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Exploring diversity in cryptorhynchine weevils (Coleoptera) using distance-, character- and tree-based species delineation

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ABSTRACT

Species boundaries are studied in a group of beetles, the western Palaearctic Cryptorhynchinae. We test for congruence of 'traditionally' identified morphospecies with species inferred through parsimony networks, distance-based clustering and the ultrametric tree-based generalized mixed yule-coalescent (GMYC) approach. For that purpose, we sequenced two variable fragments of mitochondrial DNA (CO1 and 16S) for a total of 791 specimens in 217 species of Cryptorhynchinae. Parsimony networks, morphology-calibrated distance clusters and the different tree-based species inferences all achieved low congruence with morphospecies, at best 60%. Although the degree of match with morphospecies was often similar for the different approaches, the composition of clusters partially varied. A barcoding gap was absent in morphospecies-oriented distances as well as for GMYC species clusters. This demonstrates that not only erroneous taxonomic assignments, incomplete lineage sorting, hybridization, or insufficient sampling can compromise distance-based identification, but also differences in speciation rates and uneven tree structure. The initially low match between morphospecies and the different molecular species delineation methods in this case study shows the necessity of combining the output of various methods in an integrative approach. Thereby we obtain an idea about the reliability of the different results and signals, which enables us to fine-tune sampling, delineation technique and data collection, and to identify species that require taxonomic revision.

1. Introduction

The analysis of genetic data holds special promise for the taxonomy of morphologically cryptic taxa (e.g. Knowlton, 2000; Sweijd et al., 2000; Parsons and Shaw, 2001; Eyualem and Blaxter, 2003; Blaxter et al., 2004; Ciniglia et al., 2004; Huang et al., 2007; Andrés-Sánchez et al., 2009; Schönhofer and Martens, 2010; Malausa et al., 2011). We here focus on beetles from such a group, the western Palaearctic Cryptorhynchinae, in which many species can be morphologically recognized only based on male genitalia (these sometimes being ambiguous as well). Usually larvae are impossible to identify morphologically, as well as adult females in many cases. With more than 6000 described species worldwide, the weevil subfamily Cryptorhynchinae is one of the largest subfamilies of weevils (Curculionoidea) (the family Curculionidae comprises around 100,000 species; Alonso-Zarazaga et al., 2010). They often show mimetic coloration, especially in temperate zones. Many species of Cryptorhynchinae are apterous. This is unusual in Pterygota (winged insects), out of which only about 5% extant species are flightless (Whiting et al., 2003), but it is more widespread in weevils. Western Palaearctic representatives of the subfamily currently comprise around 360 often endemic species (Stüben, 2010) and almost all of these were subject to recent, i.e. modern, taxonomic revisions (Savitsky, 1997; Stüben and Behne, 1998; Stüben, 1998, 1999a,b, 2003, 2004; Bahr, 2000; Stüben and Germann, 2005). Their body size ranges between 1.2 and 10 mm and their larvae develop in stressed or dying lignified parts of plants. In some regions, such as the northern part of Europe, Cryptorhynchinae are considered potentially valuable bioindicators for old, undisturbed woodlots (Strejcek, 1989; Stüben, 2005). However, their difficult identification due to poor interspecific morphological variation has so far impeded their use in conservation, ecology or forestry. Previous studies that used a combination of molecular and morphological evidence focusing on the systematics of western Palaearctic Cryptorhynchinae (Astrin and Stüben, 2008, 2009, 2011; Stüben and Astrin, 2010a,b) attempted to overcome these problems. However, the match of species boundaries based

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on 'traditional' morphological data (morphospecies criterion) and on DNA sequence data (genospecies criterion) have so far been explored only in a few limited cases in this group, as has the reciprocal insight that can be derived from these two delineation approaches.

In this group of cryptorhynchine weevils our aim was to test for congruence of 'traditionally' derived species boundaries through morphology and species limits inferred exclusively from the variation of DNA sequences. For that purpose, we sequenced two variable fragments of mitochondrial DNA (CO1 and 16S). Our analysis compares the number of recognized morphospecies with the putative species clusters obtained from mtDNA data through distance-based clustering (Meier et al., 2006), parsimony networks (Templeton, 2001) and the tree-based generalized mixed yule-coalescent approach (Pons et al., 2006; Fontaneto et al., 2007). The two latter methods are based on a phylogenetic species concept (which one exactly, see e.g. Wheeler and Meier, 2000, remains to be defined) and 'species boundaries' are derived from observed DNA sequence variation alone. Distance-based clustering, on the contrary, is connected to extrinsic determinations (e.g. morphological identifications and therefore a different species concept) in order to establish the respective best-fitting divergence threshold. It has its foundation in the practical criterion of finding a barcoding gap (the lack of overlap between infraspecific and interspecific molecular divergence) as an indicator of established species entities (Hebert et al., 2003a,b) and is computationally the simplest of the three methods. The general mixed Yule-coalescent (GMYC) model (Pons et al., 2006; Fontaneto et al., 2007) attempts to infer species boundaries as a shift in branching rates on a tree with multiple species and populations. Branching patterns within the species reflect neutral coalescent processes (Kingman, 1982), whereas branching among clusters reflects isolated lineage evolution (i.e. speciation; Yule, 1924). GMYC exploits the predicted difference in branching rate under the 2 modes of lineage evolution (coalescence vs. speciation), determining the point with the highest likelihood for the transition (Pons et al., 2006; Fontaneto et al., 2007). Thus, the goal is to recover independently evolving lineages as GMYC species clusters. These were subsequently tested for a gap between intraspecific and interspecific genetic distances in comparison to results based on established morphospecies. If we assume here that the mtDNA tree is congruent with the species tree and that GMYC species clusters represent 'perfect' phylospecies, we can test for the existence of an error-free barcoding gap in GMYC species. The tentative use of GMYC species clusters to detect a potential barcode gap is detached from prior taxonomic assignments and voucher identifications and lacking, excessive or incompletely sorted genetic divergence.

The existence of a 'barcoding gap' (Meyer and Paulay, 2005; Astrin et al., 2006; Dalebout et al., 2007; Wiemers and Fiedler, 2007; Lahaye et al., 2008; Meier et al., 2008; Kerr et al., 2009; Robinson et al., 2009) is crucial for successful species identification by DNA barcoding (e.g. Hebert et al., 2003a,b; Kerr et al., 2009). A key premise of DNA barcoding is that a gap between interspecific and intraspecific divergence can be used to identify unknown individuals (Hebert et al., 2003a,b). Typically, a threshold pairwise distance is defined based on a database or 'barcode library' (Hebert et al., 2004). Samples whose genetics distances are below the threshold are defined as conspecific (Hebert et al., 2003a, 2004; Blaxter, 2004; Lefébure et al., 2006).

Several case studies have found such a gap in their dataset (e.g. Hogg and Hebert, 2004; Barrett and Hebert, 2005; Monaghan et al., 2005; Vences et al., 2005; Ward et al., 2005; Astrin et al., 2006; Hajibabaei et al., 2006; Huang et al., 2007; Mikkelsen et al., 2007; Eaton et al., 2010). However, the absence of a gap in other studies (e.g. Meyer and Paulay, 2005; Meier et al., 2006; Elias et al., 2007; Wiemers and Fiedler, 2007; Meier et al., 2008) have led some authors to caution against the use of a simple distance threshold-oriented barcoding approach. So far it is unclear why some studies detect a barcoding gap while others do not (Wiemers and Fiedler, 2007). Meyer and Paulay (2005) assume that insufficient sampling on both the interspecific and intraspecific level are responsible for the barcoding gap, while others argue that the main reason for an overlap can be found in inappropriate assumptions underlying a sequence from the DNA library (i.e. poor identification, alpha-taxonomy or incompatible species criteria). Additional error sources can come from exceptionally high or low genetic variability (often based on population demography), incomplete lineage sorting (Zachos, 2009; but see Knowles and Carstens, 2007), hybridization after introgression events (Ballard and Whitlock, 2004; Mallet et al., 2007; Petit and Excoffier, 2009) and, in mitochondrial DNA, nuclear mitochondrial pseudogenes (numts; Bensasson et al., 2000; Pons and Vogler, 2005; Buhay, 2009), endosymbionts (Hurst and Jiggins, 2005; Weinert et al., 2007; Whitworth et al., 2007; Duron et al., 2008; Raychoudhury et al., 2009) or heteroplasmy (Matsuura et al. 1991; Magnacca and Brown, 2010).

