

Integrative taxonomy of Iberian *Merodon* species (Diptera, Syrphidae)

^{1,*}XIMO MENGUAL, ²GUNILLA STÅHLS, ³ANTE VUJIĆ &
¹M^a ÁNGELES MARCOS-GARCÍA

¹ Centro Iberoamericano de la Biodiversidad (CIBIO), Universidad de Alicante, E-03080 Alicante, Spain. E-mail: xmengual@ua.es, marcos@ua.es

² Finnish Museum of Natural History, PO Box 17, FI-00014 University of Helsinki, Finland. E-mail: Gunilla.stahls@helsinki.fi

³ Department of Biology and Ecology, Faculty of Science, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia and Montenegro. E-mail: antev@ib.ns.ac.yu

* *Corresponding author*

Abstract

The genus *Merodon* Meigen, 1803 (Syrphidae, Diptera) with more than 50 European species is primarily distributed in the Mediterranean region, with 34 species occurring in the Iberian Peninsula. The morphological variation found within some species from the Iberian Peninsula prompted us to test their taxonomic status by integrating morphological and molecular data. We generated partial sequences of the mitochondrial protein-coding gene cytochrome *c* oxidase subunit I (COI), the nuclear internal transcribed spacer (ITS2) region, and the D2 region of the nuclear 28S ribosomal RNA gene. COI and ITS2 sequences were obtained for most included taxa. The variability of the COI sequences showed great difference between the studied species groups, exhibiting an interspecific range from 0.29% to 12.5% between ingroup taxa. Closely related taxa of the *aureus* complex (e.g. *M. quercetorum* and *M. legionensis*) presented identical COI sequences. The obtained ITS2 sequences showed low intraspecific variability, only a few taxa presented more than one genotype. Species status and delimitation was discussed for all taxa in light of morphological and molecular character information available. Using the obtained sequence data of COI and 28S we inferred the phylogenetic relationships of the included taxa using

parsimony analysis. Separate analysis of the COI sequences identified four partly well-supported clades within *Merodon*, the *desuturinus*, *albifrons*, *nigritarsis* and *aureus* groups. Combined analysis of the COI and 28S genes produced a topology similar to the COI topology.

KEY WORDS: *Merodon*, Syrphidae, integrative taxonomy, molecular data, phylogenetic relationships, intraspecific variation.

INTRODUCTION

The genus *Merodon* Meigen, 1803 (Syrphidae, Diptera) with more than 50 European species is a speciose genus of hoverflies, with highest number of species occurring in the Mediterranean region. From the Iberian peninsula 34 species of *Merodon* are recorded (Marcos-García *et al.* 2002; Marcos-García *et al.* in press), of these ca 50% are identified as taxa endemic to the Iberian Peninsula (Marcos-García *et al.* in press). Data about adult habitats, visited flowers, flight periods and Spanish distributions for particular species are provided by Marcos-García (1985a; 1985b; 1989; 1990a; 1990b). Marcos-García *et al.* (in press) revised all taxa occurring on the Iberian Peninsula (including type studies), provided a key for species identification and a zoogeographical discussion. Vujic *et al.* (in prep.) studied the subgeneric relationships of *Merodon*, and provided additional data on morphological variability of taxa, based on analysis of species and specimens from a broad geographical distribution. These comprehensive studies are the platform for the present study.

The morphological intra-specific variability is well known in some *Merodon* species, e.g. *Merodon equestris* (Fabricius, 1794) that exhibits distinctive colour morphs, and *M. aeneus* Megerle in Meigen, 1822 that exhibits a wide range of colour varieties (Sack 1932). *M. tricinctus* Sack, 1913 presents a high intra-specific variability in the shape of the anterior lobe of the surstyli of the male genitalia (Popov, 2000). Marcos-García *et al.* (in press) describe intraspecific morphological variability for nine species from the Iberian Peninsula.

Analysis of mtDNA sequence data has been used extensively to study the evolutionary relationships both within and among species. Since mitochondrial DNA sequences frequently evolve faster than do nuclear sequences (e.g. Simon *et al.* 1994), the number of variable and informative sites is often greater for mtDNA than for

nuclear loci. MtDNA is particularly useful for species-level and genus-level analyses, as demonstrated in a large number of studies of animal evolutionary relationships (e.g. Caterino & Sperling 1999; Scheffer & Wiegmann 2000; Caterino *et al.* 2001; Ståhls *et al.* 2003, 2004; Arévalo *et al.* 2004; Ståhls 2006).

DNA sequence information, from mitochondrial or nuclear genes, might not always correspond with species recognized by traditional morphological and ecological criteria. The sole use of the barcoding-sequence, a 650 bp fragment of the 5' end of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) has been suggested a species identification system for most of life (Hebert *et al.* 2003, 2004a,b, Savolainen *et al.* 2005). Multiple recent papers summarize the pros and cons of DNA barcoding and review and discuss the issue from many points of view (e.g. Barrett & Hebert 2005, DeSalle *et al.* 2005, Ebach & Holdrege 2005, Hebert & Gregory 2005, Prendini 2005, Rubinoff & Holland 2005, Wheeler 2005, Will *et al.* 2005, Rubinoff 2006). The present study has produced COI sequences that constitute a contribution towards a 'barcode' database for the genus *Merodon*. The COI-3' region here employed is, however, not the 'barcoding fragment' (COI-5' region, the "Folmer fragment") employed in the barcoding framework. A comprehensive set of universally conserved primers were published by Simon *et al.* (1994) for the COI gene, and the primers working well for various genera of Syrphidae typically excluded the COI-5' region. Studies on phylogenetic relationships of taxa of Syrphidae have included a 560 bp COI-3'-fragment to 1128 nt (constituting a large part of the ca 1540 bp in total) of the COI gene analysed in conjunction with other datasets (e.g. Pérez-Bañón *et al.* 2003, Ståhls *et al.* 2004, Milankov *et al.* 2005, Rojo *et al.* 2006).

Dayrat (2005) coined and defined the term "Integrative taxonomy" as the science that aims to delimit the units of life's diversity from multiple and complementary perspectives. In short, in traditional way morphologists describe hypothetical morphospecies based on the observed morphological variation of the taxa under study. These morphospecies are then submitted to the filter of other approaches and additional data (molecular, ecological, etc.). Using this integrative approach, it is implied that the resulting species hypothesis will be better founded as they are based on more comprehensive data from multiple sources. The study of intra- and interspecific variation will always remain the core of integrative taxonomy, but will be limited by practicalities such as taxa available for morphological study are not

always available for molecular study. Will *et al.* (2005) used the term in a way consistent with this view, but expressed having different views in details of implementation strategy. As was suggested by Rubinoff (2006), an integrated approach that uses mtDNA and nuclear DNA in conjunction with morphology and ecology is better able to access different avenues of inheritance, producing more accurate results that are essential when assessing and managing biodiversity.

