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Integration of conflict into integrative taxonomy: fitting hybridization in species delimitation of *Mesocarabus* (Coleoptera: Carabidae)

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Abstract

In species differentiation, characters may not diverge synchronously, and there are also processes that shuffle character states in lineages descendant from a common ancestor. Species are thus expected to show some degree of incongruence among characters, and therefore taxonomic delimitation can benefit from integrative approaches and objective strategies that account for character conflict. We illustrate the potential of exploiting conflict for species delimitation in a study-case of ground beetles of the subgenus *Carabus (Mesocarabus)*, where traditional taxonomy does not accurately delimit species. The molecular phylogenies of four mitochondrial and three nuclear genes, cladistic analysis of the aedeagus, ecological niche divergence, and morphometry of pronotal shape in more than 500 specimens of *Mesocarabus* show that these characters are not fully congruent. For these data, a three-steps operational strategy is proposed for species delimitation by (1) delineating candidate species based on the integration of incongruence among conclusive lines of evidence, (2) corroborating candidate species with inconclusive lines of evidence, and (3) refining a final species proposal based on an integrated characterization of candidate species based on the evolutionary analysis of incongruence. This procedure provided a general understanding of the reticulate process of

hybridization and introgression acting on *Mesocarabus* and generated the hypothesis of seven *Mesocarabus* species, including two putative hybrid lineages. Our work emphasizes the importance of incorporating critical analyses of character and phylogenetic conflict to infer both the evolutionary history and species boundaries through an integrative taxonomic approach.

Introduction

The aims of taxonomy are the discovery, classification, naming and identification of the fundamental units of biodiversity (i.e., species), that are central to other biological disciplines (Dayrat 2005; Valdecasas *et al.* 2008). However, delimiting these units is a challenging task owing to a number of processes that cause them to be truly indistinct (Hey 2006) or to difficulties in identifying separate lineages along the divergence continuum (Mallet 2008; Padial *et al.* 2010). The current trend known as integrative taxonomy has been recently applied to delineate species where a traditional pattern-based taxonomy failed to accurately find their limits, such as species complexes resulting from recent radiations, or groups showing conflict arising from morphological stasis (e.g., Bond & Stockman 2008; Vieites *et al.* 2009; Gebiola *et al.* 2012; Arribas *et al.* 2013). A particularly challenging case for species delimitation is provided by taxa where character incongruence is not the result of different levels of resolution offered by particular markers, but rather the imprint of both past and recent hybridization events in a reticulate process of lineage formation, with conflicting distribution of characters that may obscure the evolutionary history and hinder species delineation (Dowling & Secor 1997; Funk & Omland 2003; Petit & Excoffier 2009; Cardoso *et al.* 2009). Indeed, hybridization is generally accepted as a common and widespread evolutionary phenomenon in plants leading to frequent introgression (Arnold 1997) and speciation (Coyne & Orr 2004), and molecular markers suggest that the same processes are relatively frequent in animals too (Mallet 2005, 2008). Beyond the obvious implications of these processes in the evolutionary history of the species involved, their relevance for taxonomy is that *the conflict that they generate is real*; it is not an analytical artifact that

can be dealt with using statistical or quantitative arguments, or circumvented by examining additional sets of characters, as generally proposed by integrative taxonomy. Yet, such conflict needs to be incorporated in the description of species diversity in the same terms as other evidence (Schlick-Steiner *et al.* 2010).

The subgenus *Mesocarabus* includes apterous beetles occupying a variety of habitats, from sea shores and dry steppes to alpine forests across the western Palearctic region, and it has been subdivided in five putative species (Serrano 2003; Turin *et al.* 2003): the western European *Carabus problematicus* Herbst, the Iberian *C. dufourii* Dejean, *C. lusitanicus* Fabricius and *C. macrocephalus* Dejean, and the North Moroccan *C. riffensis* Fairmaire. However, there is a lack of consensus on species delimitation in *Mesocarabus*. For instance, the Iberian endemics *C. lusitanicus* and *C. macrocephalus* have been considered a single species, or more commonly referred to as the *"C. lusitanicus* species complex*"* (Toulgöet & Lassalle 1983; Deuve 2004). Moreover, the high degree of local morphological differentiation resulted in the description of dozens of infra-specific taxa, many of them found close or within contact zones between putative species, and showing intermediate morphological characteristics that hinder their systematic placement and the recognition of species limits, with taxonomists frequently invoking the misleading concept of 'transition forms' (Toulgöet & Lassalle 1983). Consistent with the historical difficulties in studying this group using a traditional taxonomic approach (e.g., Toulgöet & Lassalle 1983; Casale & Kryzhanovskij 2003), molecular phylogenetics on this subgenus has provided evidence of reticulation (Andújar *et al.* 2012a) and artificial hybridization has been demonstrated among members of the subgenus (Puisségur 1987). These observations strongly suggest that hybridization is a primary confounding factor for the taxonomy of *Mesocarabus* and a prominent process in its evolution, and thus it should be critically incorporated into any attempt to species delimitation. Therefore, *Mesocarabus* presents an illustrative example on how to integrate conflict into integrative taxonomy.

We have incorporated multiple lines of evidence to explore species boundaries within the subgenus *Mesocarabus*, proposing a procedure on how to consider the pattern of incongruence in the delineation of candidate species by taking into account the recognition of introgression events and the occurrence of putative hybrid lineages. Molecular data from nine gene fragments (6700 nt; 251 specimens) as well as environmental and morphological data were studied in an ensemble of more than 500 *Mesocarabus* specimens across most of the geographic range of the subgenus and including most of its subspecific taxonomic entities. Our dense sampling and intense data collection allow us (1) to standardize a procedure to delineate candidate species implicitly integrating information of character incongruence, (2) to generate meaningful evolutionary hypotheses for the discordance among lines of evidence determining the extent of hybridization and mitochondrial introgression in the subgenus *Mesocarabus*, and (3) to advance species delimitation in *Mesocarabus* based on the previously recognized patterns and processes.

Material and methods

Sampling of specimens

We analyzed a total of 511 *Mesocarabus* specimens from 216 localities and initially classified them in five species and 19 subspecies. Sampling covered almost the entire geographic range of the subgenus and was especially intensive in the Iberian Peninsula, where all subspecies accepted by recent authors were collected (Turin *et al.* 2003; Deuve 2004) (Table S1; Fig. S1). Sampling also included populations of *C. problematicus* from Spain, France, Italy, Belgium, Great Britain, Czech Republic and Germany. Two specimens of *C. riffensis* from North Morocco, the sister group of European taxa (Andújar *et al.* 2012a), were used as outgroup in all analyses.

Sampling of molecular data

DNA was extracted and sequenced from 251 *Mesocarabus* specimens from 137 localities (Table S1) using the methods described by Andújar *et al.* (2012a). Nine DNA fragments were sequenced, corresponding to seven ribosomal and protein coding genes from the mitochondrial (*nd5, cox1*-a*, cox1*-b *cob, rrnL*) and nuclear (*LSU*-a*, LSU*-b*, ITS2, HUWE1*) genomes, with a total character length of approximately 6700 nt (Table S2 shows accession numbers and Table S3 primer information). The 3'-end of the locus *HUWE1* includes a fragment of a length-variable intron; heterozygotic sequences for this marker were excluded from the analyses and occasional double peaks in chromatograms of the other markers were coded using the IUPAC ambiguity code.