2. Materials and methods

2.1. Specimen sampling and sequencing

We sampled multiple specimens for known morphospecies from as many localities as possible throughout Europe, focusing especially on southern and western Europe, where cryptorhynchine diversity is highest. Additionally, we collected very comprehensively on the Macaronesian islands (especially the Canarian and Madeiran archipelagos) and North Africa (see map in Fig. 1).

In total, we analyzed sequences from 791 individuals belonging to 217 species and 25 genera (see Appendix 2) of Cryptorhynchinae. A proportion of 25% of the data have already been analyzed in previous studies with a different focus (see Appendix 1 for details). Since many species are extremely rare, many of these have been collected in small numbers. Species with wide or even pan-European distributions have been sampled from their entire range, as far as this was possible (see Fig. 2).

Two outgroup taxa were included in order to root the phylogenetic trees. Both belong to the same family as the ingroup (Curculionidae), but represent distinct subfamilies: *Coeliodes* sp., Ceutorhynchinae, and *Cionus* sp., Curculioninae.

Appendix 1 lists the collecting and vouchering information for the analyzed material as well as the corresponding GenBank accession numbers. Frozen voucher specimens in ethanol and extracted genomic DNA are deposited at the biobank of the ZFMK (Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany).

We extracted DNA with the Nucleo Spin Tissue kit (Macherey–Nagel, Düren, Germany) from samples preserved in ethanol or from dried material. Therefore, we used either 2–3 legs, head and prothorax, or in some cases the whole weevil, depending on size and conservation of the sample. We amplified and sequenced two fragments of mitochondrial DNA. These included the 5′ end of the cytochrome *c* oxidase subunit 1 gene (CO1; used in animal barcoding studies, cf. Hebert et al., 2003a,b) and part of the mitochondrial ribosomal large subunit or LSU gene (16S). PCR reaction mixes (50 µl) contained 125 nmol $MgCl_2$, 5 µl 10x PCR-buffer, 25 pmol of forward and reverse primer each, 5 pmol dNTPs, 1.75 units of *Taq* polymerase, and 5 µl total undiluted DNA template. The lab chemicals were purchased from Sigma–Aldrich (Steinheim, Germany). We used the Qiagen (Hilden, Germany) Multiplex PCR kit in cases where the regular protocol failed. PCR primers were taken from Astrin and Stüben (2008) (LCO1490-JJ-al from Astrin and Stüben, 2011). Primer sequences were as follows:

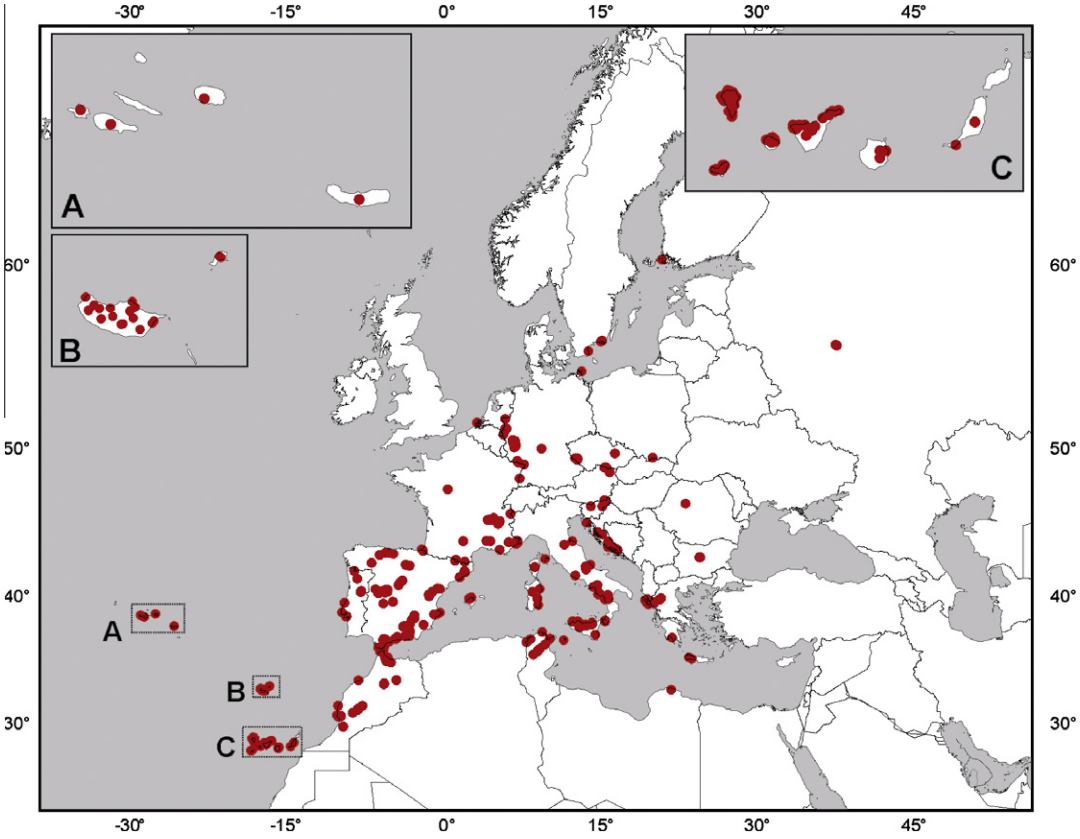

Fig. 1. Collecting. Collecting areas for this study are indicated as dots on the map.

Fig. 2. Individuals per species. Diagram showing the number of individuals (ordinate) sampled per species (abscissa). The mean number of represented individuals per species is 3.6.

LCO1490-JJ (CO1 forward, fw) 5′-CHACWAAYCATAAAGATATYGG-3′,
LCO1490-JJ-al (CO1 alternative fw, alt fw) 5′-TAYTCHACYAAYCAYAAAGAYATYGG-3′,
HCO2198-JJ (CO1 reverse, rev) 5′-AWACTTCVGGRTGVCCAAARAATCA-3′,
16S-ar-JJ (16S fw, erroneously as "rev" in Astrin and Stüben 2008) 5′-CRCCTGTTTATTAAAAACAT-3′,
16Sar-JJ-al (16S alt fw) 5′-CCTGTWTATTAAAAACATGGC-3′,
16S-1472-JJ (16S rev) 5′-AGATAGAAACCRACCTGG-3′,
16S1472-JJ-al (alt rev) 5′-GGTCCTTTCGTACTAA-3′.

Thermal cycling was performed on blocks of the type GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR programs followed the 'Touch Down' routine or, in case of CO1, a combination of 'Touch Down' and 'Step Up' routine. For 16S: first cycle set (15 repeats): 35 s denaturation at 94 °C, 35 s annealing at 55 °C (−1 °C per cycle) and 60 s extension at 72 °C. Second cycle set (25 repeats): 35 s denaturation at 94 °C, 35 s annealing at 40 °C and 50 s extension at 72 °C. For CO1: same as for 16S, but annealing temperatures at 70 °C and 55 °C, with a decrease of 2 °C per cycle in the first cycle set. Double stranded sequencing was carried out by a sequencing facility (Macrogen, Seoul, South Korea) on ABI 3730XL sequencers.

2.2. Alignment and data analysis

DNA sequence alignment for CO1 was performed using the MUSCLE ver. 3.6 programme (Edgar, 2004a,b), run with default parameters. The 16S alignment was run on the European Bioinformatics Institute web server (http://www.ebi.ac.uk/Tools/msa/mafft/) using MAFFT ver. 6.7 (Katoh et al., 2002; Katoh and Toh, 2008). The default MAFFT alignment parameters were used: gap opening and extension penalties at 1.53 and 0.123, respectively, the number of refinement iterations and progressive tree rebuilds set to maximum (at 100).

