ACTA ENTOMOLOGICA MUSEI NATIONALIS PRAGAE
Published 15.xii.2014 Volume 54(2), pp. 741–757 ISSN 0374-1036
http://zoobank.org/urn:lsid:zoobank.org:pub:5CB81C03-0C5F-4220-AD2B-1C97F74C30D3

DNA barcoding applied: identifying the larva of *Merodon avidus* (Diptera: Syrphidae)

Andrijana ANDRIĆ[1,*], Branko ŠIKOPARIJA[1], Dragana OBREHT[1], Mihajla ĐAN[1], Jelena PRERADOVIĆ[1], Snežana RADENKOVIĆ[1], Celeste PÉREZ-BAÑÓN[2] & Ante VUJIĆ[1]

[1] Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 2, RS-21000 Novi Sad, Serbia; e-mails: andrijana.andric@dbe.uns.ac.rs, branko.sikoparija@dbe.uns.ac.rs, dragana.obreht@dbe.uns.ac.rs, mihajla.djan@dbe.uns.ac.rs, jelenaprerad86@gmail.com, snezana.radenkovic@dbe.uns.ac.rs, ante.vujic@dbe.uns.ac.rs

[2] CIBIO Research Institute (Iberoamerican Center for the biodiversity), University of Alicante, Apt 99, E-03080 Alicante, Spain; e-mail: celeste.perez@ua.es

*) Corresponding author.

Abstract. The immature stages of phytophagous hoverflies and their plant hosts are mostly unknown. This paper describes the larva of *Merodon avidus* Rossi, 1790 (Diptera: Syrphidae), based on material collected from Đerdap National Park in Serbia. Larvae were found in the bulbs of *Ornithogalum* L. (Hyacinthaceae) and in the surrounding soil. DNA barcoding was used to identify the species and scanning electron microscopy was used to describe the morphological characteristics of *Merodon avidus*. Reared material previously identified as *Merodon constans* (Rossi, 1794) is shown to belong to *M. hurkmansi* Marcos-García, Vujić & Mengual, 2007.

Key words. Diptera, Syrphidae, *Merodon*, hoverflies, immature stages, larval morphology, DNA barcoding, Serbia, Palaearctic Region

Introduction

Hoverflies (Syrphidae) are one of the most diverse and species-rich dipteran families (Rotheray & Gilbert 2011, Thompson 2010). Although this group is relatively well-known, current knowledge is based mostly on studies of adult insects. A targeted search for eggs and larvae is difficult because breeding and ovipositing sites for most species are imprecisely recorded or unknown (Hurkmans 1993, Rotheray 1993). In addition, descriptions of immature stages are only available for a few species, because larvae which have been successfully reared all-too-often remain undescribed (Rotheray & Gilbert 2011).

In contrast to adults, which usually feed on pollen or nectar, hoverfly larvae have a wide spectrum of feeding habits, including phytophagy, mycophagy, zoophagy and saprophagy (Rotheray & Gilbert 2011). Syrphid species grouped in the same higher taxonomic category usually share similar larval feeding habits (e.g. immature stages of the tribe Merodontini are phytophagous). Thus, this could possibly indicate the importance of larval feeding habits for our understanding of evolutionary and phylogenetic relationships, since morphological innovation is often associated with feeding modes (Rotheray 1993, Rotheray & Gilbert 1999).

Phytophagous syrphid larvae feed on the tissues of living plants such as bulbs, stems, roots, rhizomes, tubers, leaves and cambium. This close relationship with plants makes hoverfly species with phytophagous larvae good bioindicators of environmental stress (Sommaggio 1999) and habitat quality (Rotheray & Gilbert 2011). However, food plants and immature stages have been identified for less than 8 % of known phytophagous hoverflies (e.g. *Eumerus* Meigen, 1822, *Cheilosia* Meigen, 1822 and *Merodon* Meigen, 1803) (Rotheray & Gilbert 2011). In one of the most comprehensive studies on phytophagous syrphid larvae, Stuke (2000) investigated the morphology of larvae from the genus *Cheilosia*, including food plants and larval characters in phylogenetic reconstructions. In general, research on the biology and ecology of immature hoverfly stages is necessary to address the needs of rare species, as well as manage pests or economically beneficial species.

The hoverfly genus *Merodon* belongs to the subfamily Eristalinae, tribe Merodontini (Eumerini *sensu* Ståhls et al. 2009; *Merodon* Meigen, 1803 is older than *Eumerus* Meigen, 1822, thus, Merodontini is the correct name of the tribe), with more than 160 species distributed over the Palaearctic and Afrotropical Regions (Ståhls et al. 2009). Although the systematics and evolution of this species-rich genus has recently received detailed attention (Francuski et al. 2011; Marcos-García et al. 2007, 2011; Mengual et al. 2006; Milankov et al. 2009; Ståhls et al. 2009; Vujić et al. 2011, 2012), the taxonomic status and identification of many *Merodon* species still needs to be clarified. A particular problem is the lack of knowledge on the ecology and larval development of *Merodon* species.

Merodon adults feed on the flowers of many plants with underground storage organs, building anthecological relationhips with both wild and cultivated monocotyledonous geophytes (Hurkmans 1993, Marcos-García et al. 2007, Petanidou 1991, Ståhls et al. 2009). These plants have fleshy subterranean storage organs which are usually the only part of the plant that survives unfavorable climate conditions (winter, drought), and hence are suitable for safe larval development.