Sequence alignment, data combinability and phylogenetic analyses

Protein coding DNA sequences were aligned using MAFFT 6.240 (Katoh *et al.* 2002) with the L-INS-i method, while rRNA genes were aligned using Q-INS-i (Katoh & Toh 2008), a structural-aided alignment algorithm known to outperform non-structural methods (Letsch *et al.* 2010). Correct amino acid translation of protein coding genes was checked in MEGA 4 (Tamura *et al.* 2007). Concatenated matrices were initially obtained by combining (1) five mitochondrial gene fragments (*MIT*: 251 specimens, 3684 nt), (2) three nuclear ribosomal fragments (*RIB*: 206 specimens, 2373 nt), (3) all nuclear fragments, i.e. *RIB* plus *HUWE1* (*NUC*: 241 specimens, 3016 nt) and (4) all genes (*ALL*: 251 specimens, 6700 nt). Each concatenated dataset included only specimens with at least three loci available. Data combinability was assessed using a sequential procedure, first testing for phylogenetic congruence among DNA fragments and subsequently testing for recombination within phylogenetically congruent datasets. Individual and concatenated datasets with mutually consistent phylogenetic signal were objectively identified by exploring their fit to optimal tree topologies obtained for alternative datasets using SH tests (Shimodaira & Hasegawa 1999), as implemented in PAUP* 4.0 (Swofford 2003) with 1000 replicates and the RELL option. Tree topologies were

subsequently obtained using Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) for each individual and concatenated dataset, partitioning by gene (but not codon position, due to low variation in first and second codon positions; Andújar *et al.* 2012a) and with their optimal model of evolution as estimated using jModelTest (Posada 2008) under the Akaike information criterion. The analyses consisted of two independent runs for 50 million generations, sampling trees every 1000 generations. The standard deviation of split frequencies and the mean and effective sampled size (ESS; after removing the initial 25% of trees) of likelihood values computed with Tracer 1.5 (Rambaut & Drummond 2007) were checked to assess the convergence of results. The strict consensus trees were obtained discarding 25% of the initial trees and node posterior probabilities (PP) were interpreted as support values. Finally, for each set of phylogenetically congruent markers, we tested for the presence of recombination using both the PHI test (Bruen *et al.* 2006) as implemented in SplitsTree 4.13.1 (Huson & Bryant 2006), and the methods RDP (Martin and Rybicki 2000), GENECONV (Padidam *et al.* 1999) MAXCHI (Maynard Smith 1992) and CHIMAERA (Posada and Crandall 2001) as implemented in RDP4 (Martin *et al.* 2010) with Bonferroni correction and a highest acceptable *P-value* of 0.05 as recommended. Concatenated data were further split in non-recombining subsets if evidences of recombination were found.

Molecular phylogenetic and dating analyses

Bayesian calibration analyses were run in BEAST 1.6.1 (Drummond & Rambaut 2007) for each subset of congruent data. The age of the root was set at 10.9 Ma (95% confidence interval: 8.9-13.3; gamma prior: shape=68.49, scale=0.1604) for the Miocene split between *C. riffensis* and European *Mesocarabus* (Andújar *et al.* 2012b, 2014). Two independent runs of 50 million generations sampling every 8000 generations were performed on each analysis, using a constant size coalescent prior, an uncorrelated lognormal clock and partitioning by gene. The best fitting model of evolution as obtained in jModelTest was applied to each gene. Additionally, every nucleotide substitution class was modelled

empirically (Andújar *et al.* 2012b) as a prior uniform probability function ranging from 0 to 10, the rate of molecular evolution between 0 and 1. Trace plots and ESS of likelihoods were checked using Tracer 1.5 to assess the convergence of independent runs. Subsequently, trees were pooled removing 25% of samples as initial burn-in, and consensus trees were obtained using median values for branch lengths in LogCombiner 1.5.4 and TreeAnnotator 1.5.4 (Drummond & Rambaut 2007). Node posterior probabilities were interpreted as support values.

Additionally, maximum likelihood (ML) analyses were conducted in RAxML 7.0.3 (Stamatakis 2006) on the selected datasets with a GTR+G model for each gene partition and 100 random starting trees runs to find the optimal topology. Node support (BS) was calculated with 1000 bootstrap pseudoreplicates and the same tree search strategy. RAxML analyses were run on CIPRES (Miller *et al.* 2010)

Cladistic analysis of aedeagal characters

The last abdominal segments, including genital organs, of 184 male specimens from 116 localities (Table S1) were digested overnight with 1 ng/µL Protein kinase K (Promega, Madison, USA). The endophallus was everted from the median lobe of the aedeagus by injecting toothpaste through the basal orifice (Berlov 1992) and it was subsequently dried and preserved (ZAFUMU coll.). Lateral and sagittal images of the everted sac were taken with a Spot Insight Firewire digital camera (Sterling Heights, USA) adapted to a Zeiss Stemi 2000C Trinocular Zoom Stereomicroscope (Thornwood, USA). Description of inflated endophalli followed Ishikawa's (1978) terminology, modified for *Mesocarabus* by Anichtchenko (2004). Specimens were classified based on eleven characters related to presence/absence and shape of lobes of the endophallus and the distal part of the penis (Fig. S2). Cladistic analyses were conducted in PAUP* with 100 random sequence addition heuristic searches for the most parsimonious trees, with TBR branch swapping. An initial analysis was run with unweighed

characters and unordered state transformations of binary and multistate characters. Character weights were estimated *a posteriori* using the maximum value of the rescaled consistency index and iterative analyses were conducted until character weights stabilized (Farris 1989; Carpenter 1988). *C. riffensis* was used as outgroup and the strict consensus tree of all most parsimonious trees was computed. Node support was assessed using 1,000 bootstrap pseudoreplicates (Felsenstein 1985).

Morphometric study of pronotal shape

Morphometric elliptic Fourier analyses of dorsal outlines of pronota were conducted for 297 male specimens from 154 localities (Table S1). Digital images of each pronotum were obtained as before. Pronotal dorsal profiles were automatically extracted and used to calculate normalized Elliptic Fourier Descriptors (EFDs) for 60 Fourier harmonic ellipses using the *ChainCoder* and *Chc2Nef* programs in the Shape 1.3 package (Iwata & Ukai 2002). EFDs were normalized based on the first harmonic ellipse in order to standardize size, orientation and the starting point for tracing the contour of pronotum relative to the major axis of the first ellipse. Principal Component Analyses (PCA) and inverse Fourier reconstructions allowed visualizing shape changes associated to each PCA component, using all EFDs coefficients with *PrinComp* (Iwata & Ukai 2002). The statistical evaluation of differences in pronotal characteristics relative to species hypotheses was attempted based on MANOVA analyses for the main PCA components. In addition, ANOVA and *post-hoc* tests with Tukey's correction for each PCA component were used to compare pairs of candidate species. All analyses were conducted in R with the library 'stats' (R Development Core Team 2010).

Niche modeling

Climatic data were obtained at a spatial resolution of 2.5 arc-minutes from WORLDCLIM 1.3 (http://www.worldclim.org; Hijmans *et al.* 2005). To avoid problems due to multi-colinearity of

predictive variables in the modeling process, Spearman correlations (R≥0.9) were used to reduce the number of bioclimatic variables in the studied region, i.e. including the entire distribution range of *Mesocarabus* (latitude: 72.0N-33.5N; longitude: 25.0E-26.5W). The records for species occurrence were the same sampling localities of the specimens used in the previous morphological and molecular analyses (215 localities; Table S1) and 75 additional localities obtained from the literature and the GBIF database (http://www.gbif.org/; Table S4). We used MaxEnt (Phillips *et al.* 2006) to estimate potential distributions of candidate species. Niche overlap between pairs of candidate species with contiguous distributions was calculated using those potential distribution estimates and ENMTools (Warren *et al.* 2010) based on Schoener's (1968) D metric, ranging from 0 (no niche overlap) to 1 (full niche overlap). Because niche differences may result from spatial autocorrelation of the explanatory environmental variables (i.e., background environmental divergence; Warren *et al.* 2008), we conducted the background similarity test procedure as implemented in ENMTools. The "background area" of each candidate species was adjusted to the habitat available for each studied entity (Warren *et al.* 2010), defined here as the known distributional area of each putative taxon.

Integrative taxonomic approach

Incongruence across different sources of data conveys information about the evolutionary history of the studied lineages and therefore is relevant for species delineation. Assuming an underlying evolutionary concept of species (Simpson 1961), we propose a sequential three-steps procedure for species delimitation based on the general structure suggested by Schlick-Steiner *et al.* (2010) but with explicitly defined steps to enable investigation of any system with character incongruence resulting from intricate evolutionary histories. This species delimitation procedure includes: (1) objective delineation of candidate species based on *integration of incongruence* among lines of evidence capable of defining groups (termed *conclusive disciplines* by Schlick-Steiner *et al.* 2010), (2) corroboration of candidate species by incorporating *inconclusive* (*sensu* Schlick-Steiner *et al.* 2010)

lines of evidence, and (3) integrated characterization of candidate species using an evolutionary framework including all lines of evidence, with a final explicit proposition of species.