ModelTest ver. 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004), implementing the Bayesian (BIC; Schwarz, 1978) and standard AIC (Akaike, 1974) information criterion, identified the general time reversible (GTR; Lanave et al., 1984) + proportion of invariable sites (I) + gamma distribution (Γ) model of nucleotide substitution (or one of its subsets) as the most suitable model for both markers. We concatenated the sequence data from the different partitions using BioEdit ver. 7.0.4.1 (Hall, 1999) and filled up terminal gaps in slightly shorter sequences by the character N.

Pairwise genetic distances were calculated in PAUP* ver. 4.0b10 (Swofford, 2002) and summarized for intra- and interspecific distances with PASW Statistics ver. 18 (SPSS).

Model-based phylogenetic analysis was performed on the complete dataset. Maximum Likelihood (ML; Felsenstein, 1973) searches were performed in PhyML ver. 2.4.4, (Guindon and Gascuel, 2003) and RAxML ver. 7.2.5 (Stamatakis et al., 2005). We also conducted a Bayesian analysis using parallel MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004), which we ran for 20 million generations. All reconstructions used a GTR + I + Γ model (as selected by ModelTest) and estimated the parameters directly from the data. Bayesian MCMC (Yang and Rannala, 1997) and RAxML analyses were performed with partitioned data (Brandley et al., 2005) and included 10,000 bootstrap replicates. Data were partitioned by separating the combined matrix into the two gene loci 16S and CO1, and further separating CO1 into one partition for codon positions 1 + 2 and another for codon position 3. Models were estimated for each partition. Tracer 1.4.1 (Rambaut and Drummond, 2007) was used to graphically determine stationarity and convergence of runs.

2.3. Grouping procedures and species delimitation

As a prior for species delimitation, sequence variation of specimens was subdivided into subgroups using statistical parsimony analysis (Templeton, 2001). This procedure partitions the data into networks of closely related haplotypes connected by changes that are non-homoplastic with a 95% probability. For mtDNA of insects, these networks usually group haplotypes around species-level (Templeton, 2001; Wilder and Hollocher, 2003; Cardoso and Vogler, 2005; Pons et al., 2006; Ahrens et al., 2007; Hendrich et al., 2010). Statistical parsimony networks were determined using TCS v.1.3 (Clement et al., 2000). Separate analyses were conducted on CO1 and 16S. A third analysis was conducted for both fragments in combination using only those individuals with both partitions.

We also used generalized mixed Yule-coalescent (GMYC) modeling for estimating species boundaries directly from the phylogenetic tree (Pons et al., 2006; Fontaneto et al., 2007), produced with the combined mitochondrial data and for each mitochondrial marker alone. This procedure exploits the differences in the rate of lineage branching at the level of species and populations, recognizable as a sudden increase of apparent diversification rate when ultrametric node height (distance to tips) is plotted against the log number of nodes in a lineage-through-time plot (Nee et al., 1992). We use here a single threshold value for our input tree (Monaghan et al., 2009) which has been already applied successfully to selected groups of organisms (Pons et al., 2006; Ahrens et al., 2007; Fontaneto et al., 2007; Monaghan et al., 2009). The script ('GMYC') of this method is freely available as part of the 'splits' package, which contains tools for delimiting species and automated taxonomy at many levels of biological organization (https://www.r-forge.r-project.org/projects/splits/), for the R environment (R Development Core Team, 2009). Subsequently we tested for the robustness of yields of species number estimates examining the alternative phylogenetic trees obtained from PhyML, RAxML and MrBayes searches and applying different algorithms to produce the GMYC input tree (i.e. the ultrametric tree) using non-parametric rate smoothing (NPRS; Sanderson, 1997), penalized likelihood as implemented in r8s ver. 1.7 (Sanderson, 2003) and a new algorithm, PATHd8 (Britton et al., 2007).

For the resulting clock-constrained input trees, relative ages of nodes were used. For the performance of penalized likelihood as implemented in r8s, trees were fully resolved in Tree Edit v1.0a10 (Rambaut and Charleston, 2001) using an arbitrary branch length of 0.000001 for polytomies. The root of the input tree was pruned and the ingroup root node set to 1. Since the cross validation in r8s was not feasible due to the large size of the tree, we applied a range of different smoothing factors for the linearization of the tree. For one run of PATHd8 we also used absolute ages for nine nodes as calibrated in Stüben and Astrin (2010a).

To compare the efficiency of character-, tree- and distance-based approaches for taxon delineation, the SpeciesIdentifier program (Meier et al., 2006) from the TaxonDNA v.1.6.2 package (http://taxondna.sourceforge.net/) was used to compute, cluster and categorize pairwise uncorrected distances between sequences at user-predefined thresholds ("Cluster" function) based on valid taxonomic names in the sequence titles. SpeciesIdentifier groups all distances from a clique (where all individuals are connected to each other by distance values below the threshold) into a single cluster, as it does with quasi-cliques (where some individuals are connected to each other indirectly, i.e. some distances in the cluster infringe the threshold). Subsequently we determined the percentage of exact match between the resulting clusters and the morphospecies represented in the dataset.

3. Results and discussion

We obtained 862 new mitochondrial sequences (452 sequences for 16S and 410 for CO1). We used another 645 sequences from Astrin and Stüben (2008, 2009, 2011) and Stüben and Astrin (2010a,b) (Appendix 1). Aligned sequence lengths were 658 base pairs (bp) for CO1 and 593 bp for 16S. Sequence length in the ribosomal large subunit gene varied because of insertions/deletions ('indels'; no gaps were present in the CO1 alignment). Thus, the longest sequence in 16S counted 544 bp, the shortest 529 bp (disregarding missing bases). The concatenated alignment of the combined markers was 1251 bp long. The median of sequence variation was 14.7% in 16S and 19.6% in CO1. Using PhyML with the mtDNA of the full dataset of 791 specimens we obtained a well resolved tree with a likelihood score of ln$L$ = −80348.26. Almost all genera were monophyletic (see Section 3.1.) and the tree topology was widely consistent with previous results (Astrin and Stüben, 2008, 2009, 2011; Stüben and Astrin, 2010a,b). Morphospecies (including 61 singletons in our dataset) were monophyletic in all but 19 cases for PhyML (marked by a "+" in Fig. 3). In MrBayes, 21 cases of non-monophyletic morphospecies occurred and in RAxML, 22 (see below). Morphospecies singletons all had unique haplotypes with only a few exceptions (five cases in 16S and four in CO1).

3.1. Tree topology and monophyly of taxa

The three different software programs used to infer phylogenetic relationships (MrBayes, PhyML, RAxML) delivered different topologies mainly at the deeper nodes of the tree. The relationships among the cryptorhynchine genera were not completely resolved, although an 'Atlantic clade' (Astrin and Stüben, 2008; see arrow on first page of Fig. 3) was always recovered as monophyletic with high support. Bootstrap, posterior probability and approximate likelihood ratio test (aLRT) values were in general low at nodes separating cryptorhynchine genera.