Although scarce, the available data on the immature stages of phytophagous hoverflies show a subtle relationship between species and their food plants, including adjustments in behavior, ecology and functional morphology relating to particular circumstances (Rotheray & Gilbert 2011). In their study of Syrphidae phylogeny, Rotheray & Gilbert (1999) concluded that morphological innovation is associated mainly with feeding modes and that major structural changes can occur in mouthparts, the thorax, the anal segment and locomotory organs. Phytophagous hoverfly larvae show numerous features that enable them to adapt to specific feeding modes, and unique specializations that help them adjust to specific situations (Rotheray 1993). All previously described *Merodon* larvae were found feeding in the bulbs and rhizomes of geophytes (Heiss 1938, Hodson 1932, Ricarte et al. 2008, Rotheray 1993,

Table 1. Published information about *Merodon* species larvae including status and host plants.

Species	Status of larva	Host plant	Reference
M. equestris (Fabricius, 1794)	described	*Iris* L., *Narcissus* L.	Heiss (1938), Hodson (1932)
M. bombiformis Hull, 1944	described	*Gladiolus* L.	Stuckenberg (1956)
M. luteihumerus Marcos-García, Vujić & Mengual, 2007	described	*Urginea maritima* (L.) Baker	Ricarte et al. (2008)
M. hurkmansi Marcos-García, Vujić & Mengual, 2007*	described	*Muscari comosum* (L.) Miller	Ricarte et al. (2008)
M. nigritarsis Rondani, 1845	reared but not described	*Hyacinthella pallasiana* (Steven) Losinskaja	Stepanenko & Popov (1997)
M. eques (Fabricius, 1805)	reared but not described	*Narcissus* L.	Pehlivan & Akbulut (1991)
M. alexandri Popov, 2010	not described	Hyacinthaceae [1]	Popov (2010)
M. avidus (Rossi, 1790)	not described	*Muscari* (L.) Miller [2]	Reemer & Goudsmits (2004)
M. loewi van der Goot, 1964	not described	*Ornithogalum* [3]	Hurkmans (1988)
M. armipes Rondani, 1843	not described	*Muscari*, *Ornithogalum* L. [3]	Speight (2012)
M. rufus Meigen, 1838	not described	*Anthericum* L. [3]	Speight (2012)
M. cinereus (Fabricius, 1794)	not described	*Crocus* L. [3]	Speight (2012)
M. dobrogensis Bradescu, 1982	not described	*Scilla autumnalis* L. [3]	Speight (2012)
M. flavus Sack, 1913	not described	*Narcissus* L. [3]	Speight (2012)

Species identification: * erroneously published as *M. constans* (Rossi, 1794) in Ricarte et al. (2008).
Host plant association: [1] bulb-feeding observed, [2] oviposition observed, [3] association suspected.

Stuckenberg 1956) (Table 1). Other studies describing the biology of immature stages or the behavior of adult insects towards plants, such as oviposition, also indicate a *Merodon* - geophyte connection (Hurkmans 1988; Pehlivan & Akbulut 1991; Popov 2001, 2010; Reemer & Goudsmits 2004; Speight 2012; Stepanenko & Popov 1997) (Table 1).

To date the immature stages of only four *Merodon* species have been described (Table 1). All known *Merodon* larvae develop in the underground bulbs and rhizomes of geophytes (i.e. Amaryllidaceae, Iridaceae and Hyacinthaceae) or the surrounding soil. Rotheray (1993) stressed that the larvae of some *Merodon* species could be considered horticultural or agricultural pests, particulary *M. equestris* (Fabricius, 1794). However, although they are commonly known as "large bulb flies" or "narcissus bulb flies" (Hurkmans 1993), their food plants in natural conditions remain to be discovered. In fact, host plants for the immature stages of most *Merodon* species are unknown, and even if larvae have been found, there are difficulties associated with their determination. In particular, there are few identification manuals or keys available for early *Merodon* stages; hence identification requires rearing, which could take months (Rotheray & Gilbert 2011).

The most problematic component associated with studying immature insect stages is their identification to species level. Rearing is considered to be the only infallible method for correctly linking larvae with their respective adults. However, the process of rearing is time intensive, and it is difficult to achieve appropriate conditions for many species (Caterino & Tishechkin 2006, Popov 2001). DNA barcodes have been proposed as an alternative approach

for the identification of arthropod larval stages. Previous studies using DNA barcodes include the identification of immature terrestrial and aquatic arthropods (e.g., Caterino & Tishechkin 2006, Ekrem et al. 2007, Miller et al. 2005). A general conclusion from these studies is that identification is more successful when a comprehensive DNA barcode library is available. The mitochondrial gene cytochrome c oxidase I (COI) was successfully used for DNA barcoding in *Merodon* taxonomy and phylogeny (Mengual et al. 2006, Milankov et al. 2009, Radenković et al. 2011, Ståhls et al. 2009, Vujić et al. 2012) and the resultant COI barcode libraries could be used for molecular identification of *Merodon* larvae.

Over the course of long-term research of hoverfly fauna in Đerdap National Park (Serbia), three larvae of the genus *Merodon* were found associated with bulbs of *Ornithogalum umbellatum* L. (Hyacinthaceae). The aim of the present study was the identification of larvae using DNA barcoding coupled with detailed morphological descriptions. A detailed morphological description of hoverfly larvae identified as *Merodon avidus* Rossi, 1790 (Diptera: Syrphidae) was performed.