In the first step, specimens are grouped based on each independent line of evidence according to an explicit criterion. In our case, the anchor to delimit groups were taxonomic attributions and the groups were the main supported clades obtained from phylogenetic analyses of mitochondrial, nuclear ribosomal, and *HUWE1* data as well as the morphology of the aedeagus. Subsequently, based on the obtained patterns, the specimens are clustered across all lines of evidence (i.e., these showing a unique and the same combination of group assignments) and considered as members of the same candidate species. In our case, candidate species were also corroborated using SpedeSTEM (Ence & Carstens 2011) based on the phylogenetic trees of mutually congruent datasets. This approach estimates both the species tree and support for proposed candidates species in a single analysis based on the maximum likelihood of coalescent models (Carstens *et al.* 2013).

In the second delimitation step, candidate species are further assessed by incorporating inconclusive lines of evidence, aiming at identifying additional support for their separation. For example, pronotal morphometry and ecological niche similarity do not define groups in the case of *Mesocarabus*. In the last step, information about all lines of evidence is integrated in an evolutionary framework interpreting the potential processes generating the observed phylogenetic patterns. Ideally, individuals grouped in each candidate species (i) are monophyletic for a particular marker, (ii) show coherent geographic ranges, and (iii) show significant differences in ecological, morphological, behavioural or physiological traits. This systematic procedure leads to a final species proposal that takes into account incongruence and the plausible evolutionary history for the group, which critically helps to interpret the observed inconsistence among characters. In other words, knowledge about the evolutionary history of candidate

species becomes an integral part of their delimitation, and conflict is resolved by means of evolutionary criteria.

Results

Molecular data and trees

A summary of the characteristics of aligned sequence data is shown in Table 1. Protein coding mitochondrial genes showed no length variation, whereas the *rrnL* mitochondrial ribosomal fragment ranged from 727 to 729 nt, therefore requiring two gapped positions in the aligned matrix. The optimal substitution model for every mitochondrial gene and their combination was a GTR+G+I. The *LSU*-a nuclear ribosomal fragment showed no length variation and the observed variability was best described under a GTR model of evolution. In turn, *LSU*-b (844-880 nt) and *ITS2* (515-541 nt) alignments required 50 and 33 gapped positions, respectively, and were both consistent with a GTR+G model of evolution. The nuclear protein coding *HUWE1* gene showed a stop codon and eleven gapped positions close to its 3'-end; the best substitution model for this marker was a GTR+G+I.

SH tests revealed no significant incongruence among individual mitochondrial genes and the combined *MIT* or *ALL* Bayesian consensus trees, or among individual nuclear ribosomal gene data and the combined *RIB* and *NUC* trees ($P>0.05$ in every case). However, individual and combined mitochondrial datasets were significantly incongruent with *NUC* and *RIB* phylogenies ($P<0.001$), and nuclear datasets were incongruent with the *MIT* tree ($P<0.001$) and the *ALL* tree ($0<P<0.028$; except for *LSU*-a gene: $P=0.294$). The *HUWE1* gene dataset was incongruent with *MIT*, *RIB*, *NUC* and *ALL* phylogenies ($P<0.001$), and all datasets were incongruent with the *HUWE1* tree ($P<0.001$) (Table 2). In turn, the PHI test failed to find evidences for recombination among mitochondrial fragments (*MIT,* $P=0.9997$) or within each of the *LSU*-a, *LSU*-b and *ITS2* fragments ($P>0.05$). However, the PHI test

resulted in significant evidence for recombination in the three ribosomal DNA fragments (*RIB*, *P*=0.0054), and specifically in the pairwise combination of *LSU*-a and *LSU*-b (*P*=0.0004), but not in the combination of *LSU*-b with *ITS2* (p=0.5018). This pattern appears to be related to the order in which these respective fragments appear in the genome. Conversely, RDP, GENECONV, MAXCHI and CHIMAERA tests do not detect any statistically significant event of recombination (*P-value* with Bonferroni correction > 0.05). Because of the non conclusive results about the existence of recombination in the *RIB* dataset, we duplicate further analyses using two partition schemes: i) including three independent datasets; *MIT, RIB* and *HUWE1* datasets; and ii) including four independent datasets; (1) *MIT*, *LSU*-a, *LSU*-b+*ITS2* and *HUWE1*. Phylogenetic analyses resulted in the same candidate species for both partition schemes, and in the following text we refer to the three-dataset partition in the main text (Figs 1 and S3-S5), while results for the four-dataset partition are shown in Supplementary Materials (Fig. S6-S9).

Phylogenetic analyses on the combined mitochondrial *MIT* dataset resulted in a tree with high support for most nodes and generally matching the geographic source of samples (Figs 1a and S3). Clade A_{MIT} (PP=1.00; BS=100%) clustered all samples of *C. dufourii dufourii*. Clade B_{MIT} (PP=1.00; BS=100%) included all *C. macrocephalus* except for most specimens of the subspecies *barcelecoanus* and *breuningi*. The remaining specimens constituted a clade C_{MIT} (PP=1.00; BS=89%) that clustered all the samples of *C. problematicus* and *C. lusitanicus*, plus most specimens of the previously mentioned subspecies of *C. macrocephalus.* Within clade C_{MIT}, up to eleven geographically concordant clades received high support (Clades $C1_{MIT}$-$C11_{MIT}$; PP=1.00; BS>98%). For instance, the samples of *C. problematicus* were grouped in three different clades: $C2_{MIT}$ from the eastern Pyrenees, $C7_{MIT}$ from the western Pyrenees, and $C1_{MIT}$ from other European localities. *C. dufourii baguenai* from southeastern Iberia constituted clade $C10_{MIT}$, sister to a parapatric clade $C11_{MIT}$, including most *C. lusitanicus helluo* and *C. lusitanicus albarracinus* specimens from eastern Iberia. Most specimens of *C. macrocephalus breuningi* formed clade $C4_{MIT}$, and those of *C. macrocephalus barcelecoanus* and *C.*

macrocephalus macrocephaloides were clustered in clade $C6_{MIT}$, together with the subspecies *bolivari, brevis* and *lusitanicus* of the *C. lusitanicus* complex (Fig. 1a).

Bayesian and maximum likelihood phylogenetic analyses for *RIB* dataset showed four main highly supported clades corresponding to European populations of *Mesocarabus* (Figs 1b and S4). Clade A_{RIB} (PP=0.95; BS=78%) grouped all *C. problematicus* specimens, while all the Iberian samples of *Mesocarabus* clustered as the sister group with high support (PP=0.93; BS=99%). Among Iberian *Mesocarabus,* clade B_{RIB} (PP=1.00; BS=93%) clustered all specimens of *C. dufourii*, with those classified in the subspecies *baguenai* forming a well-supported lineage (PP=1.00; BS=97%). Clade C_{RIB} (PP=0.99; BS<50%) included all *C. macrocephalus* specimens. Within this clade, the populations of *C. macrocephalus breuningi* south of Miño and Sil rivers (North Portugal and Ourense province) were retrieved as a highly supported group (clade $C1_{RIB}$; PP=1.00; BS=87%), the same as the specimens of the subspecies *barcelecoanus* (clade $C2_{RIB}$; PP=1.00; BS<50%). Finally, clade D_{RIB} (PP= 0.99; BS<50%) grouped all subspecies of *C. lusitanicus*.

Phylogenetic analyses of the *HUWE1* gene retrieved a Clade A_{HUWE} (PP=0.91; BS<50%) grouping all the specimens of *C. problematicus*, a clade B_{HUWE} (PP=0.98; BS=76%) clustering all of *C. dufourii dufourii*, and a clade C_{HUWE} (PP=1.00; BS=98%) with all of *C. dufourii baguenai* and three specimens of *C. lusitanicus helluo* (Figs 1c and S5). Deeper nodes and the remaining clades received low support. Overall, the comparison of the obtained trees showed that incongruence mainly affected the phylogenetic positions of lineages including specimens classified as *C. dufourii baguenai* Breuning*, C. macrocephalus breuningi* Csiki, *C. macrocephalus barcelecoanus* Vacher de Lapouge, *C. macrocephalus macrocephaloides* Jeanne and *C. problematicus* (Fig. 1).