The aim of the present study is to employ the concept of integrative taxonomy, as the use of DNA data from multiple sources in conjunction with morphological characters and available distribution information to be contrasted with previously established species boundaries based on morphological taxonomy only. We employed mitochondrial COI sequences of the 3' region (hereafter COI) in conjunction with DNA characters from two additional gene regions, the D2 expansion region of the nuclear ribosomal 28S rRNA gene (28S) and the nuclear internal transcribed spacer two region (ITS2) in addition to morphological characters for a sample of taxa from a geographically restricted region, the Iberian Peninsula. We were particularly interested in contrasting the observed morphological variation in particular taxa with molecular variability. We discuss our results in light of all the molecular data (particularly for the 3' fragment of the COI gene) and morphological data that were available. We also estimated the phylogenetic relationships among the included taxa based on the available information of COI and 28S gene sequences.

MATERIAL AND METHODS

Taxon sampling

Specimens used for molecular analysis were sampled mainly from Alicante region (SE Spain) with some samples from other regions of Spain, and additionally two specimens from Greece and one from Andorra (Table 1). For species identification we used the key of Marcos-García *et al.* (in press). Specimens of 17 species out of the total of 34 species occurring in Spain could be obtained for molecular work. Multiple specimens (up to nine) for each species were used when possible (Table 1). The specimens used for molecular study were also used for morphological study of Marcos-García *et al.* (in press) and there included in the Materials section with an indication for each specimen that was used for the present study. DNA voucher

specimens were deposited in CEUA (University of Alicante, Spain), MZH (Zoological Museum of the Finnish Museum of Natural History, Helsinki, Finland) or NS (University of Novi Sad, Serbia & Montenegro) (Table 1).

Molecular characters

The three gene regions were generated for partly different and overlapping purposes. The mitochondrial COI and the nuclear ITS2 region have been used for species delimitation and been shown to be taxonomically informative for measuring levels of intraspecific variability in Syrphidae and relationships among closely related species (e.g. Ståhls & Nyblom 2000, Ståhls *et al.* 2004, Milankov *et al.* 2005, Massetti *et al.* 2006), while the combined sequence data of COI and nuclear ribosomal 28S have been informative for interspecific and generic level comparisons (e.g. Ståhls *et al.* 2003, Ståhls 2006).

Laboratory methods

DNA was extracted from 1–3 legs or other parts of single individuals of dry, pinned specimens using the NucleoSpin Tissue DNA Extraction kit (Machery–Nagel) according to manufacturer’s protocol and re-suspended in 50 µl ultrapure water. PCR amplifications were carried out in 25 µl reactions containing 2–5 µl of DNA extraction, 0,25 µl of Taq DNA polymerase, 1 µl of each dNTP 200 mM, 1 µl of each primer (10 pmol/µl), 2,5 µl PCR buffer 10x without MgCl₂, 2 µl MgCl₂ 25 mM and ultrapure water. Primers used for PCR and sequencing are listed in Table 2. PCR products were purified using the GFX PCR Purification kit (Amersham Biotech). Sequences were generated with an ABI 377 automated DNA sequencer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing kit v1.1 (Applied Biosystems). Electropherograms were inspected and forward and reverse sequences were assembled and edited for each DNA region using Sequence Navigator™ 1.0 (Applied Biosystems). All sequences were deposited in GenBank (see Table 1 for accession numbers).

Parsimony analyses

Parsimony analyses were performed separately for the COI dataset and the combined COI+28S data. If obtained COI sequences were identical between samples of a particular taxon, only one sequence was included in the parsimony analysis. For the 28S gene we sequenced only one specimen per species in most cases. The 28S sequences data was mainly used for the combined parsimony analysis. As the sequence length variation among ingroup taxa was low (4 nucleotides), we were confident to align the 28S sequences by eye.

We used the program NONA v2.0 (Goloboff 1993) for the parsimony analyses (command line: hold 100000; mult*500; hold/200). All characters were equally weighted, and gaps were treated as missing data. NONA spawn from Winclada (Nixon 2002) was used for calculating evidential support for different clades using Bremer support values (branch support) and bootstrap support values (1000 replications; mult*20; hold/2). The Bremer support value for a particular clade indicates the number of extra steps from the most parsimonious tree at which the clade fails to be resolved as successively longer trees are examined. A high numerical value indicates good support. Non-parametric bootstrapping involves resampling data with replacement, and was calculated using 1000 replicates. *Eumerus etnensis* van der Goot, 1964 (Eumerini) was used as outgroup (GenBank accession number AY533315 for COI and AY540907 for 28S).

RESULTS

Molecular markers

COI. For the COI we obtained 784 nucleotide characters for 44 samples representing 17 putative ingroup taxa. The mean AT content was 71.6%. Uncorrected pairwise divergences between ingroup taxa were calculated for the COI gene, and ranged between 0.29% (samples X33*M. arundanus* Marcos-García, Vujic *et* Mengual and X77*M. obscuritarsis* Strobl *in* Czerny & Strobl, 1909) and 12.5% (samples X11*M. elegans* Hurkmans, 1993 and X15–18*M. unguicornis* Strobl *in* Czerny & Strobl, 1909). Uncorrected pairwise divergence between the outgroup and all ingroup taxa was highest for *M. unguicornis* (14.9%). These levels are similar to the divergences

between species found in other genera of Syrphidae (Ståhls et al. 2004, Ståhls 2006) for the same gene.

ITS2. For the ITS2 region we obtained 333–496 nucleotides for 41 samples representing 13 ingroup taxa. In five taxa we obtained the ITS2 sequence for a single specimen. The ITS2 was only used for intraspecific comparisons in all taxa with multiple sequences, with the aim of surveying and scoring the number of intraspecific genotypes. The highest number of intraspecific genotypes was found for *M. albifrons*, while five taxa showed identical ITS2 sequences. Alignment between both ingroup taxa and between ingroup and outgroup for this highly variable gene regions was an ambiguous and difficult task.

28S. For the 28S rRNA gene we pruned the obtained sequences to 375–379 nucleotides length for 22 samples representing 14 ingroup taxa. The aligned matrix consisted of 396 nucleotides of which 21 were parsimony informative.

Parsimony analyses

Separate analysis of COI

No insertions or deletions occurred in the COI dataset so alignment was unambiguous. Of the obtained 784 nucleotides, 194 sites were parsimony-informative. Parsimony analysis produced 63 equally parsimonious trees of 577 steps in length, with a consistency index (CI) of 0.53 and a retention index (RI) of 0.83. The strict consensus is shown in Fig. 1. The COI gene identified four well-defined clades within analyzed Iberian *Merodon* species, the *desaturinus*, *albifrons*, *nigritarsis* and *aureus* groups.