Material and methods

Sampling larvae. A targeted search for immature *Merodon* was conducted on the border of the Strict Nature Reserve "Ciganski potok", in Đerdap National Park, Eastern Serbia. Abundant populations of different *Merodon* species occur at this locality. Eleven species of this genus were found there during two years of research: *M. aberrans* Egger, 1860, *M. albifrons* Meigen, 1822, *M. ambiguus* Bradescu, 1986, *M. armipes* Rondani, 1843, *M. aureus* Fabricius, 1805, *M. avidus* Rossi, 1790, *M. clavipes* (Fabricius, 1781), *M. loewi* van der Goot, 1964, *M. nigritarsis* Rondani, 1845, *M. rufus* (Macquart, 1835) and *M. trebevicensis* Strobl, 1900.

Larval searches were carried out over the course of 5 field trips in 2012 (22 April, 15 May, 2 June, 15 October and 12 November) designed to coincide with the life cycle of the locally recorded *Merodon* species. An area known for its abundance of *Merodon* adults was carefully searched during each field trip. At least 10 sub-samples, each one a square metre in size, were thoroughly examined at the soil surface and underground. Soil was excavated to a depth of approximately 20 cm and sieved. Extracted biological material (i.e. insects, larvae, exuviae, bulbs, rhizomes, roots) was thoroughly examined.

Two hoverfly larvae (La, Lb) were found in bulbs of *Ornithogalum umbellatum* L. in April and one (Lc) was retrieved from the soil surrounding bulbs of the same plant species in November. During the search, a number of damaged *Ornithogalum* L. bulbs (discoloured and hollow, or filled by rotten tissue), were excavated as this potentially indicated larval feeding. Bulbs of other *Ornithogalum* species were also excavated, together with those of *Muscari* Miller, *Allium* L., *Crocus* L., *Gagea* Salisbury and *Narcissus* L., but no larvae were found. Larvae were kept frozen (-20 °C) for several days and then preserved in 70% ethanol.

Morphological examination. All collected specimens were thoroughly examined using a stereomicroscope (LEICA MZ16) and important characters were photographed using a LEICA DFC320 digital camera. The larvae fixation process differed from commonly used methodo-

logy (Rotheray 1993). Specifically, specimens were not boiled in water (which extends body length), to ensure that sample material remained undamaged for molecular analysis. Larvae dimensions are therefore only an estimate and cannot be used for comparison with specimens fixed using common procedures. Additional morphological studies were made with a Scanning Electron Microscopy (JEOL JSM-6460). For SEM examinations, larvae were fixed in 2.8% glutaraldehyde, dehydrated in increasing percentages of ethanol (30, 50, 70, 90 and 100%), extracted in chloroform, dehydrated in absolute ethanol for 24 hours and finally dried using a CPD 030 *Critical Point Dryer*, BAL-TEC, Germany. The fixed larvae were then coated in gold using a BAL-TEC, SCD 005, *Sputter coater*. The voltage used for SEM examination was 20 kV. Photographs of the main features are presented.

Morphological characters and terminology follow Rotheray (1993) and Rotheray & Gilbert (1999). Descriptions of larval characters are based on a comparative study of known larvae from the literature, as presented in Table 1.

Additional examinations were carried out on six adult specimens from the collection of the National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA. The aim of this further analysis was to identify adults that emerged from larvae described as *Merodon constans* (Rossi, 1794) by Ricarte et al. (2008).

Molecular analysis. Total genomic DNA was extracted from 50 mg of larval tissue using the procedure described by Chen et al. (2010). The universally conserved primers used for amplifying and sequencing the mtDNA COI 3'-fragment were the forward primer C1-J-2183 (5'-CAA CAT TTATTT TGA TTT TTT GG-3') (alias JERRY) and reverse primer TL2-N-3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT 3') (alias PAT) (Simon et al. 1994). The reaction mixture contained 1x PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM of each nucleotide, 1U *Taq* polymerase, 2 pmoles of each primer, and approximately 50 ng template DNA. Amplification was performed in an Eppendorf Thermocycler using the following conditions: initial denaturation at 95°C for 2 min; 29 cycles of denaturation at 94°C for 30 s each; 30 s annealing at 49°C; 2 min extension at 72°C; followed by a final extension of 8 min at 72°C. Amplification products were checked for the expected product size using standard 1.5% agarose gel electrophoresis. The remaining product was purified using Exonuclease I and Shrimp Alkaline Phosphatase enzymes according to the manufacturer's instructions (Fermentas, Lithuania). All sequencing reactions were performed using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Sequences were generated on an ABI 3730*xl* DNA Analyzer (Applied Biosystems, Foster City, CA).