At 'intermediate' (within-genus) phylogenetic level, the markers show a better signal, which is reflected by high or maximal support values for clades that correspond to cryptorhynchine genera. Fig. 3 (corresponding to the PhyML reconstruction) shows all genera as monophyla with the following exceptions: *Acalles, Calacalles, Dendroacalles,* and *Torneuma*. *Acalles edoughensis* appears sister to *Montanacalles nevadaensis*. Consequently, it seems to be clearly (also morphologically) not a member of the genus *Acalles*. The genus *Elliptacalles* is nested within *Calacalles*, separating the continental and Macaronesian subgenera of *Calacalles*. This is not supported by any known synapomorphy in morphological characters. The clade of *Torneuma* includes the specimens of the genera *Paratorneuma* and *Paratyphloporus*. The position of *Dendroacalles euphorbiophilus*

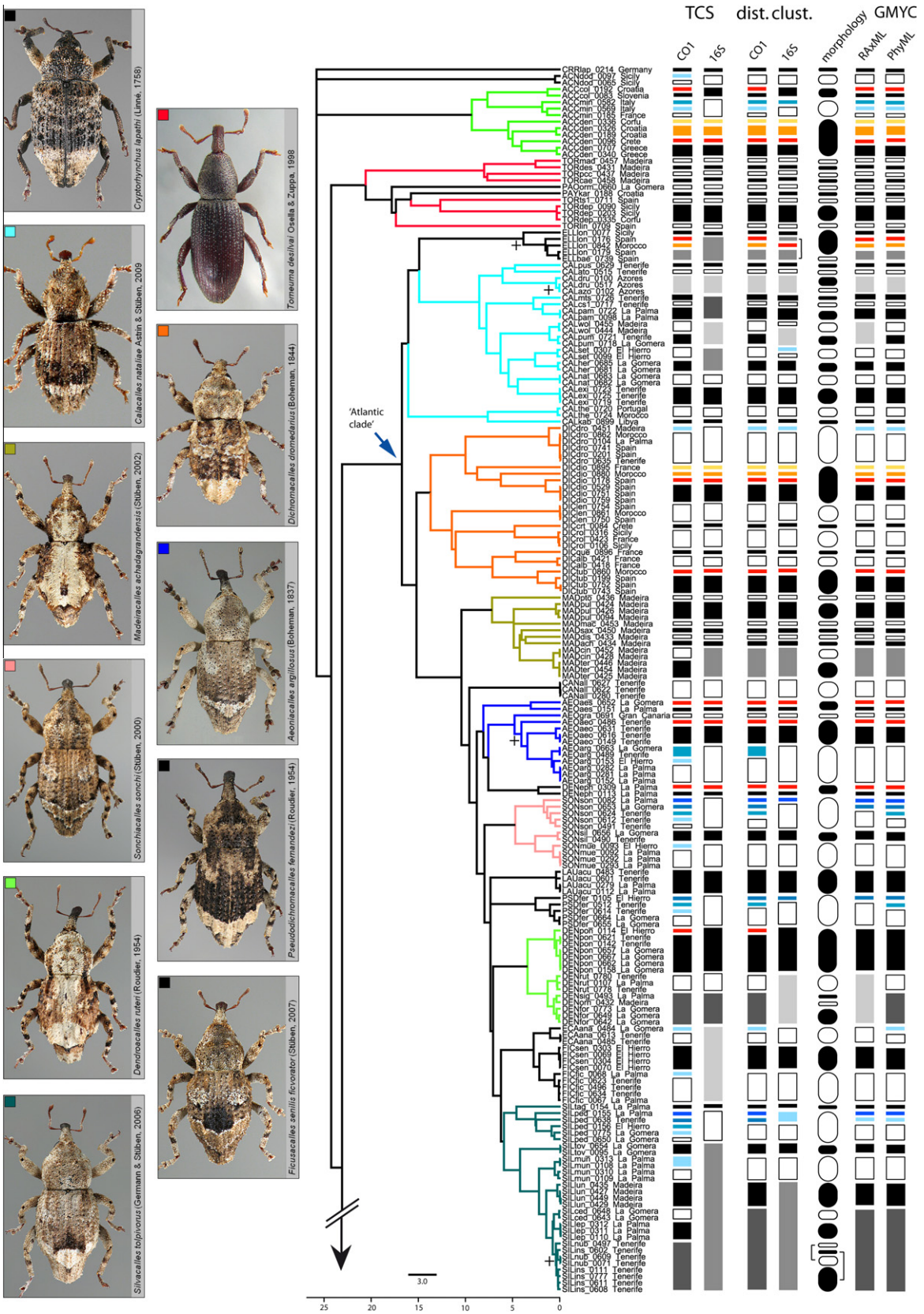

Fig. 3. Group clusters as obtained through the different delineation methods are shown on the right side. Columns 1–2: TCS (CO1, 16S); columns 3–4: distance clustering with SpeciesIdentifier (CO1, 16S); column with rounded fields: according to morphology; last two columns: GMYC modeling (RaxML and PhyML, relative ages). Results are shown next to a mitochondrial PhyML tree that is based on taxa with no missing partition. Taxon names are abbreviated (abbreviations resolved in Appendix 1). No nodal support values are shown on the tree as proprietary values of the PhyML reconstruction would be misleading. Here, the focus lies on the synoptic comparison of various methods used for species delimitation. Branches within genera share the same color. These colors match those of the squares next to the genus representatives whose habitus (dorsal view) is depicted on the left side. The "+" signs mark cases of non-monophyly in species and subspecies taxa (here not included: cases with identical haplotypes or one partition missing, but see Section 3.1.).

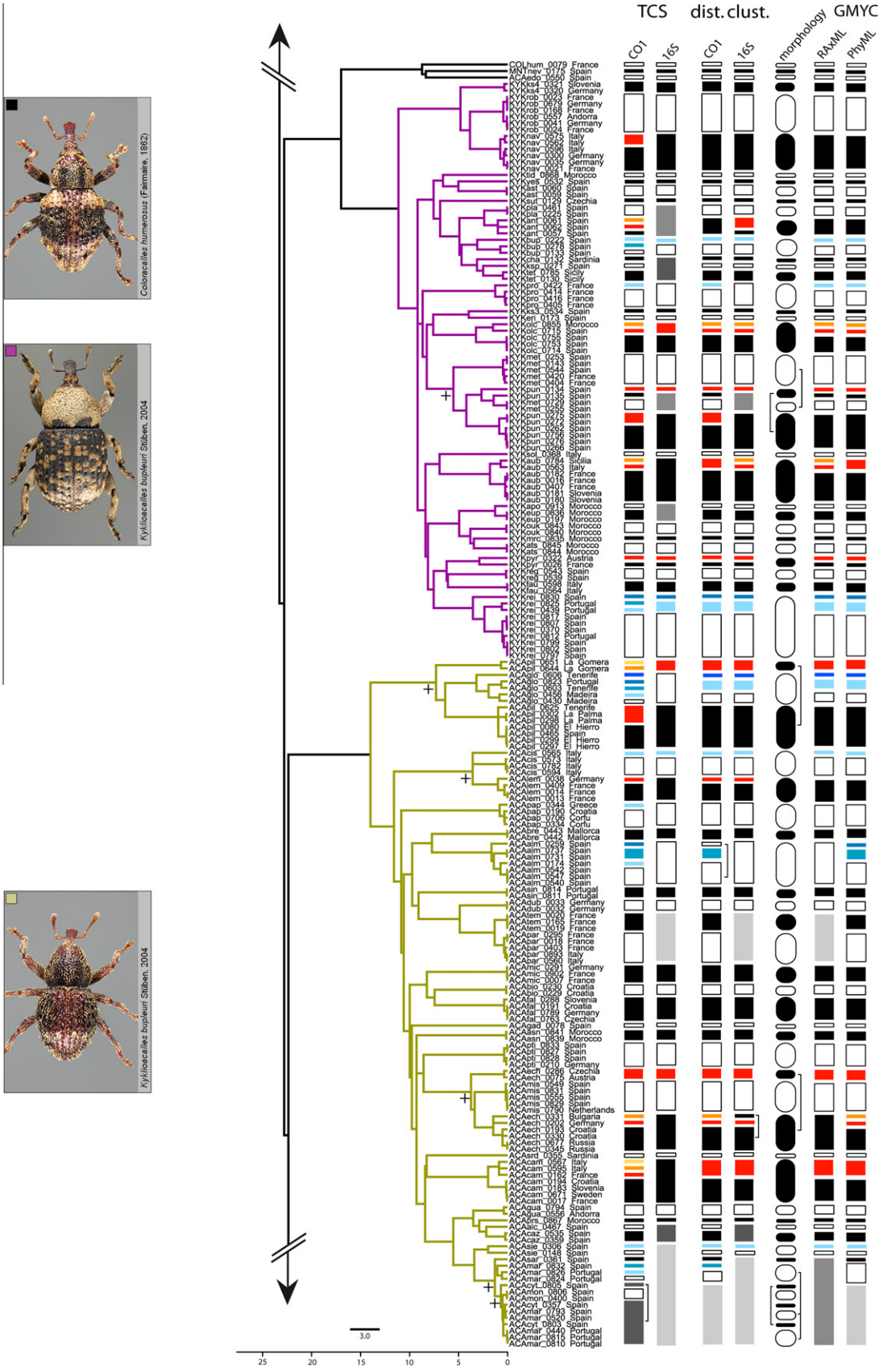

Fig. 3 (*continued*)

is a result of conflicting mitochondrial signal between the partitions (see Stüben and Astrin, 2010a).