Combined analysis

The 28S sequences were manually aligned as sequences varied with only 4 nucleotides between ingroup taxa. Specimens, for which sequences of both COI and 28S were obtained, were used for this analysis. Three species lacking 28S sequence, *M. antonioi*, *M. legionensis* or *M. unicolor*, were also included using only COI. Parsimony analysis of the combined COI and 28S data produced 9 equally parsimonious trees of 682 steps in length (CI=0.56, RI=0.66), with a topology

resolving the same groups as in the separate analysis of COI. Fig. 2 shows the strict consensus tree.

Integrative taxonomy of *Merodon* spp.

desaturinus group

This group comprises three members in the Palaearctic area (Vujic *et al.* in prep), of these only one taxon, *M. cabanerensis* Marcos-García, Vujic *et* Mengual, occurs on the Iberian Peninsula. Uncorrected pairwise divergences of COI between *M. cabanerensis* and included members of the *albifrons* group range from 7.91 to 9.06%. Parsimony analysis resolved this taxon as sister group to the *albifrons* group, a placement that is in agreement with similarity of some morphological characters (Vujic *et al.* in prep.).

albifrons group

M. albifrons group in Iberia is the most diverse and contains 14 species (Marcos-García *et al.* in press). This study includes five taxa.

We obtained three male and two female specimens that by morphological characters were identified as *M. albifrons* Meigen, 1822 (samples X1–X4 from Spain: Alicante and S534 from Greece: Lesvos). The COI sequences were identical among the four samples from Alicante, these samples were obtained from two locations of ca 12 km distance (Foia Ampla in Agres and El Menetjador in Alcoi). The uncorrected pairwise divergence between the Spanish *albifrons* and the *albifrons* from Greece was 3.32%. 28S sequences were obtained for one sample from Spain and the one from Greece and were identical. The ITS2 fragment was obtained for four specimens of *M. albifrons* from Spain and produced three different genotypes, with variability in a dinucleotide repeat region, AT_(1–5) (Fig. 3). This variability we interpret as intraspecific. The divergence of the COI for the included Spanish *albifrons* and the *albifrons* from Greece (3.32%) is in conflict with the identical morphology and 28S sequences. The 28S gene is more conservative and accumulates change more slowly, which is generally also the case with morphological characters. This could explain our results. *M. albifrons* is widely distributed in the Mediterranean area, and several hundred specimens have been studied, but the observed slight morphological variability was not so striking as to suggest the presence of cryptic taxa, except for *M. hurkmansi*

Marcos-García, Vujic *et* Mengual from Algeria (Marcos-García *et al.* in press). Although DNA sequence data was obtained from 2–5 specimens (28S vs. COI + ITS2, respectively), we conclude that the samples from Spain and Greece probably represent different taxa. To confirm this result additional samples from the Mediterranean area will have to be collected for both molecular and morphological study.

The study included three specimens of *M. geniculatus* Strobl in Czerny & Strobl, 1909 and one specimen of *M. antonioi* Marcos-García, Vujic *et* Mengual from Spain. The recent taxonomic study of Iberian *Merodon* (Marcos-García *et al.*) discovered and described two cryptic taxa close to *M. geniculatus*, and the present study includes one of these taxa, *M. antonioi*. All three *M. geniculatus* specimens used for DNA sequencing share the same morphological characters, and agree with the holotype of *M. geniculatus*.

The parsimony analysis resolved samples S546 + X34 as sister group to X5 + S545 (Figs. 1 and 2). Two *geniculatus* specimens differed with only one nucleotide change (0.13%) (samples S564 and X34), and the third specimen (X5) was resolved as sister taxon of *M. antonioi* (S545) with an uncorrected pairwise divergence of COI which was 1.15%, while it was 3.06% between X5 and X34. The 28S sequences were obtained for samples X5 and X34 and differ by 2 nucleotide changes. The ITS2 sequences were obtained for samples X34, X5 and S545, these sequences are all distinct and private (e.g. 18 gaps were required to manually align X5 and X34 ITS2 sequences, while comparisons of these with sample S545 require at least 30 gaps + some nucleotide changes). The magnitude of differences between the *M. geniculatus* and *M. antonioi* samples for the different gene regions is in agreement with the general description of 28S being the more conservative, the ITS2 being fast evolving and the evolutionary rate of COI being intermediate between 28S and ITS2. The apparent molecular divergence of the COI, 28S and ITS2 sequences of the *geniculatus* samples that were resolved in two different lineages suggests the presence of an additional morphologically cryptic taxon (not agreeing with any of the recently described taxa), while *M. antonioi* is distinct in both morphological and molecular characters from its sister “taxon” (X5). Morphological differences between *Merodon geniculatus* and *M. antonioi* include the length of basoflagellomere, structure of hind

legs and shape of cercus of male genitalia. These species have sympatric populations in Cabañeros National Park, Spain (our samples S546 and S545).

For *M. obscuritarsis* we obtained COI sequences for five specimens, 28S sequences for three specimens and ITS2 sequences for nine specimens. The intraspecific uncorrected divergence between these specimens ranged from 0.14 to 0.89% for the COI gene, the sequence samples for 28S and ITS2 were identical for respective gene region. This taxon is morphologically variable. The recently described taxon *M. arundanus* that parsimony analysis resolved as sister to *M. obscuritarsis* (specimen X29), is morphologically clearly different from *M. obscuritarsis*. Marcos-García *et al.* (in press) described differences e.g. in male genitalia, colour and length of body hairs and structure of integument. Uncorrected pairwise divergences of the COI ranged from 0.28 to 0.89% between the *M. obscuritarsis* samples and *M. arundanus*. *M. arundanus* differs from *M. obscuritarsis* by five nucleotide changes for the 28S, and by two indels and three nucleotide changes for the ITS2. The intraspecific divergences for COI for samples of *M. obscuritarsis* and the interspecific divergences between *M. arundanus* and *M. obscuritarsis* were completely overlapping. The levels of divergences between the different gene regions are surprising and not agreeing with conclusions outlined for the *M. geniculatus* samples (previous section).

nigritarsis group

This is a very diverse group, especially in eastern Mediterranean area. Many taxa belonging to this group were revised by Hurkmans (1993). Only 9 were registered in the Iberian Peninsula (Marcos-García *et al.* in press).

All taxa in this group were supported as distinctive species by both morphological and molecular characters. Interspecific divergences of the COI ranged from 5.89 to 8.47% between the included taxa.

For *M. nigritarsis* Rondani, 1845 we obtained COI sequences for five specimens, from two separate localities of 6.5 km distance, and all were identical. The ITS2 sequences were obtained from four specimens and these were also identical. This taxon is widely distributed in southern and central Europe.

For *M. elegans* we obtained COI and ITS2 sequences for two specimens, and these were identical for respective gene region. The distributional range for *M. elegans* is Spain and Northwest Africa.