Sequences were aligned using the ClustalW algorithm (Thompson et al. 1994) as implemented in BioEdit 7.0.9.0. (Hall 1999), with final adjustments performed manually. The final data set contained 69 mtDNA COI sequences, with a final alignment length of 506 bp. The dataset used contained three larval sequences (labeled as La, Lb, and Lc; KM507178-KM507180), together with the following sequences retrieved from GenBank: *Merodon aberrans* (HE653240); *M. albifrons* (KC763576, KC763587, KC763553, DQ386320); *M. armipes* (DQ885917, DQ885918); *M. aureus* (DQ387906, DQ387913, DQ387917, DQ387922); *M. avidus* sequences (GeneBank: DQ845109-DQ845133, HE653243); *M. bicolor* Gil Collado, 1930 (DQ845135, DQ845136, DQ386329 – note, the

latest accession number is listed in GenBank as *M. avidus*, however, personal communication with the authors suggests that this specific sequence belongs to *M. bicolor*); *Merodon clavipes* (HE653247); *M. desuturinus* Vujic, Simic, & Radenkovic, 1995 (DQ387899); *M. italicus* Rondani, 1845 (HE653253); *M. loewi* (DQ885923-DQ885928); *M. nigritarsis* (DQ386323-DQ386327, HE653258); *M. pruni* (Rossi), 1790 (HE653260); *M. rufus* (KM-507174-KM507177); *M. trebevicensis* (DQ885919-DQ885922); and *M. velox* Loew, 1869 (HE653265). In addition, the mtDNA COI sequence of *Eumerus flavitarsis* Zetterstedt, 1843 (AY212782); *E. etnensis* van der Goot, 1964 (AY533315) and *Alipumilio avispas* Vockeroth, 1964 (AY261709) were used as outgroups.

Phylogenetic analysis was performed using Neighbor-joining (NJ) and Maximum Likelihood (ML) criteria, and trees were created in MEGA 6.0. (Tamura et al. 2013). The evolutionary model used for the ML analysis was the Tamura-Nei model (TN93+G) of nucleotide substitution as defined in MEGA 6.0 (Tamura et al. 2013). The ML tree was based on a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Statistical support of internal nodes was calculated with 1000 bootstrap repetitions.

Results

Identification based on molecular analysis

All three larval mtDNA COI sequences were identical. Based on BLAST analysis, larval sequences showed similarity to adult *M. avidus* sequences published in GenBank within a range of 98.72–100%.

Next, we used the 3'-region of the mitochondrial gene cytochrome c oxidase I (COI) to cluster those larval sequences with all available relevant published sequences of different *Merodon* species. All sequences included in the dataset are distinguished by 0–14.5% COI gene sequence divergence among 15 *Merodon* species. For the sake of brevity only the ML tree is presented since ML topology is identical to that recovered in the NJ tree (Fig. 1).

All larval COI sequences clustered together with the *Merodon avidus* sequences retrieved from GenBank with high bootstrap support (bootstrap value 90). The bootstrap value for the *Merodon avidus* cluster in the NJ tree was 98. Taxa with multiple sequences were clustered with high bootstrap values, i.e. *M. avidus*, *M. bicolor*, *M. nigritarsis*, *M. aureus*, *M. rufus* and *M. trebevicensis*. In the case of *M. albifrons* the bootstrap value was moderately high compared with other species (74) and it might indicate a high intraspecific molecular variability for this marker, as indicated by Mengual et al. (2006). A similar situation is found in *M. aureus*. On the other hand, our results show that COI barcoding is not useful to distinguish between *M. armipes* and *M. loewi* species, and both taxa are recovered intermixed in the tree with high bootstrap support values. A similar result was reported by Milankov et al. (2008a).

Morphological description

Two of the larvae (La and Lb) were identified as L2 instars and one (Lc), with visible primordia of pupal spiracles (Rotheray 1993), was identified as an L3 instar. A detailed

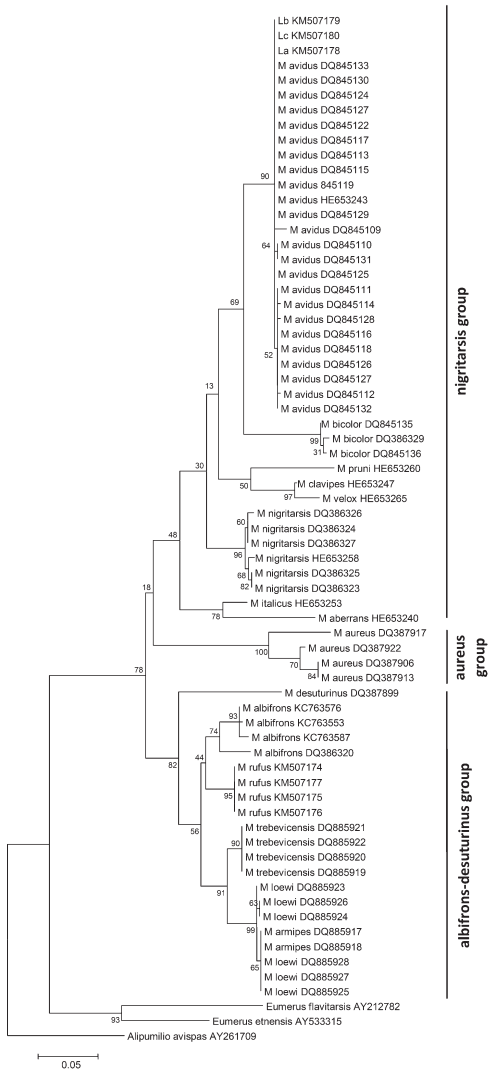

Fig. 1. Molecular phylogenetic tree generated using the Maximum Likelihood criterion. Bootstrap support values are shown next to nodes.

description is given below based on the second instar larva (La). Specific morphological traits that differed notably between the examined specimens are indicated.

Overall appearance (Fig. 2A). Length 8.6 mm and greatest width 3.1 mm (La larva). "Short tailed" larva with external mouth-hooks and sclerotised mandibular lobes. Uniformly beige to brown in colour. Subcylindrical in cross-section, tapered anteriorly, with the anal segment inclined dorsally. Integumental vestiture well-developed, with short, blunt and slightly sclerotised spicules smaller on the ventral surface. Prolegs absent. Anal segment with two pairs of lappets.