Cladistic analysis of aedeagal characters

A total of eleven parsimony informative characters were identified, including four binary and seven multistate characters describing the presence and shape of particular lobes of the endophallus and anatomical features in the apical area of the penis (Table S5 and Fig. S2). Parsimony analysis of the resulting data matrix (Table S6) yielded six most parsimonious trees (length=29 steps; consistency index=0.62; retention index=0.98), and their strict consensus as well as the geographic distribution of the main clades obtained is shown in Fig. 2, highlighting bootstrap support values above 50%.

The apex of the aedeagi of *C. riffensis* and *C. problematicus* were clearly differentiated from these in specimens of populations endemic to the Iberian Peninsula. The Iberian clades, albeit some with low statistical support, corresponded to populations identified as (1) *C. dufourii* (round short apex and lobe V entire), (2) *C. lusitanicus* clade (longer, less rounded apex and divided ventral lobe V), (3) *C. macrocephalus* clade (very short apex, entire lobe V and rounded lobe VB), (4) *C. macrocephalus breuningi* clade, grouping populations from North Portugal and South Galicia (genitalia similar to that of *C. lusitanicus* but with sharper apical angle of median lobe, less prominent ligule and reduced dorso-apical lobes), and (5) *C. macrocephalus macrocephaloides* clade including specimens from Puerto de Lizárraga (penis with intermediate characteristics between those found in specimens of the *C. macrocephalus* and *C. lusitanicus* clades) (Fig. 2; Table S7).

Step-wise species delimitation

1. Candidate species delimitation using conclusive evidence. Figure 3 shows a summary of groups obtained for *Mesocarabus* using phylogenetic criteria for each independent line of evidence (ILE). The initial set of five hypothetical species was grouped in different ways for each character system, either in a congruent (e.g., each ILE relative to *C. riffensis*) or in an incongruent fashion. Incongruence

necessarily produced two alternative outputs (or combinations of both): split of an initial hypothesis (e.g., ILE1 and ILE3 for *C. dufourii*) or merging of hypotheses (e.g., ILE3 for *C. lusitanicus* and *C. macrocephalus*). Specimens sharing a unique combination of characters (i.e., combination of group assignments) defined by these lines of evidence were clustered and proposed as nine candidate species. Five matched nominal species as defined by the current taxonomy of *Mesocarabus* (*C. dufourii, C. lusitanicus, C. macrocephalus*, *C. problematicus* and *C. riffensis*; candidates *dufourii, lusitanicus, macrocephalus*, *problematicus* and *riffensis* respectively) and the other four candidate species grouped mainly specimens assigned to named subspecies (*C. dufourii baguenai, C. macrocephalus barcelecoanus*, *C. macrocephalus breuningi* and *C. macrocephalus macrocephaloides;* candidates *baguenai*, *barcelecoanus*, *breuningi* and *macrocephaloides* respectively). SpedeSTEM analyses supported this decision, with the model considering nine candidate species (LnL=-40359.01) significantly better (AIC=10.60) than the best nested model considering eight species (LnL=-40365.32). Two levels of conflict among characters appeared in this ranking of candidate species: (1) discrepancies in group boundaries among the character systems, with individuals within a single candidate species (*riffensis*) appearing as monophyletic for the four datasets, and (2) a reticulated pattern of mtDNA relationships among the groups resulting from this marker and those retrieved by other lines of evidence. The latter is not problematic for species delimitation but it is extremely important to understand the evolutionary scenario that originated the independent evolutionary lineages in *Mesocarabus*. The circumscription of the former type of conflict to a particular subset of (taxonomically coherent) samples and seemingly generated by mtDNA data, is suggestive of these being introgressed samples.

2. Corroboration of candidate species with inconclusive evidence. Candidate species defined in the previous step were corroborated against morphological and ecological traits, assessing for statistically significant differences among groups within each of the hypotheses of sample subdivision (Fig. 3).

Candidate species *macrocephaloides* included a single population, and it was therefore excluded from statistical analyses.

The first three components of pronotal shape ordination captured 85.8% of shape variation. The effect of these three components was visualized using inverse reconstructions with mean and extreme values (±2 standard deviation for each principal component) (Fig. S10). The first axis mainly explained the variance in the pronotal width/length ratio; the second axis was related to the curvature of the apical margin while the third axis mostly reflected variance in the prominence of the basal lobes of the pronotum. MANOVA analyses including these first three PCA components as variables revealed significant differences in pronotal shape among the European candidate species (P<0.001; Table 3). The integrated results of post-hoc tests using Tukey's correction with a 95% confidence level for the three axes showed significant differences in pronotal shape for all candidate species with the exception of candidates *macrocephalus* and *barcelecoanus* which were indiscernible (Figs 4 and S11).

Projected maps using the MaxEnt algorithm generated potential distribution data for each candidate species (Fig. S12). Niche divergence was found between candidates *lusitanicus* and both *macrocephalus* and *dufourii* (P=0.09 and 0.02, respectively; Figs 4 and S13). Conversely, niche conservatism was found in the other paired comparisons ($P\leq$0.1), all of them involving candidate species affected by phylogenetic incongruence.

3. Data integration and species delimitation. The status of candidate species was further assessed by integrating all the available information in an evolutionary framework (Fig. 5). As discussed below, these results are the basis for a final proposition of six European species of *Mesocarabus.* Four of them agree with these more widely accepted based on traditional taxonomy: *C. problematicus*, *C.*

macrocephalus, *C. dufourii* and *C. lusitanicus*. Two additional species with a putative hybrid origin and initially described as subspecies of *C. lusitanicus* are hypothesized: *C. breuningi* Csiki 1927 **stat. nov.** and *C. baguenai* Breuning 1926 **stat. nov.** Two candidate species are discarded as valid species: candidate *barcelecoanus* is recognized as a population of *C. macrocephalus* affected by mitochondrial DNA introgression from *C. lusitanicus*, and candidate *macrocephaloides* is recognized as a population of *C. lusitanicus* (*C. lusitanicus macrocephaloides* (Jeanne 1972) **comb. nov.**) characterized by morphological and genetic features revealing the possibility of hybridization with nearby populations of *C. macrocephalus.*

Discussion

Hybridization as a consistent source of taxonomic conflict in Mesocarabus

The multidisciplinary study of non-random character incongruence patterns provides the key to understanding the evolutionary history of species diversification and assisting in objective species diagnosis. The applicability of congruence-based protocols to species delineation in taxa subject to complex evolutionary processes has been questioned (Valdecasas *et al.* 2008; Cardoso *et al.* 2009), and only few recent proposals have considered them explicitly (e.g., Schlick-Steiner *et al.* 2010). Our study on *Mesocarabus* illustrates how the integrative exploration of these divergent patterns can be very important to attempt the recognition of independent evolutionary units (i.e., species in this case). Morphological and nuclear gene-based groups showed two types of discrepancy with groups based on mitochondrial genes, both in the recognition of limits between groups and the relationships among these groups (Fig. 3). An operational inclusion of conflict among conclusive lines of evidence resulted in the recognition of nine putative species among European *Mesocarabus*, four of them in good agreement with traditional species-level taxonomic assignments (Fig. 3), but also identified particular types of conflict and the specific groups of samples affected. Specimens responsible for incongruence among independent lines of evidence are always found in intermediate geographic locations between

the ranges of main clades diagnosed and hypothesized as species, a situation that fits the expectation for hybridization events (Hare & Avise 1998; Gómez-Zurita & Vogler 2006). The integrated study of multiple lines of evidence from an evolutionary perspective allows us to propose that hybridization is a frequent process in the diversification of *Mesocarabus*, where we could pinpoint the different stages in the continuum of the speciation process where hybridization can eventually lead to entities recognizable as species, such as *C. baguenai* and *C. breuningi*.