Milankov *et al.* (2001) studied populations on the Balkan Peninsula and separated two taxa, *M. avidus* A and *M. avidus* B, based on allozyme data combined with morphological data. The single specimen included in the present analysis agrees with the morphological concept of *M. avidus* B of Milankov *et al.* (2001). The uncorrected pairwise divergence of COI between sampled specimens of *avidus* A and *avidus* B was 6.16% (unpublished data). Taxonomic status and species delimitation of all taxa of the *M. avidus* complex will be presented separately (Milankov *et al.*, in prep.). *M serrulatus* Wiedemann in Meigen, 1822 is the most widespread taxon of genus *Merodon*, with a distribution range including Russia (Altai Mountains), Ukraine (Black Sea) and all of the Mediterranean area. We obtained COI sequences for four samples (three localities, Table 1), and these were identical. The intraspecific COI divergence of three *M. serrulatus* specimens from Russia, Spain and Greece ranged from 0.0 to 0.37% (unpublished data). ITS2 sequences were obtained for eight samples from two localities, and were identical. 28S sequences from two samples needed one indel to be aligned.

aureus group

Species group of *aureus* includes a large number of taxa spread in all Mediterranean area, with many local endemic species. Five of seven Iberian species belonging to *aureus* group are endemics, with the exception of *M. funestus* and *M. chalybeus* (Marcos-García *et al.* in press).

M. funestus is morphologically more different from other taxa in the group, the uncorrected pairwise divergences between *M. funestus* and other taxa of the *aureus* group ranged from 8.94 to 11.34%. The two obtained COI sequences of *M. funestus* didn't show differences. Parsimony analysis resolved *M. unguicornis* and *M. funestus* (Fabricius, 1794) as sister taxa. The uncorrected pairwise sequence divergence of COI between these taxa was 10.11%. *M. funestus* is widely distributed in the Mediterranean area.

The two included specimens of *M. unicolor* Strobl in Czerny & Strobl, 1909 showed a COI divergence of 1.25%. The morphology of these specimens is similar, but the COI divergence indicated the presence of morphologically cryptic species, but we await additional data with a broader geographic representation to further explore this

hypothesis. We could not obtain sequences from other loci for this taxon. *M. unicolor* is distributed in the western Mediterranean area.

The COI sequences of the two included specimens of *M. chalybeus* Wiedemann in Meigen, 1822 showed one nucleotide difference (28S and ITS2 sequences were obtained for one specimen only). *M. chalybeus* is known from Spain, southern France and the former Yugoslavia area.

Marcos-García *et al.* (in press) described *M. quercetorum* and *M. legionensis*, with type localities in Puerto Honduras (Hervás, Cáceres, Spain) and Murias de Paredes (León, Spain). The COI sequences of the three *M. quercetorum* and the single *M. legionensis* sample were identical. The manual alignment of obtained ITS2 sequence between samples X19+X20 vs. X21 of *M. quercetorum* required only one indel. The ITS2 sequence was not obtained for *M. legionensis*. The two taxa can be separated based on some morphological characters, e.g. pilosity of abdomen and hind legs. Further morphological and molecular study of additional specimens will shed more light on present status of taxa.

DISCUSSION

The subgeneric division of genus *Merodon* is in preparation (Vujic *et al.*). Based on the present study we identify four monophyletic groups: *desuturinus*, *albifrons*, *nigritarsis* and *aureus* groups that agree with morphology. The phylogenetic informativeness of 28S rRNA gene is limited, due to the conservative nature of the gene, as this dataset exhibited only 21 variable and parsimony informative sites. Hence, combined analysis of COI and 28S sequences produced similar topology as separate analysis of COI (Figs. 2 and 1, respectively).

DNA Barcoding vs Integrative taxonomy

Several studies have demonstrated the utility of DNA barcodes (sequences) to diagnose species, reveal cryptic species, link different life stages of local faunas, identify parasites and their invertebrate disease vectors, and in forensics and pest management (e.g. Palumbi & Cipriano 1998; Symondson 2002; Baker *et al.* 2003;

Besansky *et al.* 2003; Whiteman *et al.* 2004; Miller *et al.* 2005; Smith *et al.* 2005; Smith *et al.* 2006). But also problems using DNA barcodes have been revealed, e.g. mitochondrial introgression between taxa, recent speciation followed by incomplete lineage sorting or interbreeding (Palumbi & Cipriano 1998; Scheffer & Wiegmann 2000; Croucher *et al.* 2004; Bachtrog *et al.* 2006; Kaila & Ståhls 2006).

The need for using an integrative taxonomy approach for species delimitation was pointed out by Dayrat (2005), Rubinoff & Holland (2005), and Will *et al.* (2005). Our obtained results for the genus *Merodon* exemplify three possible ‘cases’ (situations) in an integrative taxonomy framework, with datasets showing contradictory or congruent signal. We present and discuss these in the following.

Morphology and DNA in concordance

Species with several sequenced specimens for one or several loci showing concordance of “taxonomic signal” of morphology and sequences were *M. nigritarsis*, *M. elegans*, *M. serrulatus*, *M. unguicornis*, *M. funestus*, *M. chalybeus* and *M. quercetorum*.

These are the cases where DNA barcoding would be applicable, at least for samples collected on the Iberian Peninsula, where molecular COI barcodes with high probability would identify only one species. We agree with DeSalle *et al.* (2005) that the COI sequences of few specimens, however, may not be (or are not likely to be) representative of the possible variability of the species as a whole, especially for taxa with widespread distributions. The species listed above presented uncorrected pairwise distances ranging between 6.80% (*M. ottomanus* vs. *M. serrulatus*) and 12.50% (*M. elegans* vs. *M. unguicornis*), these magnitudes of difference are clearly distinct.

Different morphology with identical DNA sequences

The second situation is when the morphological data support two different taxa, but COI sequences are identical or almost identical. This is the case of *M. legionensis* and *M. quercetorum*, two recently described species (Marcos-García *et al.* in press) that have sympatric populations and are morphologically very similar yet discernible using some diagnostic characters. This could indicate mitochondrial introgression between

the taxa, or speciation followed by incomplete lineage sorting. Introgression between animal species has been statistically supported, taxonomically widespread and far more common than generally recognized (see review by Funk & Omland, 2003). There are multiple examples of introgression in insects (see Funk & Omland, 2003; Shaw, 2002; Bachtrog *et al.* 2006) and in other arthropods like spiders (Croucher *et al.* 2004).

In this particular case COI barcodes would fail in the identification or delimitation of species. In the absence of additional sources of information on species limits, mitochondrial barcoding necessarily relies on some combination of mitochondrial monophyly and genetic distances to indicate probable species (Scheffer *et al.* 2006). In these cases of mitochondrial introgression or incomplete lineage sorting, the species-level tree will show no resolution for the closest species and DNA barcoding will fail in the delimitation of species. Cryptic taxa are defined by molecular characters comparing them with well-known and well-studied taxa based on morphological, ecological and biogeographical characters, because we need to refer them to already studied taxa.

Species with intraspecific variability of DNA sequences but similar morphology

Species that show great intraspecific variability in their mitochondrial DNA but are morphologically identical, are in the present study represented by *M. geniculatus* and *M. albifrons*.

