**, 173–187.
- Rohlf, F.J. & Archie, J.W. (1984) A comparison of Fourier methods for the description of wing shape in mosquitoes (Diptera: Culicidae). *Systematic Zoology*, **33**, 302–317.
- Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.
- Schott, C. (2003) *Spermophagus calystegiae* (Lukjanovitch et Ter-Minassian, 1957), *Bruchus occidentalis* Lukjanovitch et Ter-Minassian, 1957, *Bruchidius pusillus* (Germar, 1824) en Alsace (Coleoptera, Bruchidae). *Bulletin de la Société Entomologique de Mulhouse*, **59**, 9–14.
- Southgate, B.J. (1979) Biology of the Bruchidae. *Annual Review of Entomology*, **24**, 449–473.
- Spalik, K. & Downie, S.R. (2001) The utility of morphological characters for inferring phylogeny in Scandiceae subtribe Scandicinae (Apiaceae). *Annals of the Missouri Botanical Garden*, **88**, 270–301.
- Tatsuta, H., Mizota, K. & Akimoto, S.-I. (2004) Relationship between size and shape in the sexually dimorphic beetle *Proso-pocoilus inclinatus* (Coleoptera: Lucanidae). *Biological Journal of the Linnean Society*, **81**, 219–233.
- Ter-Minassian, M.E. (1968) A new species of the genus *Bruchus* L. (Coleoptera: Bruchidae) from the fauna of USSR. *Revue d'Entomologie de l'URSS*, **47**, 181–183.
- Thioulouse, J., Chessel, D., Dolédec, S. & Olivier, J.M. (1996) ADE-4: a multivariate analysis and graphical display software. *Statistics and Computing*, **7**, 75–83.
- Udayagiri, S. & Wadhi, S.R. (1989) Catalog of Bruchidae. *Memoirs of the American Entomological Institute*, **45**, 1–301.
- Wendt, H. (1993) *Bruchus ecalcaratus* K. Daniel, 1906 – ein Synonym zu *Bruchus rufimanus* Boheman, 1833 (Coleoptera, Chrysomeloidea, Bruchidae). *Deutsche Entomologische Zeitschrift*, **40**, 161–165.
- Zampetti, M.F. (1993) Una nuova specie di *Bruchus* del Libano (Coleoptera, Bruchidae). *Fragmenta Entomologica*, **24**, 215–218.