The combined evidence supports the recognition of *C. baguenai* as a lineage of mosaic origin found in the contact zone between *C. dufourii* and *C. lusitanicus*, an idea reinforced by the morphometric analysis of pronotal shape, which places *C. baguenai* at a significantly different but intermediate position between the other two species (Fig. S11). A similar scenario applies to *C. breuningi*, which is found in the contact zone between two well-differentiated species of *Mesocarabus* (*C. lusitanicus* and *C. macrocephalus*), concomitant with intermediate or mosaic morphological and genetic characteristics. Both species showed significant phylogenetic conflict between mtDNA and rRNA data and have been recorded in taxonomic literature as problematic intermediate forms between otherwise well-characterized species (Toulgoët & Lassalle 1983; Anichtchenko 2004). Fixed, diagnostic genetic differences and gene monophyly for several molecular markers (interpreted as the result of long interruption of gene flow with other populations of *Mesocarabus*), morphological differences relative to any other *Mesocarabus* species in characters of the aedeagus and pronotum, and a relatively deep Pleistocenic divergence of both lineages, are consistent with their treatment as separate species, and ensemble evidence points at interspecific hybridization explaining their evolutionary origin.

C. macrocephalus barcelecoanus and *C. macrocephalus macrocephaloides* exemplify an intermediate stage between the evolutionary consolidation of independent taxa of hybrid origin (as proposed for the species above) and ongoing hybridization with local introgression (as in the cases described below).

These two lineages, recognizable from a morphological point of view and with intermediate phenotypes between otherwise well-established species, bear the signal of their hybrid origin in the form of classic mitochondrial capture (García-París *et al.* 2003), but they do not show so clear distinctive traits compelling their treatment as independent evolutionary units, as described for *C. baguenai* and *C. breuningi*.

Finally, there are a few other examples of conflict in our dataset that support introgressive hybridization as a common phenomenon in the evolution of *Mesocarabus*. The samples of *C. problematicus*, a candidate species diagnosed by nuclear and morphological characters, grouped into three geographically congruent mitochondrial groups: one north from the Pyrenees, one in the eastern and one in the western Pyrenees. The latter clade (from Ochagavía, specimens 1513-1514) is nested with high support and only for mtDNA data as sister to the geographically contiguous eastern Iberian clade of *C. lusitanicus*. Natural hybridization between *C. problematicus* and *C. lusitanicus* seems possible because artificial interspecific crosses have been successfully obtained in the laboratory (Puisségur 1987; Deuve 2004) and, in fact, some specimens determined as morphological hybrids between these two species were cited from localities in the southern Pyrenees and the Cadena Catalana (Mollard 2011). Another specimen, classified as *C. lusitanicus latus* from La Calahorra, Granada (sample 1400) falls among other samples of this subspecies inhabiting the Betic chains for nuclear genes, but its mtDNA is clearly nested within *C. baguenai*. This sampling locality is precisely in the contact region where *C. lusitanicus, C. dufourii* and *C. baguenai* meet. Finally, two localities in the contact zone between *C. baguenai* and the eastern Iberian lineage of *C. lusitanicus*, Sierra Espuña and Sierra de la Pila, include one individual each (55 and 513, respectively) with conflicting signal for mtDNA (of *C. lusitanicus*) and nuclear genes (of *C. baguenai*). In this example of mitochondrial capture, and conversely to most other cases discovered so far in *Mesocarabus*, mtDNA does not show any divergence relative to parental populations, and in the same localities there are specimens fully concordant for every marker tested and compatible with their inclusion in *C. baguenai*. These

observations are consistent with recent or ongoing gene flow in this contact zone between *C. lusitanicus* and *C. baguenai*, species with overlapping niches (Fig. 5). These latter examples do not have in principle taxonomic implications, not even in the context of an integrative approach exploiting character conflict as attempted here, but offer additional support to the hypothesis of hybridization and introgression driving diversification in *Mesocarabus*.

Natural history of introgressive hybridization

Two intriguing and perhaps related patterns emerge from the detailed analysis of conflict in *Mesocarabus*. First, for every recorded instance of mtDNA introgression, *C. lusitanicus*, central to the other five peripheral species, acts as the donor. Second, while there is statistically significant niche segregation among *C. lusitanicus* and two of the other three canonical candidate species (*C. dufourii* and *C. macrocephalus*), there is niche conservatism with the two candidate species of putative hybrid origin (*C. baguenai* and *C. breuningi*) as well as with the introgressed populations of *C. problematicus* and *C. macrocephalus barcelecoanus*. It seems that there are evolutionary processes favouring the introgression of *C. lusitanicus* mtDNA across the contact zones between this and the other species of *Mesocarabus*. This asymmetric pattern of introgression has been reported for other taxa, e.g., the mountain hare in the Iberian Peninsula (Alves *et al.* 2008), and several non-exclusive explanations can be hypothesized which will require specific testing. Biased sex dispersal can explain unidirectional mtDNA introgression in scenarios of competitive replacement at the front of range expansions (Alves *et al.* 2008; Petit & Excoffier 2009; Currat & Excoffier 2012). Data on the dispersal activity of western European *Carabus* are consistent with males showing higher dispersal power, particularly during the breeding season (Rijnsdorp 1980; Dülge 1994; Drees & Huk 2000). Thus, the observed pattern could be explained by recurrent migration and gene flow of males from peripheral species over a *C. lusitanicus* mtDNA background, implying that males of the former species are more aggressive colonizers. Another possibility is that there are physiological and/or behavioural factors that

asymmetrically influence mating among evolutionary lineages (Funk & Omland 2003). In this case, data would suggest that the females of *C. lusitanicus* are more tolerant to allospecific mating compared to females of the related species. Alternatively, there might be positive natural selection favouring the *C. lusitanicus* mitochondrial type in the contact zones, as it has been shown that mtDNA is not always a neutral marker (Ballard & Whitlock 2004). However, a Tajima's D neutrality test for all the mtDNA data and the subsets from candidate species produced non-significant results in each case. Positive selection and physiological/behavioural asymmetries could produce patterns of genetic diversity characteristic of range expansion, and consequently are hardly distinguishable without complementary further physiological and cross-mating experiments (Alves *et al.* 2008). Moreover, this type of disequilibrium in hybrid populations has been also explained in some cases by epistatic effects due to genetic associations between cytoplasmic and nuclear genomes, which may in turn have additive effects on disequilibria caused by both assortative mating and selection (e.g., Cruzan & Arnold 1999).

Of the canonical candidate species, *C. lusitanicus* is one of the youngest splits in the phylogeny of *Mesocarabus* (Andújar *et al.* 2012a). Yet it has a considerably large distribution range in the central portion of the Iberian Peninsula, covering a variety of habitats from sea level to high altitudes, and cornering the other species to the periphery of Iberia, with non-overlapping and stricter niche requirements. This could suggest a direction of introgression gradually towards the ranges of the peripheral species. Indirect evidence provided by hybrid candidate species and also the introgressed populations of *C. macrocephalus barcelecoanus* suggest instead a different alternative.

Putative hybrid taxa and lineages in *Mesocarabus* show significant niche conservatism with both parentals (Fig. 5), while the latter significantly diverge in their niches. This may bestow an ecological selective advantage due to intermediate niche tolerance of hybrid taxa and populations (Anderson

1948; Lexer *et al.* 2003). If this were the case, the direction of the introgression (carried by these individuals) would be that of the range expansion of the hybrid lineage across ecologically intermediate and/or extreme areas for parentals. Thus, the observed geographic pattern would not be the result of introgression of the *C. lusitanicus* mitochondrial type by secondary backcrossing with one of the parentals, but the result of the expansion of the hybrid species from the initial contact zone where successful hybridization took place (in any case, a testable hypothesis). Relevant to the taxonomic issues discussed here, it should be noted that the hypothesized potential superiority of hybrid taxa in intermediate or different, maybe extreme environments for the parentals ("hybridized habitats"; Anderson 1948; Lexer *et al.* 2003) would actually favour the speciation process, despite of lack of barriers to gene flow and interbreeding with parental species (Moore 1977; Mallet 2007).