The information from the DNA sequence data for three samples of *M. geniculatus* was in conflict with the observed morphological similarity. The apparent molecular divergence of the COI, 28S and ITS2 sequences of the *geniculatus* samples (that were resolved in two different lineages, Figs. 1 and 2) suggested the presence of an additional morphologically cryptic taxon (not agreeing with any of the recently described taxa). Funk and Omland (2003) stated that “If other described species are more closely related to such “cryptic species” than the cryptic specie are to each other, a mitochondrial gene tree might hint at cryptic taxa by revealing polyphyly in the form of two phylogenetically separated clades”, and “Such cryptic species might reflect the retention of ancestral morphology”. *M. geniculatus* agrees perfectly with these predictions. Our results indicate that additional sampling of specimens from a

broad geographic distribution is necessary for discerning intraspecific variability from interspecific.

M. albifrons specimens from Spain have no differences in their COI sequences. The variability of the ITS2 can be addressed as intraspecific due to the nature of the dinucleotide repeat (loop) region (Fig. 3). COI for the included Spanish *albifrons* and the *albifrons* from Greece shows a divergence of 3.32%, being in conflict with the apparent identical morphology of them, and can indicate the presence of morphologically cryptic taxon.

Discovery of cryptic species is one of the goals that can be achieved with DNA barcodes (e.g. Hebert *et al.* 2004a; Kaila & Ståhls 2006; Smith *et al.* 2006). Thus, DNA barcoding has a potential utility to reveal taxonomic information and help to bring to view differences not expressed by morphology.

A special case in this study of species with intraspecific differences of DNA sequences is *M. obscuritarsis* because the intraspecific variability and the interspecific variability overlap completely. The recently described taxon *M. arundanus* that parsimony analysis resolved as sister to *M. obscuritarsis* (specimen X29), is morphologically clearly different from *M. obscuritarsis*, but uncorrected pairwise divergences of the COI ranged from 0.28 to 0.89% between the *M. obscuritarsis* samples and *M. arundanus*, and were overlapping with the intraspecific divergences for the *M. obscuritarsis* sequences (0.12 to 0.89%). Marcos-García *et al.* (in press) described differences in some morphological characters clearly allowing separation of the two taxa. *M. arundanus* is described from the Natural Park of Sierra de Grazalema (Andalucía), a Biosphere Reserve from 1975 by UNESCO, with the highest precipitation value of Iberia and the presence of the endemic spruce *Abies pinsapo* Boiss, a fossil species from the Tertiary. As *M. arundanus* is morphologically distinct, it is recognized as an endemic taxon and is presently found only in Grazalema (South Spain). The taxon agrees with a common pattern for endemism in the Iberian Peninsula: the areas with highest concentrations of endemic species occur in mountain areas (Martín *et al.* 2000; Castro-Parga *et al.* 1996). Thus, we hypothesize that the low divergence between the taxa both for the COI and the ITS2 region indicates that these taxa have speciated recently, but the presence of distinctive morphological diagnostic characters and level of 28S divergence suggest the opposite. This could represent a

case of incomplete lineage sorting following recent speciation. The results for the COI gene remain ambiguous until more data is obtained.

DNA barcoding rules for species delimitation don't work in these cases. A problematic issue of using a DNA barcoding approach on a single mitochondrial gene is the adoption of quite simplistic and arbitrary criteria as a percentage of uncorrected pairwise distances for determining species limits (Hebert *et al.* 2003). Many authors have discussed species delimitation boundaries in a DNA barcoding context (Sperling 2003; Will & Rubinoff 2004; Prendini 2005; Rubinoff 2006) concluding that the 3% divergence rule for insects would only conceal incongruent character distributions without solving the underlying biological problems (Sperling 2003) and the ranges for intra- and interspecific variation are still mostly unknown and will vary among groups and across gene loci (e.g. Prendini 2005). But DNA barcoding generates information, not knowledge (Ebach & Holdrege 2005). The use of a short sequence from a single marker alone can not define by itself species, but can help in the identification.

Results from recent studies discourage the approach for delimitation of closely related species using COI barcodes (Scheffer *et al.* 2005; Kaila & Ståhls 2006; Rubinoff 2006) and suggest that evidence from different sources such as morphological and ecological data and molecular evidence from more than one molecular locus should be used for species delimitation and identification (Sperling 2003; Dayrat 2005). DNA sequences between closely related, recently diverged, hybrid, or polyploid species, the very cases for which identification may be most crucial (Sperling 2003; Will and Rubinoff 2004), will often be too similar to allow their discrimination.

The present study based on DNA sequences of a set of taxa sampled from a geographically restricted region revealed both conflict and congruence between taxonomic information from morphological and molecular characters. If we had used only morphological characters, we would have failed to recognize 3 possible cryptic species (15.8%; X5M. *geniculatus*, X22M. *unicolor* and S534M. *albifrons*). If only COI sequences were used, 2 species were not identified (10.5%; X33M. *arundanus* and X55M. *legionensis*).

We are convinced that had we obtained a denser sampling of taxa and specimens it would have resulted in a higher number of conflicting cases and we could potentially have resolved existing ones. Although the obtained sequence data set (COI, ITS2,

28S) was not complete for all taxa, it showed that the process of delimiting and identifying species is potentially better understood if based on information from multiple loci in conjunction with morphology. We feel that congruent taxonomic signal of morphology and molecular data results in best supported species hypothesis. However, this study would have been improved by a more complete data set and geographically more diverse taxon sampling. This study has, however, encouraged us to continue using the integrative approach in future studies on taxonomy of Syrphidae.

ACKNOWLEDGEMENTS

The authors wish to thank Elvira Rättel for assistance with DNA sequencing and Antonio Ricarte for providing specimens for molecular work. This work was supported in part by the Ministry of Science and Environmental Protection of Serbia (Grant Number 143037), the Provincial Secretariat for Science and Technological Development (Maintenance of biodiversity–“Hot spots” on the Balkan and Iberian Peninsula), the Spanish Ministerio de Medio Ambiente (MMA-040/2002), Spanish Ministerio de Educación y Ciencia (CGL2005-0713/BOS) and Generalitat Valenciana (ACOMP06/063).

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Legends to figures

Fig. 1. Parsimony analysis of COI sequences. Strict consensus of 63 equally parsimonious trees, L=577, CI= 0.53, RI= 0.83. Open circles denote homoplasious characters and filled circles denote nonhomoplasious characters. Bremer support values are indicated above branches, bootstrap values below.