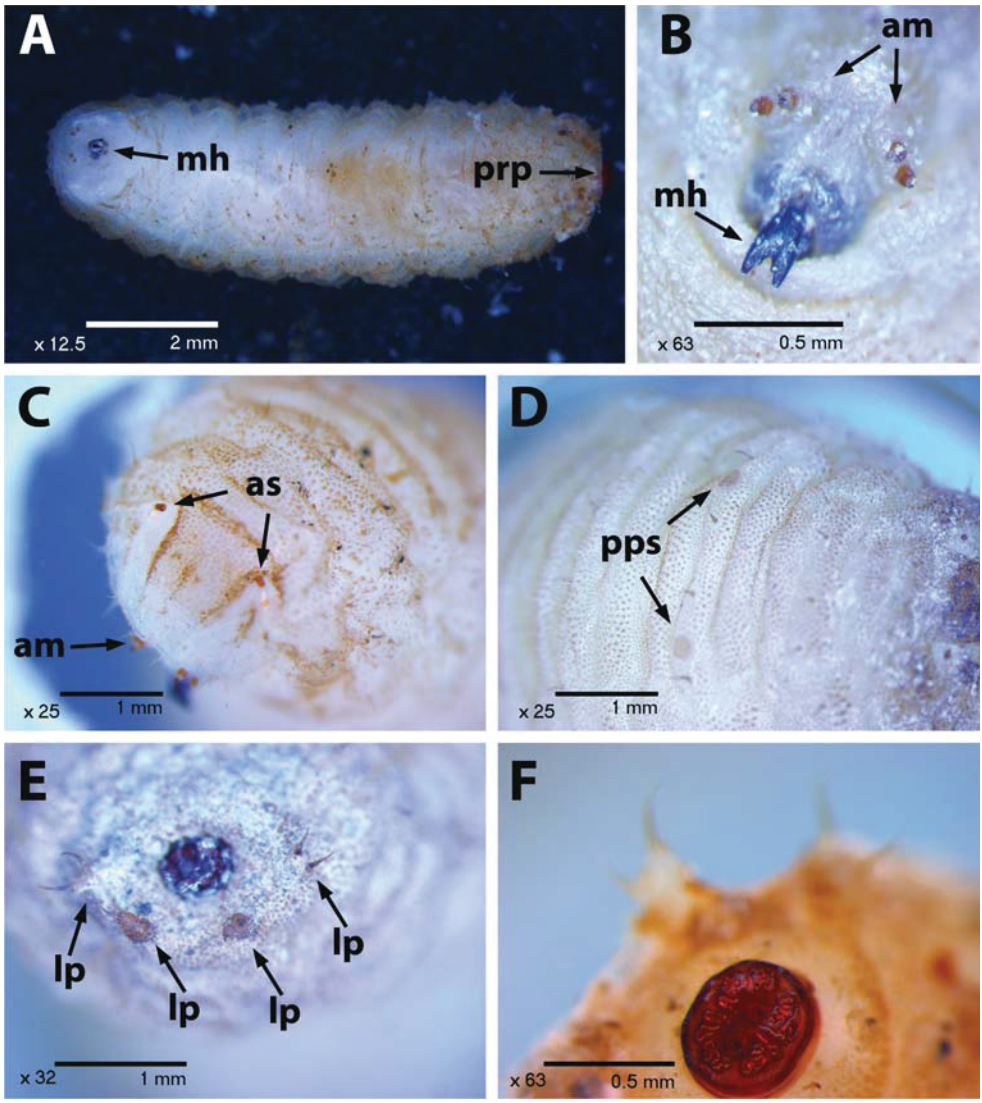

Fig. 2. Light micrographs of *Merodon avidus* Rossi, 1790 larva: A – larva in ventral view (*La*); B – head (*Lc*); C – thorax (*La*); D – primordia of pupal spiracles (*Lc*); E – lappets (*Lc*); F – posterior respiratory process (*La*). Abbreviations: am – antenno-maxillary organs; as – anterior spiracles; lp – lappets; mh – mouth hooks; prp – posterior respiratory process; pps – primordia of pupal spiracles.