All but 19 morphospecies were monophyletic in the PhyML tree (see "+" signs in Fig. 3). According to morphological evidence or,

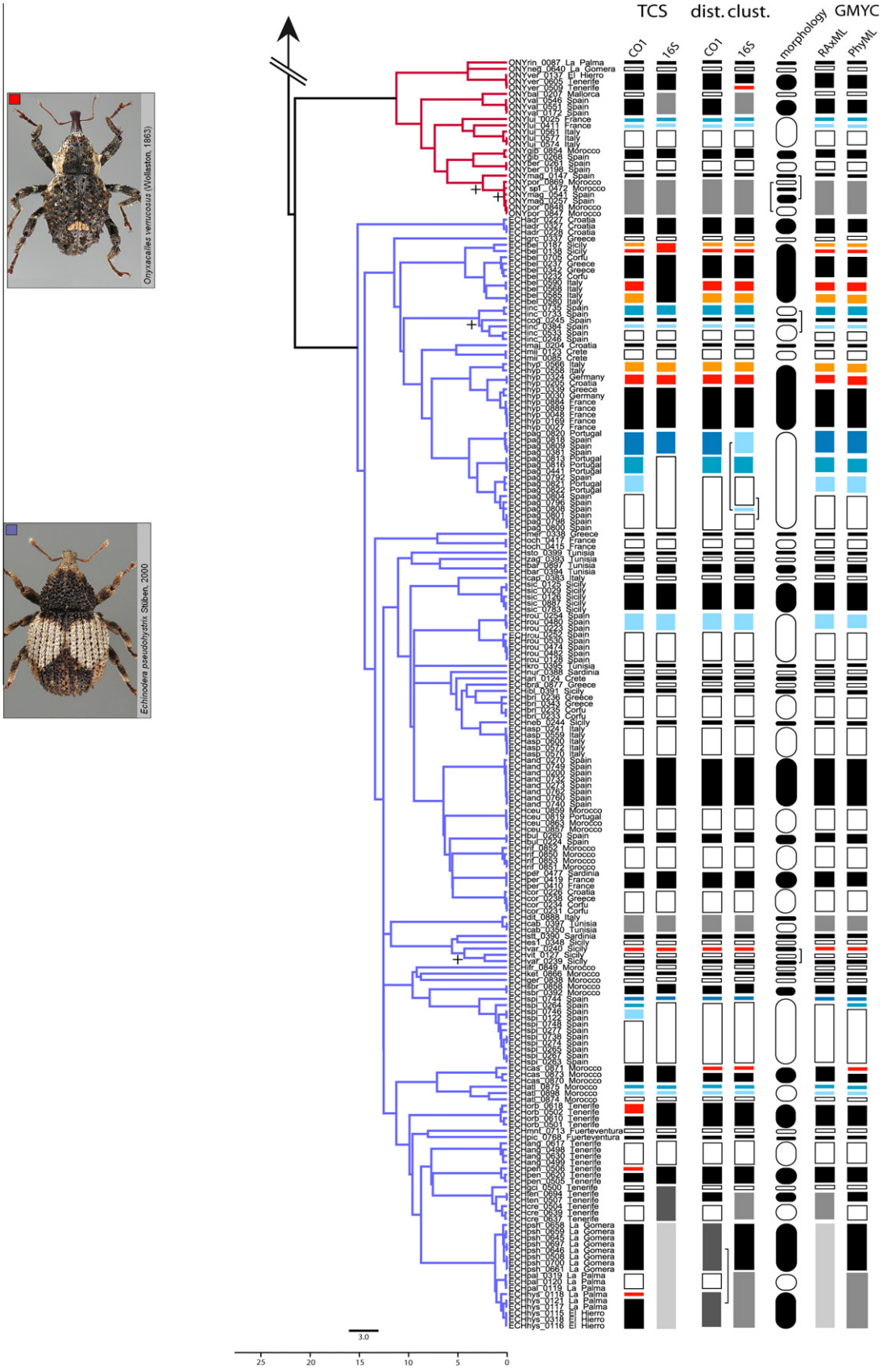

Fig. 3 (*continued*)

alternatively, according to ecological features, these cases can be grouped into two categories:

On one hand we have cases that find some correspondence in morphological or ecological evidence at a second inspection. Most

of these cases will be resolved by an updated classification: *Acallorneuma doderoi* + *Acallorneuma sabellai* (the latter not shown in the combined tree, as it lacks a CO1 sequence) appear together as one cluster of closely related species that lack reciprocal monophyly and so do *Calacalles azoricus* + *Calacalles droueti*, or *Madeiracalles terminalis* + *Madeiracalles tolpis* (the latter have identical haplotypes; as both mitochondrial partitions are identical, this case is not shown in Fig. 3, which excludes 100% identical sequences). *Acalles pilula* is not monophyletic and shows a deep genetic split between the sampled specimens. The same situation applies to *Acalles echinatus*, *Echinodera variegata*, and *Aeoniacalles aeonii*. The subspecies *Kyklioacalles punctaticollis punctaticollis* and *Kyklioacalles punctaticollis meteoricus* probably hybridize where their distributions overlap (Stüben and Astrin, 2010b). *Dichromacalles querilhaci* was not monophyletic in the reconstructions that used also taxa with missing partitions.

On the other hand, we have cases that lack support from known morphological characters or existing ecological observations and that need to be investigated further. Some might be due to misidentification of the samples, lab errors or misleading signal, as it is likely for *Silvacalles instabilis* + *Silvacalles nubilosus* (partly identical haplotypes), *Dendroacalles ruteri* + *Dendroacalles fortunatus* (partly identical haplotypes) or, with regard to the potential new species, for *Onyxacalles portusveneris* + *Onyxacalles* sp. (partly identical haplotypes) + *Onyxacalles maginaensis* (partly identical haplotypes). For other taxa, however, the possibility of synonymy has to be revisited by adding further morphological and/or molecular evidence: *Elliptacalles longus* + *Elliptacalles baeticus*, *Acalles maraoensis* + *Acalles monasterialis* (partly identical haplotypes) + *Acalles cytisi* + *Acalles sierrae* + *Acalles sarothamni*, and *Echinodera cognita* + *Echinodera incognita*.

3.2. Parsimony networks

Statistical parsimony analysis, conducted separately for 16S (477 haplotypes) and CO1 (570 haplotypes) and excluding the two outgroups, resulted in 214 and 301 networks, respectively (see Fig. 3). The connection limit (the minimum number of steps at which haplotypes are assigned to separate networks) was 10 and 11 steps for 16S and CO1, respectively. Of the 214 16S networks, 131 exclusively match a particular morphospecies, 60 match only with a part of a morphospecies while 23 networks match with two or more morphospecies. Of the 301 CO1 networks, 141 exclusively match a particular morphospecies, 152 networks match only with a part of a morphospecies and 8 match with more than one morphospecies. The combined mitochondrial dataset contain 590 haplotypes and resulted in 295 parsimony networks. These are widely consistent with those of CO1 (Fig. 3). Under this character-based approach of species delineation the more conserved gene 16S has a much better match with the morphospecies so far recognized than CO1 (Table 1). Its match is even better than any of the tree- or distance-based approaches (Table 1). However, this best match of molecular and morphological species entities is still low (61.2%). This shows that ample conflict exists between the two kinds of data or their interpretations. A considerable part of this conflict can be solved when reassessing morphological evidence in the light of divergent molecular clusters, i.e. in a 'taxonomic feedback loop' (Page et al., 2005): in almost a third of these cases (in terms of clusters; this affects almost a fifth of all analyzed morphospecies; see Appendix 3), we found substantial new morphological evidence that supports the molecular findings. Most of the remaining cases cannot be solved without considering additional evidence.