Integration of conflict into integrative taxonomy

Integrative taxonomy has been championed mainly as an exercise of data corroboration, which is both a logical and philosophically robust approach (Padial *et al.* 2010). However, proposals of integration by congruence and cumulation (and derived protocols) relegate conflict to an unavoidable nuisance intrinsic to biological diversity or specific methodology (Sites & Marshall 2004; Padial *et al.* 2009; Miralles *et al.* 2011), or in some cases conflict is not even considered explicitly (Bond & Stockman 2008). Moreover, in the search for congruence among character systems, the problem of lack of resolution affecting independent lines of evidence has been frequently and arguably interpreted as conflict (e.g., Leaché *et al.* 2009; Padial *et al.* 2010). Often, as it also happens in our study, real conflict in the data appears strongly associated to a specific line of evidence, typically mtDNA data. In this case, there is a certain inclination to water down the significance of this character for species delineation invoking the well-known litany of mtDNA limitations to reflect species trees: non-neutrality, horizontal transfer, stochastic lineage sorting and introgression (Dowling & Secor 1997; Funk & Omland 2003). This type of conflict is therefore ignored in the sense that it is at most

interpreted in the light of other evidence, but not incorporated explicitly in the working hypotheses of species limits (but see Gebiola *et al.* 2012, for an example of the opposite).

Here we argue that conflict arising from the evolutionary history of a speciating lineage has the potential to disentangle some details of this evolutionary process, and also to aid in the actual recognition of independent evolutionary units (Gómez-Zurita *et al.* 2012), in this case within the framework of integrative taxonomy. This is essentially the same proposition as put forward by Schlick-Steiner *et al.* (2010): when conflict is real and it can be linked to a particular evolutionary process of known effects and extent, it can be used to demarcate testable evolutionary units. However, these authors still treat conflict mostly as an undesirable property of data that needs to be resolved by corroboration with further disciplines. Here, we demonstrate that particular types of conflict, such as mtDNA introgression, should not be necessarily resolved by corroboration. Instead it should be critically incorporated in the delimitation of testable evolutionary units, providing that this type of conflict reflects the existence of new biological entities (hybrids, in this case), with full potential to be treated as independent evolutionary units. Consider, for instance, unisexual animals, generally originated by interspecific hybridization (Bullini 1994; Simon *et al.* 2003) which may never be resolved as independent evolutionary units if delimitation ignores or is aimed at finding ways to break the tie caused by genomic mosaicism. Solving conflict should be aided by evolutionary criteria as an integral part of species delimitation, to propose candidate species previous to corroboration, while current integrative taxonomy approaches tend to use them as an interpretative final stage (Padial *et al.* 2010).

Finally, the proposition of candidate species for subsequent corroboration contains a subjective element in deciding how to group specimens within each line of evidence, for example, choosing which hierarchical level in a tree is a meaningful group. Gene tree methods such as GMYC (Pons *et*

al. 2006) may offer an objective tree-based solution to this problem (e.g., Payo *et al.* 2013). We actually explored the potential of this method as a tool for candidate species delimitation in *Mesocarabus*. However, each independent genetic partition yielded unrealistic or problematic outputs with either massive oversplitting—e.g., 74 GMYC entities based on the *MIT* dataset—or results non-significantly better than the null, single-species model—*HUWE1* datasets—, and were consequently disregarded (Table S8). In any case, data has to be linked to an initial structure and this initial decision is important. We propose using species hypotheses based on morphospecies or on a taxonomic revision, but other alternatives are possible depending on prior knowledge on the group, including temporal (e.g., diversification before the Pleistocene) or geographic boundaries. Once initial groups are defined for each independent conclusive line of evidence, our procedure to delimit candidate species considers that conflict is objective, and that it represents a useful approach to assist proposing evolutionary meaningful candidates species. These can be used for further analyses, including the application of species-tree methods based on coalescence models, relying on *ad hoc* species hypotheses, or statistical analyses based on additional inconclusive evidence, as done here. Following this systematic approach potentially provides with a proper integrative framework to study complex species groups affected by recurrent introgressive hybridization.

Conclusions

The evaluation of multiple lines of evidence, the corroboration of conflict among them and their interpretation, also considering species ranges and quantitative traits, pointed to hybridization and concomitant genetic introgression as dominant processes in the evolution of *Mesocarabus*, processes well recognized as a nuisance for taxonomic assessment. Overall, we provided an unprecedented overview on the structure and evolution of an otherwise taxonomically controversial beetle lineage. The scrutiny of conflict revealed examples of successive stages along the continuum of hybrid diversification, from polymorphic populations possibly with current interspecific gene flow between

C. lusitanicus and *C. baguenai*, to local fixation of captured mtDNA and intermediate non-diagnosable phenotypes (hybrid zone between *C. lusitanicus* and *C. problematicus*), to regional spread of clearly hybrid types as illustrated by *C. macrocephalus barcelecoanus*, ending in genetically, morphologically and ecologically diagnosable independent evolutionary entities assimilated to species (*C. baguenai* and *C. breuningi*).

Our data represents an even sampling of sources of taxonomic evidence, but it is plausible that there are datasets where some lines of evidence outweigh the others. Our approach relies on phylogenetic hypotheses as practical summaries of data based on each conclusive independent line of evidence, thus makes irrelevant the number of characters which these trees were built from, and emphasizes on the origin and the biological meaning of evidences. Recognizing incongruence (or congruence) among these independent lines of evidence will have an effect in phrasing the candidate species, but their final proposal as a defensible species will depend on the subsequent interpretation of the whole available evidence. Integrative taxonomy and the effort to standardize taxonomic practice under a convenient and generally accepted evolutionary species concept represent a notable advance to the species problem. As befits an approach that is in its initial and exploratory stages, it will benefit from additional integration by incorporating both character and phylogenetic conflict and congruence to a best founded evolutionary species delineation. We expect our contribution to help in this global initiative.

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Data Accessibility

Final DNA sequence alignments, BEAST input files and normalized Elliptic Fourier Descriptors of pronotum outlines are available in Dryad (doi:10.5061/dryad.4g832). Genbank accessions for DNA sequences, calibrated phylogenetic trees obtained in BEAST for *MIT*, *RIB*, *LSU*-a*, LSU*-b+*ITS2* and *HUWE1* datasets, sampling localities, location of studied specimens, detailed phylogenetic trees, occurrence data used in climatic niche analyses and morphological data on aedeagus are uploaded as online Supplemental Materials.

Author Contributions

CA, PA, JS and JG-Z designed the research, CA and PA obtained the data, and CA, CR and PA conducted the analyses. CA and JG-Z led the writing and all authors contributed to the discussion of results and the writing.

References

Alves PC, Melo-Ferreira J, Frieyas H, Boursot P (2008) The ubiquitous mountain hare mitochondria: multiple introgressive hybridization in hares, genus *Lepus*. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**, 2831-2839.

Anderson E (1948) Hybridization of the habitat. *Evolution*, **2**, 1-9.

Andújar C, Gómez-Zurita J, Rasplus JY, Serrano J (2012a) Molecular systematics and evolution of the subgenus *Mesocarabus* Thomson 1875 (Coleoptera, Carabidae, *Carabus*) based on mitochondrial and nuclear DNA. *Zoological Journal of the Linnean Society,* **166**, 787-804.

Andújar C, Serrano J, Gómez-Zurita J (2012b) Winding up the molecular clock in the genus *Carabus* (Coleoptera: Carabidae): assessment of methodological decisions on rate and node age estimation. *BMC Evolutionary Biology,* **12,** 40.

Andújar C, Soria-Carrasco V, Serrano J, Gómez-Zurita J (2014) Congruence test of molecular clock calibration hypotheses based on Bayes factor comparisons. *Methods in Ecology and Evolutio,* **5**, 226-242.

Anichtchenko AV (2004) Notas taxonómicas sobre el subgénero *Mesocarabus* Thomson, 1875 (Coleoptera, Carabidae) de la Península Ibérica. Primera nota. *Boletín de la Sociedad Española de Entomología*, **28**, 89-103.

Arnold ML (1997) *Natural hybridization and evolution*. Oxford University Press, Oxford, UK.

Arribas P, Andújar C, Sánchez-Fernández D, Abellán P, Millán A. (2013) Integrative taxonomy and conservation of cryptic beetles in the Mediterranean region (Hydrophilidae). *Zoologica Scripta*, **42**, 182-200.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729-744.

Berlov O (1992) Preparati permanenti a secco dell'endofallo nel genere *Carabus* L. (Coleoptera, Carabidae). *Bollettino della Società Entomologica Italiana*, **124**, 141-143.

Bond JE, Stockman AK (2008) An integrative method for delimiting cohesion species: finding the population-species interface in a group of Californian trapdoor spiders with extreme genetic divergence and geographic structuring. *Systematic Biology*, **57**, 628-646.