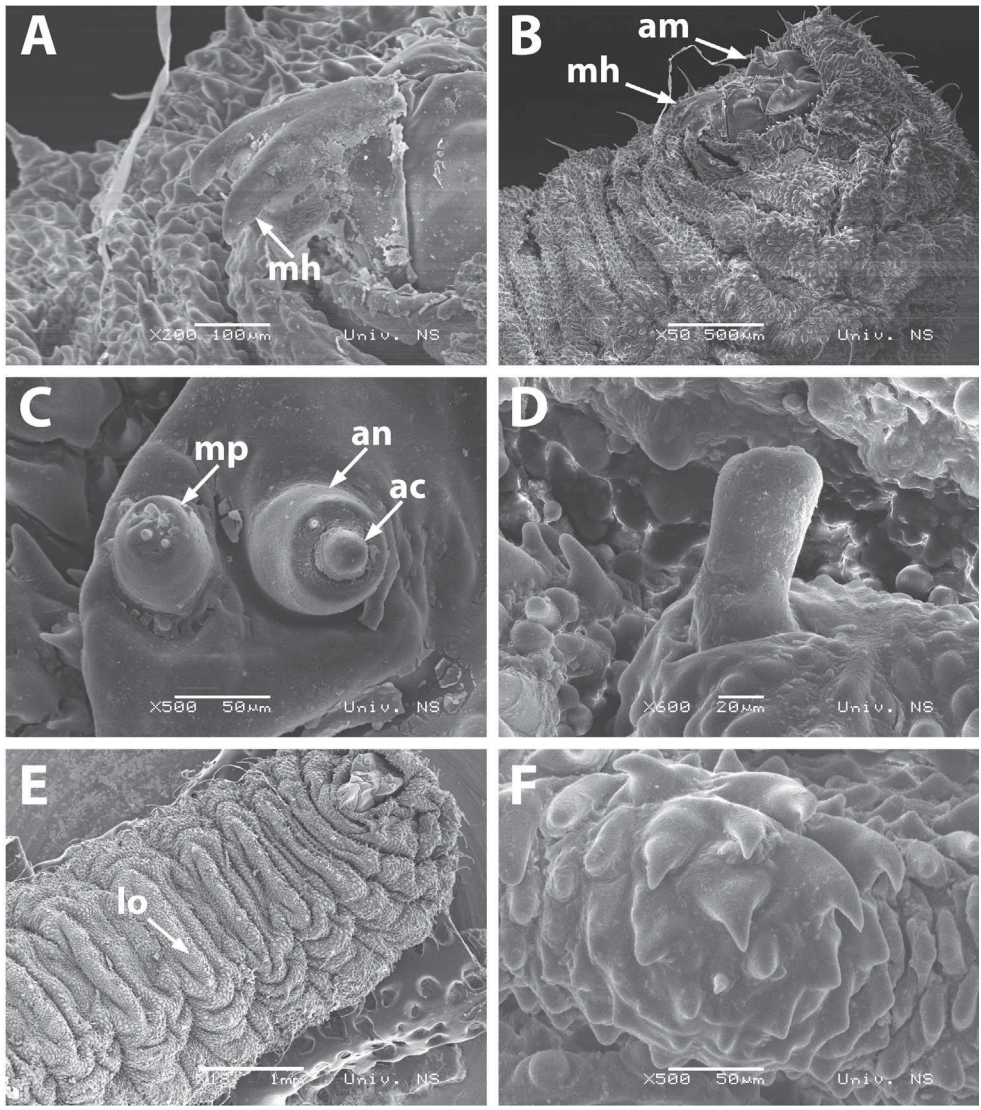

Fig. 3. SEM micrographs of *Merodon avidus* Rossi, 1790 larva (*La*): A – mandibles; B – head; C – antenno-maxillary organs; D – anterior spiracle; E – larva in ventral view; F – locomotory organ. Abbreviations: ac – antennal cone; am – antenno-maxillary organs; an – antenna; lo – locomotory organs; mh – mouth hooks; mp – maxillary palp.

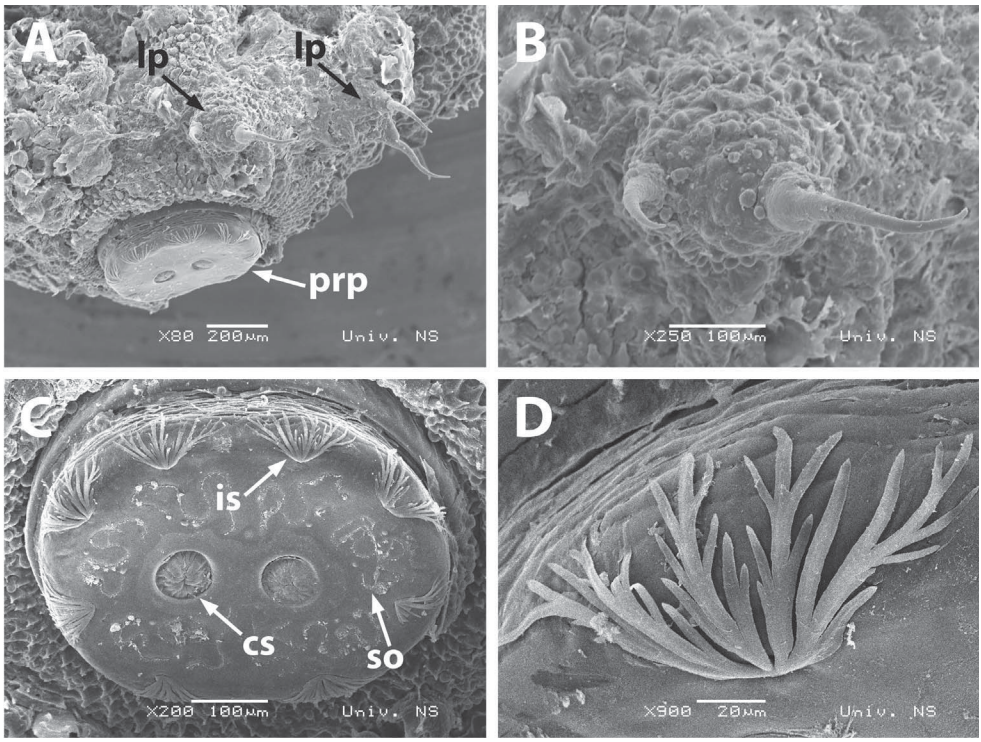

Fig. 4. SEM micrographs of the anal segment of *Merodon avidus* Rossi, 1790 larva (*La*): A – anal segment; B – lappet; C – posterior respiratory process; D – inter-spiracular setae. Abbreviations: cs – central scars; is – inter-spiracular setae; lp – lappets; prp – posterior respiratory process; so – spiracular openings.