3.3. Barcoding gap and cluster analysis by genetic distance

Statistically analyzing uncorrected (*p*-) distances within and between valid morphospecies delivered very different results for each of the two mitochondrial markers used (see Table 3). However, both markers (CO1, 16S) coincided in showing a wide overlap of inter- and infraspecific distances for morphologically delimited species. Both lack a barcoding gap when all observations are considered: the smallest interspecific distances are always 0.0% (see Table 3). This compromises the direct use of distances in the present dataset for exact descriptive taxonomy (DNA taxonomy), e.g. in the hypothetical case that potentially new species should be added to see if all their pairwise distance comparisons fall into the 'interspecific' range. However, DNA barcoding focuses on routine species reidentifications rather than species descriptions and therefore builds on already existing (more or less appropriate) taxonomies, thus making a low proportion of erroneous determinations acceptable, as they would incur in any other reidentification system.

Distance-based clustering in SpeciesIdentifier was performed with thirteen (0.5–12.0%) and eight (0.25–6.0%) different thresholds for CO1 and for 16S respectively (see Appendix 4). The highest taxonomic accuracy was achieved at a clustering threshold of 3% for CO1 and at 1% for 16S, yielding 260 and 233 species clusters respectively. Interestingly, a threshold of 3% in CO1 is often used as standard in insect barcoding. The match with morphospecies is similar for both genes (Table 1). CO1 shows a higher number

Table 1
Comparison of results of the different DNA sequence-based species delineations (1) in terms of clusters, (2) regarding their match to morphospecies (lumps and splits are given as total or partial *mismatches* from morphospecies, i.e. not the 'raw' number of clusters in a split morphospecies nor the 'raw' number of morphospecies included in a lumped cluster). (3) Comparison of match between DNA-based clusters.

	Number of clusters	Splits vs. morphology	Lumps vs. morphol.	Match with morphol.	TCS CO1	TCS 16S	Distance clustering CO1	Distance clustering 16S	GMYC PhyML (rel. age)	GMYC RAxML	GMYC MrBayes (best)
TCS											
CO1	301	102	11	46.8	–	71.9	88.7	81.3	87.2	85.5	85.5
16S	214	37	39	61.2	71.9	–	78.7	83.7	81.2	85.3	80.3
Distance clustering											
CO1 (3%)	260	70	14	54.2	88.7	78.7	–	88.8	96.7	92.7	94.9
16S (1%)	233	58	21	54.1	81.3	83.7	88.8	–	90.5	93.6	91.0
GMYC											
CO1 individually	259	68	16	45.6							
16S individually	262	68	20	45.4							
PhyML (abs. age)	259	69	14	55.2							
PhyML (rel. age)	251	65	16	55.4	87.2	81.2	96.7	90.5	–	95.7	96.5
RAxML	244	60	10	56.1	85.5	85.3	92.7	93.6	95.7	–	94.7
MrBayes (best)	255	66	15	55.3	85.5	80.3	94.9	91.0	96.5	94.7	–
r8s (sf 1.6)	260	70	14	45.8							
NPRS (run 1)	265	76	16	49.4							

Table 2
GMYC clusters. The number of GMYC clusters (N_{GMYC}) and confidence interval (CI) using single and combined partitions under different tree building and linearization methods and applying a single threshold model. Likelihood values are presented for null (L_0) and GMYC (L_{GMYC}) models. All datasets used relative ages for tree linearization (i.e. root = 1) except "abs. age"; NPRS run 1 (r1): weight rate differences across root; NPRS r2: weight rate difference at root with mean; NPRS r3: weight rate difference at all nodes with mean. Significance of the likelihood ratio (LR) was evaluated using a chi-square test with 3 degrees of freedom to compare GMYC and null models as implemented in the 'splits' software. $p < 0.001$; sf: smoothing factor.

		N_{GMYC}	CI	L_0	L_{GMYC}	LR
PATHd8	PhyML					
	CO1	259	253–286	3657.893	3811.922	308.058*
	16S	262	247–271	3181.163	3265.288	168.251*
	Comb (abs. age)#	259	232–270	2033.526	2190.347	313.642*
	Comb (rel. age)	251	235–269	3942.55	4099.652	314.205*
	Comb RAxML	244	220–253	4144.41	4331.352	373.883*
	Comb Bayes best	255	223–268	4118.843	4224.582	211.479*
	Comb Bayes cons.	259	249–276	4113.923	4237.07	246.294*
r8s	$Combined_{sf}$ 1.6	260	247–270	3970.039	4112.05	284.022*
	$Combined_{sf}$ 3.2	564	563–571	4007.868	4058.439	101.142*
	$Combined_{sf}$ 1000	5	1–5	3953.012	3954.677	3.329
	$CO1_{sf}1$	274	254–293	3623.752	3758.659	269.815*
	$CO1_{sf}1.6$	274	255–294	3623.894	3758.565	269.340*
	$CO1_{sf}3.2$	568	1–569	3759.041	3760.653	3.225
	$CO1_{sf}1000$	568	7–569	3711.721	3715.593	7.744
	$16S_{sf}1$	241	233–250	3162.124	3221.268	118.288*
	$16S_{sf}1.6$	241	233–250	3162.228	3221.336	118.217*
	$16S_{sf}3.2$	241	233–250	3162.303	3221.411	118.217*
	$16S_{sf}1000$	47	37–56	3118.067	3127.057	17.980*
NPRS	Combined r_1	265	227–279	3575.483	3630.816	110.666
	Combined r_2	257	226–278	3581.74	3637.503	111.527
	Combined r_3	265	236–281	3625.155	3714.957	179.603