Bruen TC, Philippe H, Bryant D (2006) A simple and robust statistical test for detecting the presence of recombination. *Genetics*, **172**, 2665-81.

Bullini L (1994) Origin and evolution of animal hybrid species. *Trends in Ecology and Evolution*, **9**, 422-426.

Cardoso A, Serrano ARM, Vogler AP (2009) Morphological and molecular variation in tiger beetles of the *Cicindela hybrida* complex: is an 'integrative taxonomy' possible? *Molecular Ecology*, **18**, 648-664.

Carpenter JM (1988) Choosing among multiple equally parsimonious cladograms. *Cladistics*, 4, 291-296.

Carstens BC, Pelletier TA, Reid NM, Satler JD (2013) How to fail at species delimitation. *Molecular Ecology*, **22**, 4369-4383.

Casale A, Kryzhanovskij OL (2003) Key to the adults. In: *The genus* Carabus *L. in Europe, a synthesis* (eds. Turin H, Penev L, Casale A), pp. 73–123. Co-published Pensoft, Sofia; European Invertebrate Survey, Leiden.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Sunderland, MA.

Cruzan M, Arnold M (1999) Consequences of cytonuclear epistasis and assortative mating for the genetic structure of hybrid populations. *Heredity*, **82**, 36-45.

Currat M, Excoffier L (2012) Modern humans did not admix with Neanderthals during their range expansion into Europe. *Plos Biology*, **2**, e421.

Dayrat B (2005) Toward integrative taxonomy. *Biological Journal of the Linnean Society*, **85**, 407-415.

Deuve T (2004) *Illustrated Catalogue of the Genus* Carabus *of the World (Coleoptera: Carabidae).* Pensoft, Sofia-Moscow.

Dowling TE, Secor CL (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593-619.

Drees C, Huk T (2000) Sexual differences in locomotory activity of the ground beetles *Carabus granulatus* L. In: *Natural history and applied ecology of carabid beetles* (eds. Branmayr P, Lövei GL, Brandmayr TZ, Casale A, Vigna Taglianti A), pp. 133-138. Pensoft, Sofia.

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Dülge R (1994) Seasonal activity of carabid beetles in wooded habitats in northwest (Coleoptera, Carabidae). In: *Carabid beetles: ecology and evolution* (Desender K, Dufrêne M, Loreau M, Luff ML, Maelfait JP), pp. 125-131. Kluwer, Dordrecht.

Ence DD, Carstens BC (2011) SpedeSTEM: a rapid and accurate method for species delimitation. *Molecular Ecology Resources*, **11**, 473-80.

Farris JS (1989) The retention index and the rescaled consistency index. *Cladistics,***5**, 417- 419.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783-791.

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, **34**, 397-423.

García-París M, Alcobendas M, Buckley D, Wake DB (2003) Dispersal of viviparity across contact zones in Iberian populations of Fire Salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. *Evolution,* **57**,129-43

Gebiola M, Gómez-Zurita J, Monti MM, Navone P, Bernardo U (2012) Integration of molecular, ecological, morphological and endosymbiont data for species delimitation within the *Pnigalio soemius* complex (Hymenoptera: Eulophidae). *Molecular Ecology*, **21**, 1190-1208.

Gómez-Zurita J, Vogler A (2006) Testing introgressive hybridization hypotheses using statistical network analysis of nuclear and cytoplasmic haplotypes in the leaf beetle *Timarcha goettingensis* species complex. *Journal of Molecular Evolution*, **62**, 421-433.

Gómez-Zurita J, Sassi D, Cardoso A, Balke M (2012) Evolution of *Cryptocephalus* leaf beetles related to *C. sericeus* (Coleoptera: Chrysomelidae) and the role of hybridization in generating species mtDNA paraphyly. *Zoologica Scripta*, **41**, 47-67.

Hare MP, Avise JC (1998) Population structure in the American oyster as inferred by nuclear gene genealogies. *Molecular Biology and Evolution*, **15**, 119-128.

Hey J (2006) On the failure of modern species concepts. *Trends in Ecology and Evolution.* **21,**447-450.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965-1978.

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, **23**, 254-67.

Ishikawa R (1978) A revision of the higher taxa of the subtribe Carabina (Coleoptera, Carabidae). *Bulletin of Natural Science Museum, Ser.A (Zool)*, **4**, 15-68.

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.

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, **30**, 3059-3066.

Katoh K, Toh H (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics*, **9**, 212.

Leaché AD, Koo MS, Spencer CL, Papenfuss TJ, Fisher RN, McGuire JA (2009) Quantifying ecological, morphological, and genetic variation to delimit species in the coast horned lizard species complex (*Phrynosoma*). *Proceedings of the National Academy of Sciences USA,* **106**, 12418-12423.

Letsch HO, Kück P, Stocsits RR, Misof B (2010) The impact of rRNA secondary structure consideration in alignment and tree reconstruction: simulated data and a case study on the phylogeny of hexapods. *Molecular Biology and Evolution,* **27**, 2507-2521.

Lexer C, Welch ME, Raymond O, Rieseberg LH (2003) The origin of ecological divergence in *Helianthus paradoxus* (Asteraceae): selection on transgressive characters in a novel hybrid habitat. *Evolution*, 57, 1989-2000.

Mallet J (2005) Hybridization as an invasion of the genome. *Trends in Ecology & Evolution*, **20**, 229–37.

Mallet J (2007) Hybrid speciation. *Nature*, **446**, 279–83.

Mallet J (2008) Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society B,* **363,** 2971-2986.

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P. (2010) RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics,* **26**, 2462-2463

Martin D, Rybicki E. (2000) RDP: detection of recombination amongst aligned sequences. *Bioinformatics,* **16**, 562-563.

Maynard Smith J. (1992) Analyzing the mosaic structure of genes. *Journal of Molecular Evolution*, **34**, 126-129.

Miller M, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway Computing Environments Workshop (GCE)* , pp. 1–8. New Orleans, LA.

Miralles A, Vasconcelos R, Perera A, Harris DJ, Carranza S (2011) An integrative taxonomic revision of the Cape Verdean skinks (Squamata, Scincidae). *Zoologica Scripta*, **40**, 16-44.

Mollard A (2011) *Le sous-genre* Carabus (Mesocarabus) *Thomson, 1875 en péninsule Ibérique*. Acorep, Paris.

Moore WS (1977) An evaluation of narrow hybrid zones in vertebrates. *The Quarterly Review of Biology*, **52**, 263-277.

Padial JM, Castroviejo-Fisher S, Kohler J, Vila C, Chaparro JC, De la Riva I (2009) Deciphering the products of evolution at the species level: the need for an integrative taxonomy. *Zoologica Scripta,* **38**, 431-447.

Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. *Frontiers in Zoology*, **7**, 16.

Padidam M, Sawyer S, Fauquet CM. (1999). Possible emergence of new geminiviruses by frequent recombination. *Virology*, **265**, 218-225.

Payo DA, Leliaert F, Verbruggen H, D'hondt S, Calumpong HP,De Clerck O. (2013) Extensive cryptic species diversity and fine-scale endemism in the marine red alga Portieria in the Philippines. *Proceedings of the Royal Society B*, **280,** 1753.

Petit RJ, Excoffier L (2009) Gene flow and species delimitation. *Trends in ecology & evolution (Personal edition)*, **24**, 386-393.

Phillips SJ, Anderson RP, Schapire RE (2006) Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, **190**, 231-259.

Pons J, Barraclough T, Gomez-Zurita J, Cardoso A., Duran D, Hazell S, Kamoun S, Sumlin W, Vogler AP. (2006). Sequence-based Species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, **55**, 595-609

Posada D (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution,* **25**, 1253-1256.

Posada D, Crandall KA. (2001) Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. *Proceedings of the National Academy of Sciences USA,* **98**, 13757-13762.

Puisségur C (1987) Hybridation plurispécifique chez les *Chrysocarabus* et *Hadrocarabus*. *Bulletin de la Société Sciences Nat*, **56**.

R Development Core Team. R: A language and environment for statistical computing. R foundation for statistical computing. 2010. Vienna, Austria.