Head (*pseudocephalon*). The mandibles possess black, sclerotised hooks which protrude from the mouth and have small accessory teeth (Fig. 2B). The mouth-hooks project downwardsalong each side of the mouth fused to the external mandibular lobes, which are brownish-black and also sclerotised (Figs 2B, 3A). The mouth-hooks separated at the apex by a distance wider than their basal width (Fig. 2B). The antenno-maxillary organs well-developed, located between the mouth and dorsal surface of the prothorax (Figs 2B, 3B). These organs consist of two pairs of cylindrical-shaped structures tipped with different types of sensilla (Fig. 3C). Each pair borne on a fleshy basal papilla as long as broad, or longer. Inner structures; the antennae, easily identified by the presence of a single antennal sensory cone and one small sensilla on the top surface. Outer structures; the maxillary palps bear several satellite sensilla (mainly mechano- and chemoreceptors), one of which protrudes from a shallow groove (Fig. 3C). The dorsal lip, the area of integument between the mouth and the antenno-maxillary organs, smooth and lacks setae. The ventral lip poorly developed.

Table 2. Comparison of the described morphological characters for known *Merodon* larvae. See Table 1 for references in which the respective larva was described.

Characters	***M. avidus***	***M. hurkmansi****	***M. luteihumerus***	***M. equestris***	***M. bombiformis***
Mouth hooks	extensively sclerotised	not extensively sclerotised	extensively sclerotised	sclerotised	heavily sclerotised
Apical mandibular hooks	with accessory teeth/hooks	without accessory teeth/hooks	without accessory teeth/hooks	with accessory teeth/hooks	–
Mouth-hooks separation at the apex	by a distance wider than basal width	by about the same distance as basal width	by less distance than basal width	by a distance wider than basal width	–
Anterior respiratory process (ARP): color	yellowish-brown	yellowish-brown	blackish-brown	brown	–
Spiracular openings at the ARP apex: number	two	two	four to five	up to five	–
Lappets	two pairs	four pairs, middle pair consisting of two separate projections, the first and third pair well developed	four pairs, middle pair consisting of two separate projections, the first and third pair barely produced	four pairs, middle pair consisting of two separate projections	–
Posterior respiratory process (PRP): color	reddish-brown	blackish-brown	black	coal-black	black
Spiracular openings at spiracular plate of PRP	highly convoluted	highly convoluted	smoothly curved	highly convoluted	looped slits, irregular and complex in shape
Spiracular setae (PRP)	well developed	well developed	developed	developed	absent
Integument	short, blunt, slightly sclerotized spicules	coated with broad setae	lacks setae	covered with minute spinules	covered with minute spinules

* published under name *M. constans* by Ricarte et al. (2008), misidentification

Thorax. Lateral lips flat and coated in dome-shaped papillae. The anterior fold, the area between the antenno-maxillary organs and the front margin of the prothorax, lacks hooks but with evenly distributed, conspicuous, yellowish-brown spicules. The dorsal surface of the prothorax with a pair of anterior spiracles (Fig. 2C) about twice as long as broad at the base (Fig. 3D), sclerotised, cylindrical in shape, yellowish-brown in color, with two oval-shaped spiracular openings that appear at the apex and completely retractile within inverted integumental pockets. The dorsal surface of the prothorax with five longitudinal grooves. Mesothoracic prolegs absent.

Abdomen. Primordia of pupal spiracles present on the dorsal surface of the first abdominal segment (Lc larva) (Fig. 2D). Prolegs absent. The locomotory organs appear as pairs of raised domes on abdominal segments 1–6, lacking planta and crochets (Figs 3E,F). The tip of the anal segment angled towards the head in profile. The anal segment with two pairs of lappets (fleshy projections), each lappet bearing a pair of small sensilla (Figs 2E, 4A,B). The posterior respiratory process (Fig. 2F) subcylindrical in shape, short and broad, wider than long; reddish-brown in color. The tip round to subelliptical in polar view. The spiracular plate has four pairs of curved and convoluted spiracular openings around two central scars (Figs 2F, 4C). Four pairs of inter-spiracular setae emerge from the edge of the spiracular plate (Fig. 4C). Each seta with at least four main branches, each with successive divisions, from the base to the tip of the seta (Fig. 4D).

Differences between the morphology of known *Merodon* larvae are given in Table 2.

Status of larva described as *Merodon constans* (Rossi, 1794)

Vujić (2011) re-examined reared adults from the larvae and puparia described by Ricarte et al. (2008) as *Merodon constans* and positively identified them as *Merodon hurkmansi* Marcos-García, Vujić & Mengual, 2007, a species from North West Africa (Marcos-García et al. 2007). Therefore, the immature stages of *M. constans* remain unknown and further discussion of these larvae will be under the name *M. hurkmansi.*

Discussion

There is no genetic benchmark to designate species, and factors such as the rate and pattern of sequence variation in the taxon under investigation and the gene region addressed have to be considered when interpreting genetic distance data for species delimitation (Bradley & Baker 2001, Johns & Avise 1998). Hebert et al. (2003a) stated that COI-based identification systems can aid in the initial delineation of species. Also, Hebert et al. (2003b) indicated that sequence divergences are closely similar in 5' COI and 3' COI regions. Based on a review of published data (Mengual et al. 2008, Milankov et al. 2009, Radenković et al. 2011, Ståhls et al. 2009, Vujić et al. 2012), both regions of the mitochondrial gene cytochrome c oxidase I (COI) were successfully used to generate DNA barcodes in *Merodon* taxonomy and phylogeny.