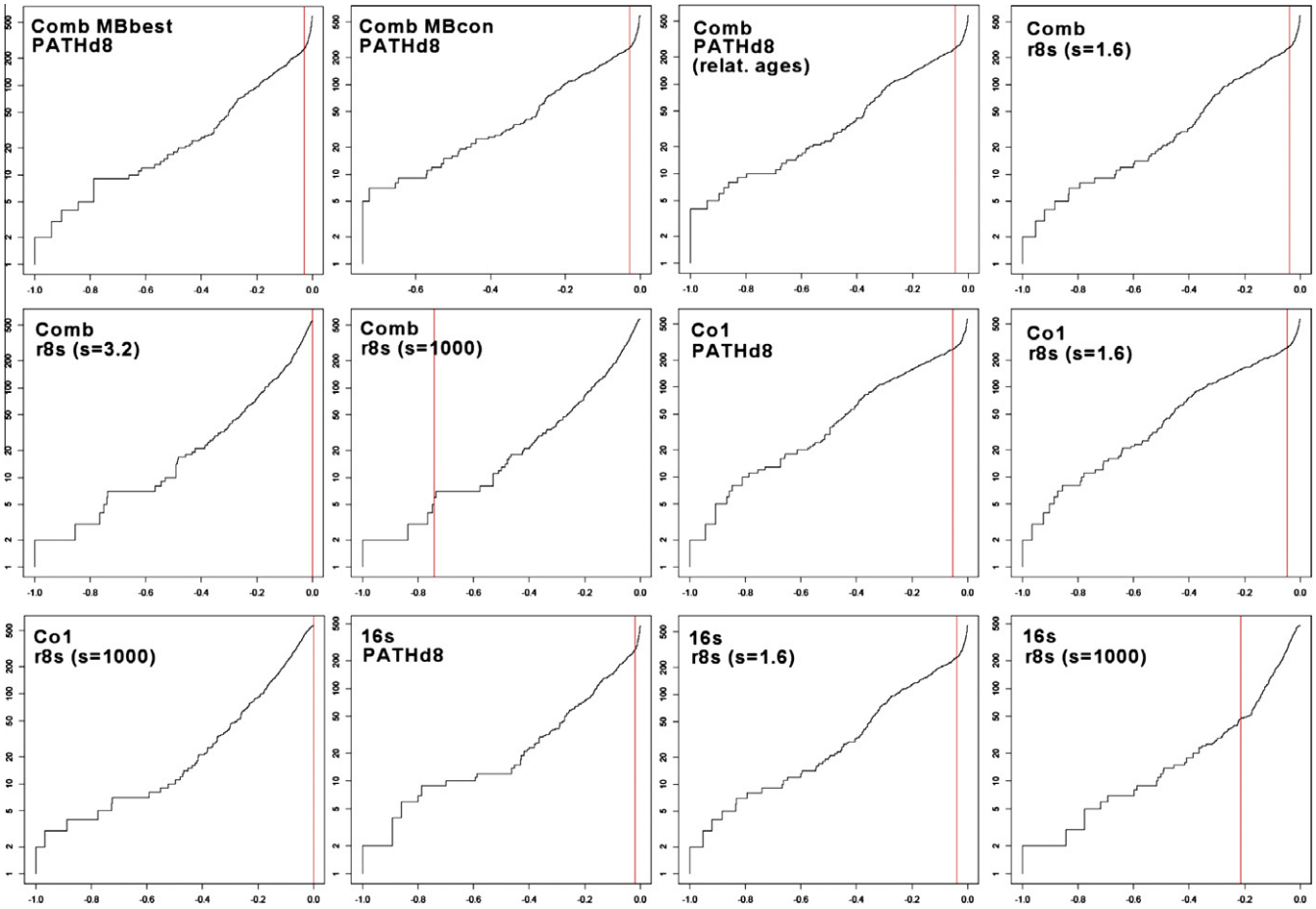

Fig. 4. 'Species cut-off'. Tree linearization and resulting lineage through time (LTT) plot structure with resulting GMYC thresholds (vertical line).

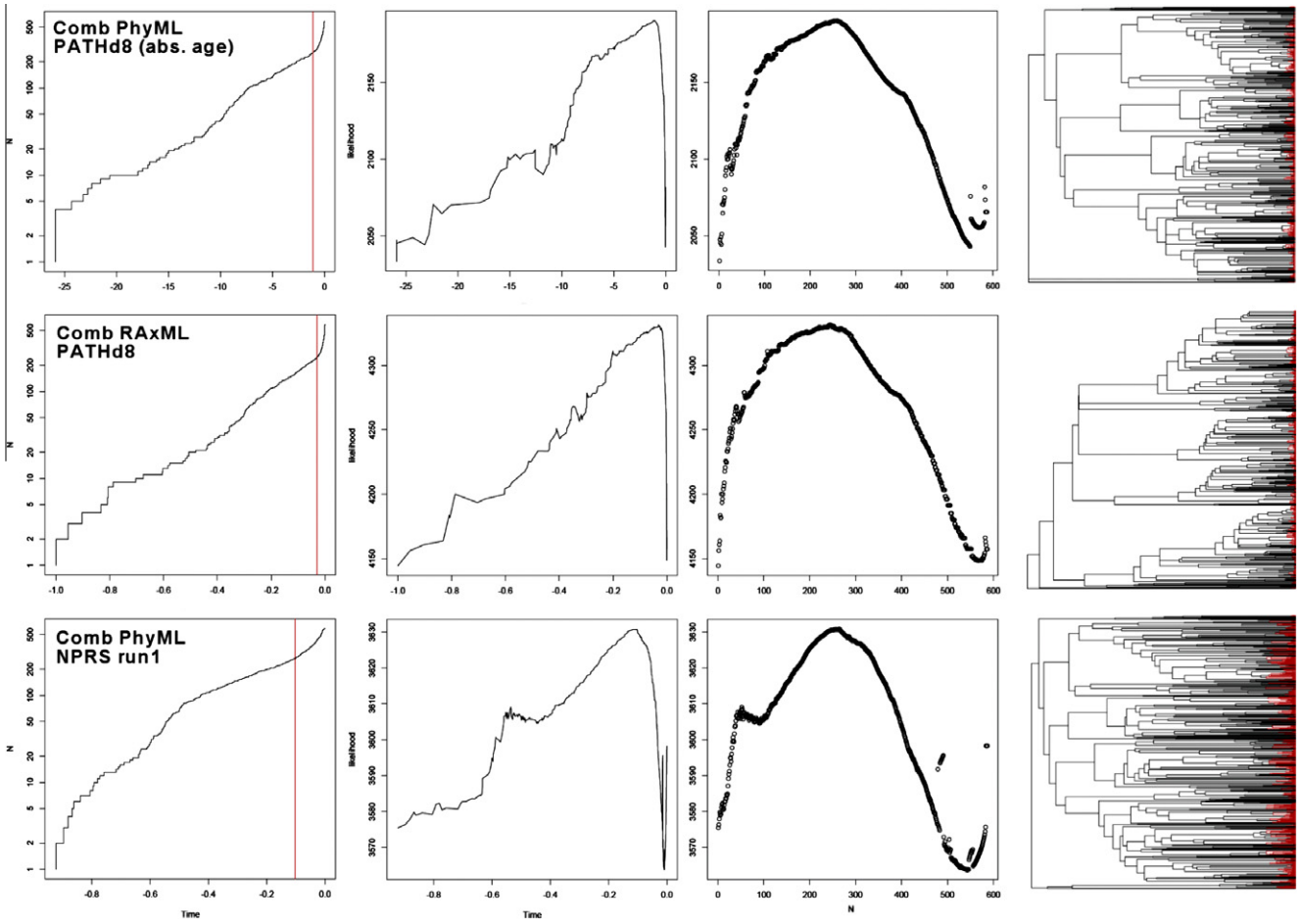

Fig. 5. The effect of the tree shape (linearization; shown by a lineage through time plot; 1st graph from left) on the GMYC modeling performance with a single threshold model (shown by a likelihood-time plot (2nd graph from left) and the likelihood surface (3rd graph from left). Red branches indicate clades under coalescence in the species tree (right) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

of additional splits, while the number of lumped clusters is greater in 16S.

3.4. GMYC clusters

For the tree-based estimation of species entities using GMYC modeling we pruned identical haplotypes from the dataset and again performed phylogenetic analyses on these data. In order to explore the robustness of the GMYC modeling results we used different tree search algorithms for this purpose as implemented in PhyML, RAxML, and MrBayes. Since the PhyML tree on combined mtDNA featured the highest number of monophyletic genera (for the dataset containing only taxa with both partitions), we use this tree to present the results of the group clusters that we obtained with the different approaches (Fig. 3).

Based on this PhyML tree, we explored the influence of the tree linearization on the output of the GMYC modeling. Therefore, we compared ultrametric input trees from NPRS, r8s using a set of various smoothing parameters, and PATHd8 (Table 2). Under the linearization using PATHd8 and NPRS, estimations of GMYC clusters for the combined dataset are quite similar. We found only slight alterations when absolute ages (from Stüben and Astrin, 2010a) were used to calibrate and linearize the tree. An interesting result is the sensitivity of GMYC clustering found with regard to suboptimal branch length estimation (e.g. incorrect smoothing parameters under penalized likelihood as implemented in r8s) (Fig. 4). This issue matters especially when cross validation cannot be performed in order to identify optimal smoothing parameters, e.g. due to large size of the tree. Here, the overall tree shape is in some cases (e.g. smoothing parameter 3.2 or 1000 for CO1 and for the combined set or 100 for 16S) not any longer compensated by the program's algorithm (as it still was under the NPRS approach, see Fig. 5) and resulted in very unlikely species boundary estimations of 5, 47, or more than 500 GMYC species (see Table 2).