Rambaut A, Drummond AJ. Tracer v1.5, Available from http://beast.bio.ed.ac.uk/Tracer. 2007.

Rijnsdorp AD (1980) Pattern of movement in and dispersal from a Dutch forest of *Carabus problematicus* Hbst (Coleoptera, Carabidae). *Oecologia*, **45**, 274-281.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574.

Schlick-Steiner BC, Steiner FM, Seifert B, Stauffer C, Christian E, Crozier RH (2010) Integrative taxonomy: a multisource approach to exploring Biodiversity. *Annual Review of Entomology*, **55**, 421-438.

Schoener TW (1968) *Anolis* lizards of Bimini: resource partitioning in a complex fauna. *Ecology*, **49**, 704-726.

Serrano J (2003) *Catálogo de los Carabidae (Coleoptera) de la Península Ibérica*. Monografías de la Sociedad Entomológica Aragonesa, Zaragoza.

Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution*, **16**, 1114.

Simon JC, Delmotte F, Rispe C, Crease T (2003) Phylogenetic relationships between parthenogens and their sexual relatives: the possible routes to parthenogenesis in animals. *Biological Journal of the Linnean Society*, **79,** 151-163.

Simpson GG (1961) *Principles of Animal Taxonomy*. Columbia University Press, New York, NY.

Sites JW, Marshall JC (2004) Operational criteria for delimiting species. *Annual Review of Ecology, Evolution, and Systematics*, **35**, 199-227.

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics (Oxford, England)*, **22**, 2688-90.

Swofford DL. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. [4]. 2003. Sunderland, Massachusetts, Sinauer Associates.

Tamura K, Dudley J, Nei M, Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**,1596-1599.

Toulgöet H, Lassalle B (1983) Vue d'ensemble sur la répartition évolutive du *Carabus (Hadrocarabus) lusitanicus*. *L'Entomologiste*, **39**, 217-238.

Turin H, Penev L, Casale A (2003) *The Genus* Carabus *in Europe. A synthesis.* Co-published by Pensoft Publishers & European Invertebrate Survey, Sofía-Moscow-Leiden.

Valdecasas AG, Williams D, Wheeler QD (2008) 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, **93**, 211-216.

Vieites DR, Wollenberg KC, Andreone F *et al.* (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences USA*, **106**, 8267-8272.

Warren DL, Glor RE, Turelli M (2008) Environmental niche equivalency versus conservatism: quantitative approaches to niche evolution. *Evolution*, **62**, 2868-2883.

Warren DL, Glor RE, Turelli M (2010) ENMTools: a toolbox for comparative studies of environmental niche models. *Ecography*, **33**, 607-611.

Table 1. Name, length and variability of molecular phylogenetic markers used to investigate species delimitation and the evolution of *Mesocarabus.*

DNA type	Gene/ fragment	N	Unique sequences	Sequence length	Aligned length	G	V	Subs. model
Mitochondrial								
Protein coding	*cox1*-a	249	211	578	578	0	171	GTR+G+I
	cox1-b	244	211	725	725	0	221	GTR+G+I
	cob	212	183	664	664	0	245	GTR+G+I
	nd5	231	205	988	988	0	328	GTR+G+I
Ribosomal	*rrnl*	245	104	727-729	729	2	97	GTR+G+I
Nuclear								

Protein coding	*HUWE1*	193	123	636-641	643	11	101	GTR+G+I
Ribosomal	*LSU*-a	246	25	939	939	0	24	GTR
	LSU-b	209	63	844-880	887	50	72	GTR+G
	ITS2	547	33	515-541	251	33	38	GTR+G

G: Gapped positions. V: Variable positions.

Table 2. Results of Shimodaira & Hasegawa (1999) tests for each individual gene studied in *Mesocarabus* and their combined datasets compared to the Bayesian phylogenetic trees obtained for the *HUWE1* and the combined *RIB, NUC, MIT* and *ALL* datasets.

	DATASET											
TREE	***rrnL***	***cox1*-a**	***cox1*-b**	***cob***	***nd5***	***LSU-a***	***LSU-b***	***ITS2***	***HUWE1***	***RIB***	***NUC***	***MIT***	***ALL***
tree_*HUWE1*	0.000*	0.000*	0.000*	0.000*	0.000*	0.002*	0.000*	0.000*	(best)	0.000*	0.000*	0.000*	0.000*
tree_*RIB*	0.000*	0.000*	0.000*	0.000*	0.000*	0.638	0.424	0.585	0.000*	(best)	0.244	0.000*	0.000*
tree_*NUC*	0.000*	0.000*	0.000*	0.000*	0.000*	0.366	0.286	0.073	0.000*	0.108	(best)	0.000*	0.000*
tree_*MIT*	0.246	0.487	0.419	0.217	0.522	0.078	0.000*	0.000*	0.000*	0.000*	0.000*	(best)	0.487
tree_*ALL*	0.279	0.371	0.337	0.207	0.374	0.294	0.008*	0.028*	0.000*	0.003*	0.033*	0.374	(best)

Table 3. Pronotal outline morphometrics in *Mesocarabus*. Results of MANOVA and ANOVA analyses for the three main axes of the pronotal outline ordination for the candidate species delineation (excluding candidate *macrocephaloides*) and for the final species proosal (see below). Df: degrees of freedom; F: F-statistiv value; *P*: statistical significance.

		Candidate species			Final species proposal		
		Df	**F**	***P***	**Df**	**F**	**P**
Three first axes (Pillai)	Taxa	6	12.529	<0.0001	5	15.641	<0.0001
	Residuals	290			291		
PC1	Taxa	6	55.035	<0.0001	5	66.183	<0.0001
	Residuals	290			291		
PC2	Taxa	6	16.358	<0.0001	5	18.748	<0.0001
	Residuals	290			291		
PC3	Taxa	6	32.475	<0.0001	5	39.090	<0.0001
	Residuals	290			291		

Figure legends

Figure 1. Ultrametric time-calibrated Bayesian trees for (A) combined mitochondrial genes (*MIT*), (B) combined ribosomal nuclear genes (*RIB*) and (C) the *HUWE1* locus of *Mesocarabus*. Numbers above nodes are posterior probabilities. Grey bars represent the 95% confidence intervals for node ages (Ma). Coloured areas and circles on nodes represent main lineages; collapsed coloured clades and points on maps highlight minor geographically concordant clades.

Figure 2. Strict consensus tree of six most parsimonious cladograms and bootstrap support (>50%) for the *Mesocarabus* male genitalia, with images of the evaginated endophalli from specimens representative of each lineage. Coloured areas on the map and coloured bars on the tree represent these lineages, generally associated with a particular taxon.

Figure 3. Candidate species delimitation with conclusive evidence. ILE0: Initial species hypothesis based on consensus taxonomic revision; ILE1-4: independent lines of evidence (ILE) named as in Figs. 1 and 2 and with their respective underlying phylogenies. Coloured bars represent candidate species based on different character combinations (labelled A-G arbitrarily in every case).

Figure 4. Corroboration of candidate species based on inconclusive evidence. (A) Distribution map of candidate species of *Mesocarabus* (see Fig. 3). (B) Differences in ordination coordinates for the three main PCA components of pronotal outlines (ILE5) among candidate species of European *Mesocarabus*. Boxplots represent medians and quartiles. Significantly different means ($P<0.05$) between species are indicated by different letters and according to Tukey's correction. (C) Pairwise background niche similarity tests (ILE6). Above diagonal: Schoener's D metric values of niche overlap. Below diagonal: D, significant niche divergence; C, significant niche conservatism, with their corresponding *P* values. (D) Summary of corroboration tests, where dotted lines joining species pairs represent non-significant differences (for ILE5) or significant niche divergence (for ILE 6).

Figure 5. Data integration and species delimitation. (A) Species delimitation in *Mesocarabus* integrating over all lines of evidence with an evolutionary interpretation of phylogenetic and character conflict (see main text). The species tree is also shown, denoting the reticulated origin of *C. baguenai* and *C. breuningi*, and mitochondrial introgression events from *C. lusitanicus* into the other species (gray dotted arrows). (B) Distribution map of final species proposal. (C and D) Results of pronotal outline morphometric analyses and background niche similarity tests as in Fig. 4 for the final proposition of European species of *Mesocarabus*.