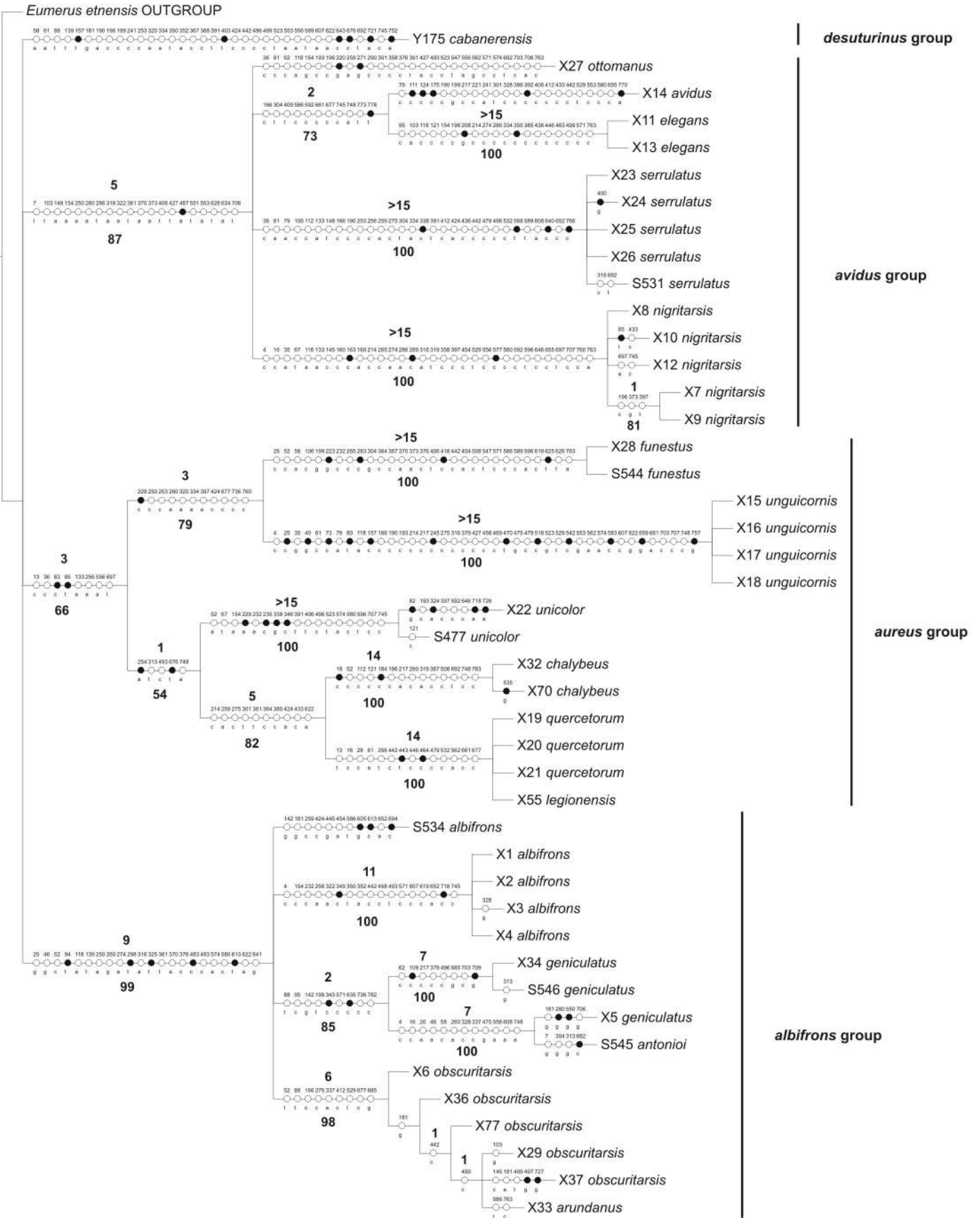
Fig. 2. Combined analysis of COI and 28S sequences. Strict consensus of 9 equally parsimonious trees, L= 682, CI= 0.56, RI= 0.66. Open circles denote homoplasious characters and filled circles denote nonhomoplasious characters. Bremer support values are indicated above branches, bootstrap values below.

Fig. 3. *M. albifrons* from Spain produced three different genotypes for ITS2 with variability in a dinucleotide repeat region, AT₍₁₋₅₎.

Table 1. Data on included specimens and GenBank accession numbers for obtained sequences.

Table 2. Primers used for amplifying and sequencing the COI, 28S and ITS2 fragments.

Fig. 1. COI analysis.