A standard log-likelihood ratio test as implemented in the GMYC software assessed whether the alternative model provides a significantly better fit than the null model of no such shift in branching process. Likelihood ratio (LR) values are not directly comparable between different data (i.e. branch lengths of different trees) because the input data (internode intervals) and thus the priors for the null model are different in each case. However, we could observe some general tendencies: the shape of the ultrametric input tree affects significantly the confidence of GMYC model estimates. In contrast to the rather similar GMYC cluster numbers and confidence intervals, we found for the LR much larger differences between the different methods of tree reconstruction and linearization, as has already been reported by Monaghan et al. (2009) for trees with clock-constraint, coalescent-relaxed clocks and Yule-relaxed clocks. In our data also penalized likelihood and nonparametric rate smoothing strongly alter the GMYC results. Interestingly, although under suboptimal smoothing parameters (r8s), LR turned to be significant for the combined data (under smoothing factor 3.2), although GMYC clusters were defined under this approach for almost each single terminal of the tree. Therefore,

Table 3
Intra- (A) and interspecific (B) pairwise distances [%] for morphospecies of Cryptorhynchinae. All distances are uncorrected (*p*-distances) except for "CO1 (K2P)". Gaps were treated as missing data. For "16S no indels", alignment positions with dubious homology (i.e. containing indels) were removed.

Morphospecies	CO1		CO1 (K2P)		16S		16S no indels		16S + CO1	
	A	B	A	B	A	B	A	B	A	B
Median	1.5	19.6	1.5	23.0	0.2	14.8	0.2	9.9	1.0	17.6
Minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	18.7	28.0	21.9	35.3	6.8	24.3	5.7	19.5	13.4	24.6
95th percentile	9.4		10.1		3.7		2.6		6.9	
5th percentile		14.6		16.4		6.8		4.6		11.3

Table 4
Intra- (A) and interspecific (B) pairwise distances [%] for the GMYC species estimated from the PhyML tree linearized in PATHd8 (see PhyML boxes in Fig. 3.; legend: see Table 3).

GMYC species	CO1		CO1 (K2P)		16S		16S no indels		16S + CO1	
	A	B	A	B	A	B	A	B	A	B
Median	1.0	19.6	1.0	23.0	0.2	14.7	0.2	10.3	0.7	17.6
Minimum	0.0	2.0	0.0	2.0	0.0	0.2	0.0	0.0	0.0	1.8
Maximum	4.7	28.0	4.9	35.3	2.2	24.3	1.8	19.7	3.0	24.6
95th percentile	3.3		3.5		0.8		0.7		2.0	
5th percentile		14.5		16.4		6.7		4.6		11.2

LR and its significance need to be used cautiously to evaluate the reliability of the GMYC species estimate. The RAxML tree has the highest likelihood ratio (see Table 2 for a comparison of LR and other values). The match with morphospecies is also highest in the RAxML tree (see Table 1 for a comparison of matches between molecular methods and morphology).

3.5. Assessment of the barcoding gap under the assumption of GMYC species units

GMYC modeling delivers phylogenetically defined tentative species clusters under the assumption that the gene tree is congruent with the species tree. GMYC species units are decoupled from misidentifications and errors in taxonomy (arising from problems in morphology), and they ignore cases of incomplete lineage sorting or of lacking differentiation in genetic markers (compared to morphology). Consequently, several sources that potentially compromise the barcoding gap are excluded a priori (see above). For this reason, GMYC modeling represents a convenient test for the theoretical feasibility of the barcoding gap approach. Therefore, we analyzed pairwise genetic distances for GMYC species (estimated using the PATHd8-linearized PhyML tree) for the individual and combined markers (Table 4). Both markers (CO1, 16S) again show a clear overlap of inter- and infraspecific distances when all observations are considered: the smallest interspecific distance lies at 2% (CO1) and 0.2% (16S) in both cases, while the largest infraspecific distances are higher than 4% in both (Table 4). This provides a clear indication of failure of the barcoding gap. While the measures of central tendency for the distance data do not vary considerably between distance datasets with underlying morphospecies and with GMYC species, the extremes partially do (Tables 3 and 4).

4. Conclusion

In this study we have shown that different DNA analysis methods can yield a mix of both congruent and contrasting results to morphological analysis (Fig3, Appendix 3). Considering these results in combination with morphology will lead – in several cases – to an updated/revised alpha taxonomy of Cryptorhynchinae. This demonstrates the benefit that can result from using multiple data sources and alternative statistical methods to obtain an idea about the reliability of the taxonomic signal.

Upcoming taxonomic work will focus in more detail on the cases of conflicting species delineations that have become apparent. As part of a taxonomic feedback loop, a first morphological reassessment already proved our dataset to include several cases with 'inadequate' taxonomy that are revealed through the application of molecular methods. This potentially affects almost a fifth of the analyzed species and could be the reason for a third of all cases of conflict with morphology (see Appendix 3). For example, all molecular analyses suggest *Aeoniacalles aeonisimilis* to be composed of two distinct species. Based on a re-examination of sequenced specimens and type material, new elytral characters have been found that allow also a distinction between two morphologically well defined forms. Consequently in this and other cases, new species are now being prepared for description. In other few cases, ecology proves that our molecular markers are not yet able to discern between young species from the Macaronesian islands, but that morphology can: *Madeiracalles cinereus*, highly specialized feeder on *Euphorbia mellifera*, is lumped by molecular methods (except TCS) into a single cluster with *M. terminalis*, which feeds in oligophagy on e.g. *Ficus* or *Spartocytisus*, but has never so far been found on *E. mellifera*. Other cases are due to misidentification of the samples (e.g. *Dendroacalles ruteri*), may be due to lab errors, or often cannot be resolved without consulting new evidence.

Our results of GMYC modeling show how the output from tree-based species delineation is affected by different competing model priors (i.e. branch length of ultrametric input trees), but that LR can be helpful to assess sensitivity and confidence of GMYC species estimates. When based on two genes instead of a single gene, coalescence-based methods of species delineation (GMYC) work better for our dataset.

Regarding the degree of match with the morphospecies so far defined in modern revisions, character-based delineation (TCS) was the most successful when applied to the more conservative ribosomal DNA marker (16S). The distance clustering results were similar to those of the best GMYC estimates (Table 1): a comparison of CO1 distance clustering and GMYC units (PhyML, relative ages) reveals that their entities differ in less than 4%.

GMYC modeling can be very helpful in descriptive taxonomy or in fast biodiversity assessments where taxa are poorly known, as it

provides us with species estimates based on molecular data alone. It offers independent evidence to revise morphology-based classification and to sort out uninformative 'noise' e.g. from morphometric data, which makes it a very valuable tool to integrative taxonomy. GMYC modeling can also provide confidence estimations. However, confidence intervals for the various results in this study vary depending on what kind of tree reconstruction, tree smoothing, etc. is used. Encompassing this uncertainty would make GMYC confidence statistics more meaningful.

Distance-based clustering is faster and easier to compute and its accuracy can be similar to GMYC clustering. This shows the potential of distance-based approaches for species reidentification, especially with very large datasets. However, it relies on external calibration (with e.g. morphospecies, see Meier et al., 2006; Hendrich et al., 2010) to be meaningful. It does therefore not provide independent hypotheses of species limits. Nevertheless, there was a surprising correspondence between the results of GMYC modeling and distance clustering when applying standard divergence thresholds for the latter (Table 1). Thus, distance approaches can deliver fast, helpful clues for integrative taxonomy.

The lack of a clear barcoding gap even with GMYC units is here not only a result of inadequate species taxonomy, incomplete lineage sorting or lacking divergence (in young species), but must also be attributed to the (uneven) branching structure of the tree (i.e. the idiosyncratic sequence evolution). It needs to be further investigated, possibly with simulations, if this is due to naturally different rates of sequence evolution (e.g. because of different dispersal activity; Papadopoulou et al., 2008, 2009) and speciation rates or rather (or also) originates from a sampling bias (Lohse, 2009).

The low match we found between different species delineations and morphospecies might partially have causes in the different species concepts used to define species. However, it also shows how necessary it is to combine the output of various methods in an integrative approach. Only in doing so we obtain an idea about the reliability of the different results (species limits) and about signals. This enables us to fine-tune sampling, delineation methods and data collection (markers), and to identify species that require taxonomic revision.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.11.018.

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