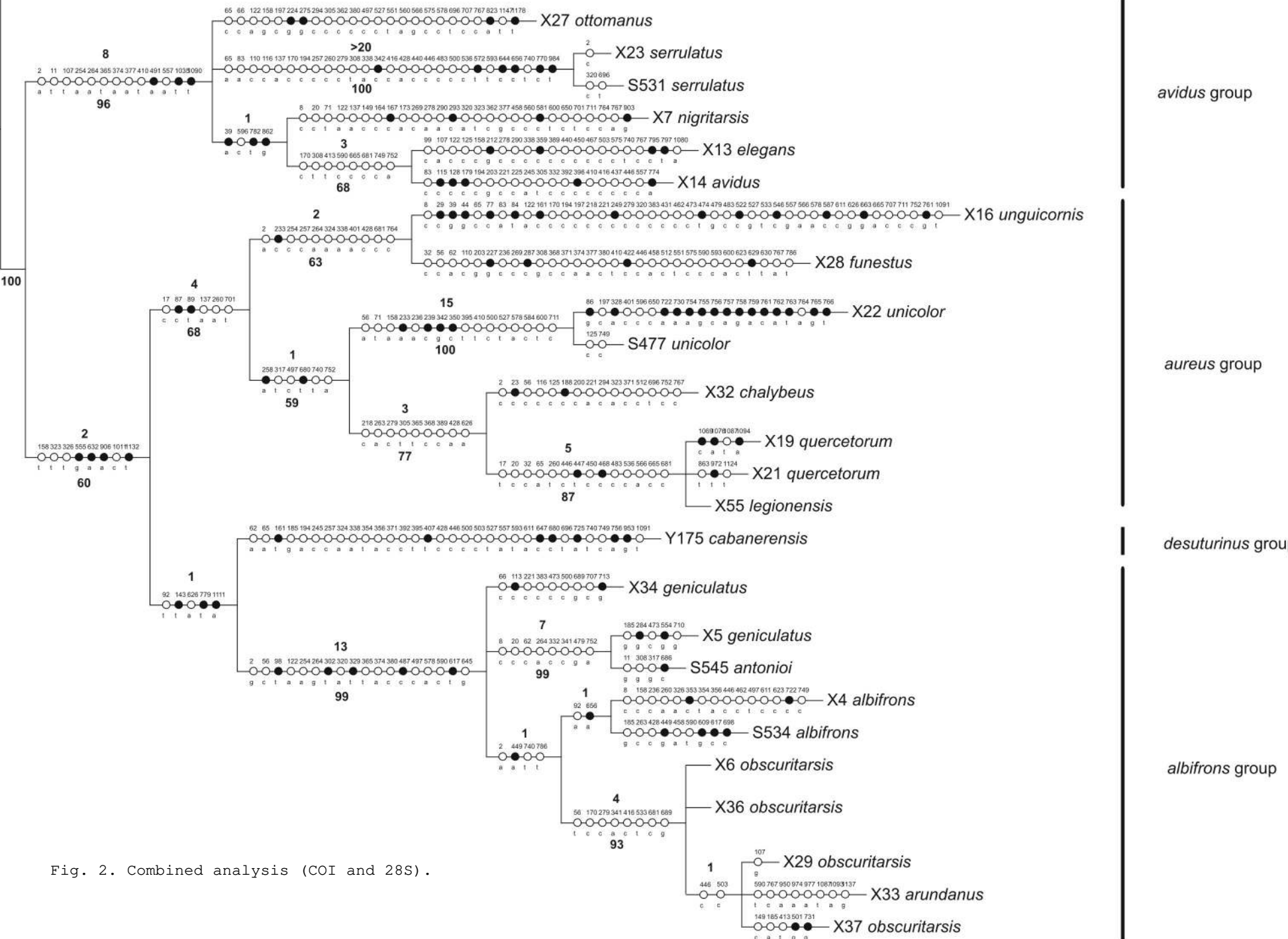


Fig. 2. Combined analysis (COI and 28S).

X1M. albifrons . . . TATATATATATATATATAT-----TAAAAAAATTTA . . .
X2M. albifrons . . . TATATATATATATATATATATATATATATATTTAAAAAAATTTA . . .
X3M. albifrons . . . TATATATATATATATATAT-----TAAAAAAATTTA . . .
X4M. albifrons . . . TATATATATATATATATATATATATAT-----TAAAAAAATTTA . . .

Fig. 3

Table 1.

Taxon	Collecting locality	Sex	Laboratory Code	Deposition	GenBank accession number COI	GenBank accession number 28S	GenBank accession number ITS2
<i>Merodon albifrons</i> Meigen, 1822	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 24-05-2002. Leg.: X. Mengual.	Male	X1	CEUA 00002091	DQ386317		DQ386376
<i>Merodon albifrons</i> Meigen, 1822	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 24-05-2002. Leg.: X. Mengual.	Male	X2	CEUA 00002088	DQ386318		DQ386377
<i>Merodon albifrons</i> Meigen, 1822	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 14-08-2001/30-08-2001. Leg.: X. Mengual.	Female	X3	CEUA 00001652	DQ386319		DQ386378
<i>Merodon albifrons</i> Meigen, 1822	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 10-09-2002/24-09-2002. Leg.: X. Mengual.	Female	X4	CEUA 00001653	DQ386320	DQ386356	DQ386379
<i>Merodon albifrons</i> Meigen, 1822	GREECE, Lesvos, 1.25 km S Pili. 140 m. 30°10'14N 26°25'11E. Olive grove complex landscape. Aegean University 0004112.	Male	S534	MZH	DQ386312	DQ386354	
<i>Merodon antonioi</i> Marcos-García, Vujić et Mengual, 2006	SPAIN, Ciudad Real, P.N. Cabañeros. 11-IX-2004. Leg. A. Ricarte.	Male	S545	MZH CEUA 00002079	DQ386314		DQ386375
<i>Merodon arundanus</i> Marcos-García, Vujić et Mengual 2006	SPAIN, Cádiz, Sierra de Grazalema, Coros, 1331 m. 20-IV-2003. N36°47' W5°22'. Leg.: W. van Steenis & E. S. Bakker.	Male	X33	CEUA 00002082	DQ386346	DQ386370	DQ386403
<i>Merodon avidus</i>	SPAIN, Ávila, Piedralaves, 18-	Male	X14	CEUA	DQ386329	DQ386361	DQ386388

<i>B sensu</i> Milankov <i>et al.</i> , 2001	07-2001. Leg.: J.R. Verdú.			00002086			
<i>Merodon</i> <i>cabanerensis</i> Marcos-García, Vujić et Mengual, 2006	SPAIN, Ciudad Real, P.N. Cabañeros. 18-03-2005. Leg.: A. Ricarte.	Male	Y175	MZH	DQ386316	DQ386355	
<i>Merodon</i> <i>chalybeus</i> Wiedemann <i>in</i> Meigen, 1822	SPAIN, Cáceres, Embalse de Guadiloba. N39° 29' W6° 17'. 16- IV-2002. Leg.: W. van Steenis.	Male	X32	CEUA 00002081	DQ386345	DQ386369	DQ386402
<i>Merodon</i> <i>chalybeus</i> Wiedemann <i>in</i> Meigen, 1822	SPAIN, Cádiz, Tarifa, Rio Jara. 08-04-2002. Leg.: W. van Steenis & E.S. Bakker.	Female	X70	W. van Steenis collection	DQ386351		
<i>Merodon elegans</i> Hurkmans, 1993	SPAIN, Alicante, Xixona, Mas de Cano, 940 m. 21-05-2001. Leg.: X. Mengual.	Female	X11	CEUA 00002099	DQ778055		DQ386386
<i>Merodon elegans</i> Hurkmans, 1993	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 02-07- 2002/16-07-2002. Leg.: X. Mengual.	Male	X13	CEUA 00002092	DQ386328	DQ386360	DQ386387
<i>Merodon</i> <i>funestus</i> (Fabricius, 1794)	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 29-05-2001. Leg.: X. Mengual.	Male	X28	CEUA 00002101	DQ386343	DQ386367	DQ386400
<i>Merodon</i> <i>funestus</i> (Fabricius, 1794)	SPAIN, Ciudad Real, P.N. Cabañeros. 2005. Leg.: A. Ricarte.	Female	S544	MZH	DQ386313		
<i>Merodon</i>	SPAIN, Valencia, Bocairant,	Male	X5	CEUA	DQ386321	DQ386357	DQ386380

<i>geniculatus</i> Strobl in Czerny & Strobl, 1909	Mas del Parral, 900 m. 04-06-2002/17-06-2002. Leg.: X. Mengual.			00002084			
<i>Merodon geniculatus</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 16-07-2002/30-07-2002. Leg.: X. Mengual.	Male	X34	CEUA 00002072	DQ386347	DQ386371	DQ386404
<i>Merodon geniculatus</i> Strobl in Czerny & Strobl, 1909	SPAIN, Ciudad Real, P.N. Cabañeros. 29-V-2004. Leg. A. Ricarte.	Male	S546	MZH CEUA 00002080	DQ386315		
<i>Merodon legionensis</i> Marcos-García, Vujić et Mengual, 2006	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 27-08-2002/10-09-2002. Leg.: X. Mengual.	Female	X55	NS	DQ386350		
<i>Merodon nigritarsis</i> Rondani, 1845	SPAIN, Alicante, Xixona, Mas de Cano, 940 m. 05-06-2001/20-06-2001. Leg.: X. Mengual.	Male	X7	CEUA 00002094	DQ386323	DQ386359	DQ386382
<i>Merodon nigritarsis</i> Rondani, 1845	SPAIN, Alicante, Xixona, Mas de Cano, 940 m. 15-05-2002/04-06-2002. Leg.: X. Mengual.	Male	X8	CEUA 00002096	DQ386324		DQ386383
<i>Merodon nigritarsis</i> Rondani, 1845	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 03-06-2002/17-06-2002. Leg.: X. Mengual.	Female	X9	CEUA 00002093	DQ386325		DQ386384
<i>Merodon nigritarsis</i> Rondani, 1845	SPAIN, Alicante, Xixona, Mas de Cano, 940 m. 23-05-2001/05-06-2001. Leg.: X. Mengual.	Female	X10	CEUA 00002095	DQ386326		DQ386385
<i>Merodon</i>	SPAIN, Alicante, Xixona, Mas	Male	X12	CEUA	DQ386327		

<i>nigritarsis</i> Rondani, 1845	de Sant Ignaci, 1020 m. 15-05-2002/04-06-2002. Leg.: X. Mengual.			00002097			
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 15-05-2002/04-06-2002. Leg.: X. Mengual.	Female	X6	CEUA 00001377	DQ386322	DQ386358	DQ386381
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 15-05-2002/04-06-2002. Leg.: X. Mengual.	Male	X29	CEUA 00001376	DQ386344	DQ386368	DQ386401
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 27-08-2002/10-09-2002. Leg.: X. Mengual.	Female	X36	CEUA 00001372	DQ386348	DQ386372	DQ386405
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Xixona, Mas de Sant Ignaci, 1020 m. 07-05-2001/23-05-2001. Leg.: X. Mengual.	Female	X37	CEUA 00001371	DQ386349	DQ386373	DQ386406
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 29-05-2001. Leg.: X. Mengual.	Male	X42	CEUA 00001375			DQ386411
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 05-06-2001/19-06-2001. Leg.: X. Mengual.	Male	X43	CEUA 00001373			DQ386412
<i>Merodon obscuritarsis</i> Strobl in Czerny	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 08-05-2001/23-05-2001. Leg.: X. Mengual.	Male	X44	CEUA 00001374			DQ386413

& Strobl, 1909							
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 24-05-2002. Leg.: X. Mengual.	Male	X45	CEUA 00001369		DQ386374	DQ386414
<i>Merodon obscuritarsis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 24-05-2002. Leg.: X. Mengual.	Male	X46=X77	CEUA 00001370	DQ386352		DQ386415
<i>Merodon ottomanus</i> Hurkmans, 1993	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 08-05-2001/23-05-2001. Leg.: X. Mengual.	Male	X27	CEUA 00002077	DQ386342	DQ386366	
<i>Merodon quercetorum</i> Marcos-García, Vujić et Mengual, 2006	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 14-08-2001/30-08-2001. Agres. Alicante. SPAIN. Leg.: X. Mengual.	Female	X19	CEUA 00002074	DQ386334	DQ386363	DQ386393
<i>Merodon quercetorum</i> Marcos-García, Vujić et Mengual, 2006	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 14-08-2001/30-08-2001. Leg.: X. Mengual.	Male	X20	CEUA 00002075	DQ386335		DQ386394
<i>Merodon quercetorum</i> Marcos-García, Vujić et Mengual, 2006	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 30-08-2001/11-09-2001. Leg.: X. Mengual.	Female	X21	NS	DQ386336	DQ386364	DQ386395
<i>Merodon serrulatus</i>	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 23-05-	Male	X23	CEUA 00001361	DQ386338	DQ386365	DQ386396

Wiedemann in Meigen, 1822	2001/05-06-2001. Leg.: X. Mengual.						
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 23-05-2001/05-06-2001. Leg.: X. Mengual.	Male	X24	CEUA 00001362	DQ386339		DQ386397
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Agres, Foia Ampla, 1060 m. 23-05-2001/05-06-2001. Leg.: X. Mengual.	Female	X25	CEUA 00001365	DQ386340		DQ386398
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 05-06-2001. Leg.: X. Mengual.	Male	X26	CEUA 00001363	DQ386341		DQ386399
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 24-05-2002. Leg.: X. Mengual.	Male	X38	CEUA 00001366			DQ386407
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 07-06-2001. Leg.: X. Mengual.	Male	X39	CEUA 00001368			DQ386408
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 23-05-2001/05-06-2001. Leg.: X. Mengual.	Male	X40	CEUA 00001364			DQ386409
<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 24-05-2002. Leg.: X. Mengual.	Male	X41	CEUA 00001367			DQ386410

<i>Merodon serrulatus</i> Wiedemann in Meigen, 1822	GREECE, Lesvos, Agiasos. Site 2, locality 6. 6-VI-2005.	Male	S531	MZH	DQ386311	DQ386353	
<i>Merodon unguicornis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Xixona, Venta de la Carrasqueta, 980 m. 23-04-2002/15-05-2002. Leg.: X. Mengual.	Male	X15	CEUA 00002104	DQ386330		DQ386389
<i>Merodon unguicornis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Caveta del Buitre 1200 m. 23-05-2001/05-06-2001. Leg.: X. Mengual.	Male	X16	CEUA 00001655	DQ386331	DQ386362	DQ386390
<i>Merodon unguicornis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Agres, Caveta del Buitre, 1200 m. 23-05-2001/05-06-2001. Leg.: X. Mengual.	Female	X17	CEUA 00001654	DQ386332		DQ386391
<i>Merodon unguicornis</i> Strobl in Czerny & Strobl, 1909	SPAIN, Alicante, Alcoi, El Menetjador, 1352 m. 15-05-2002/04-06-2002. Leg.: X. Mengual.	Female	X18	CEUA 00002087	DQ386333		DQ386392
<i>Merodon unicolor</i> Strobl in Czerny & Strobl, 1909	SPAIN, Valencia, Chelva. 10-04-1994/24-04-1994. Leg.: C. Pérez-Bañón.	Male	X22	CEUA 00002220	DQ386337		
<i>Merodon unicolor</i> Strobl in Czerny & Strobl, 1909	ANDORRA, Andorra. 10 km NE city. 17-05-2003. Leg: Halada.	Male	S477	MZH	DQ386309		

Table 2.

	PRIMER	Sequence	Source
COI	C1-J-2183 (alias Jerry)	5'-CAACATTTATTTTGATTTTTTGG-3'	Simon <i>et al.</i> , 1994
	TL2-N-3014 (alias Pat)	5'-TCCAATGCACTAATCTGCCATATTA-3'	Simon <i>et al.</i> , 1994
28S	28S(F2)	5-AGAGAGAGTTCAAGAGTACGTG-3'	Belshaw <i>et al.</i> 2001
	28S(3DR)	5'-TAGTTCACCATCTTTCGGGTC-3'	Belshaw <i>et al.</i> 2001
ITS2	ITS2A (f)	5'-TGTGAACTGCAGGACACAT-3'	Beebe & Saul, 1995
	ITS2B (r)	5'-TATGCTTAAATTCAGGGGGT-3'	Beebe & Saul, 1995