The study by Ståhls et al. (2009) suggests that DNA COI barcodes generated for *Merodon* species could be useful to determine early stages. Furthermore, DNA extraction from eggs or 1–3 instar larvae (which can be obtained from plant bulbs) facilitates association of unidentified developmental stages with adult flies, via comparison of COI barcode sequences with a barcode library.

Data obtained in the present study support the utility of COI barcode analysis for identifying species at the larval stage. All three larval COI sequences clustered together with previously published sequences for *Merodon avidus,* with high bootstrap support, enabling us to identify them as *Merodon avidus* larvae.

Clusters identified in ML tree analysis correspond to three clades within the genus *Merodon* as described by Vujić et al. (2012): the *albifrons-desuturinus* group (clusters of *M. albifrons*, *M.*

rufus, *M. trebevicensis*, *M. loewi*, and *M. armipes*), the *nigritarsis* group (clusters of *M. avidus*, *M. bicolor*, *M. pruni*, *M. clavipes*, *M. velox*, *M. nigritarsis*, *M. italicus*, and *M. aberrans*) and the *aureus* group (*M. aureus*). Despite its importance for the molecular taxonomy of syrphid flies the COI gene is not an absolutely ideal marker. As mentioned in Mengual et al. (2006) different cases of inconsistency between morphological and molecular data may occur in the genus *Merodon*. In particular, although these authors reported situations where morphology and DNA data were in agreement, they also observed cases where morphological differences were not supported by COI sequence differences, or where species with variable intraspecific DNA sequences displayed similar morphology. Milankov et al. (2008b) showed that COI failed to discriminate between the closely related species *M. aureus* and *M. cinereus* (Fabricius, 1794), and additional allozyme and morphological data were required to distinguish these species. In another case reported by Milankov et al. (2008a), the 3' COI region failed to provide sufficient resolution to define the relationship between these two species within the *albifrons-desuturinus* group cluster, comprising sequences of *M. loewi* and *M. armipes*. The same result is found in our analysis (Figure 1). Evident intraspecific variability is observed within the same clade in the *M. albifrons* cluster, which was expected given the results of Mengual et al. (2006) and Milankov et al. (2013). Subclustering in the *aureus* group with high bootstrap values might indicate the presence of different morphotypes defined in the *aureus* group by Milankov et al. (2008b). GenBank sequence DQ386329 is identified as *Merodon avidus* in the database, but personal communication with the authors suggests that this specific sequence belongs to *M. bicolor*. This may be corroborated by the high bootstrap value (99) in our analysis, a result which agrees with the high nucleotide similarity (99.35 %) between this sequence and another identified as *M. avidus* (Milankov et al. 2009).

All larvae, together with sequences of adult *M. avidus* specimens, were grouped in the *nigritarsis* group. Genetic complexity within *M. avidus* was discussed previously by Milankov et al. (2001) presenting two cryptic taxa, "*M. avidus* A" and "*M. avidus* B". The most recent work on the status of the *M. avidus* complex suggests that this taxon actually comprises several cryptic species with diversification within lineages (Milankov et al. 2009). Although the 3' COI sequence was not an adequate molecular marker for delineating potential cryptic taxa within *Merodon avidus*, it clearly separates the *M. avidus* cluster in the genus *Merodon*.

Based on field work carried out over the last two decades we conclude that many *Merodon* species appear at localities with abundant populations of *Ornithogalum* (Hyacinthaceae). In addition, species of *Ornithogalum* has been suggested as a plant host for the larvae of various *Merodon* species in other studies (Hurkmans 1988, Popov 2001, Speight 2012). Our discovery of two *M. avidus* larvae in bulbs of an *Ornithogalum* species, and one in soil surrounding *Ornithogalum,* confirms that this plant is a host for the immature stages of *Merodon avidus*. However, bulbs of other geophytes might also be used by *M. avidus*, such as *Muscari* (Hyacinthaceae), the rosette of which was observed to be an oviposition site for *M. avidus* (Reemer & Goudsmits 2004).

When compared with other genera of phytophagous larvae (e.g. *Eumerus, Cheilosia, Portevinia* Goffe, 1944) *M. avidus* larvae share morphological peculiarities indicative of feeding on firm plant tissue. Their larger and intensively sclerotised mouth-hooks are similar to those of other phytophages that feed on solid plant tissue, such as *Cheilosia grossa* (Fallén, 1817)

(Rotheray 1993). In addition, morphological features of the anal segment and posterior respiratory process are similar to those described in larvae that tunnel through plant tissues (Rotheray & Gilbert 1999). This may indicate that larvae of *M. avidus* spend much of their time feeding within the bulbs of *Ornithogalum* species, and that our observation of free larvae in the surrounding soil is a consequence of either accidental bulb destruction or mature larvae leaving the bulb prior to pupation.

The anal segment of *M. avidus* bears only two pairs of lappets, in contrast to the four pairs mentioned in descriptions of other species. This finding is important because the number of lappets has been used as a diagnostic character for higher taxonomic categories, including *Merodon* (Rotheray 1993). Rotheray & Gilbert (1999) indicated that all syrphid taxa have three pairs of lappets (middle pair fused together), with the exception of *Merodon* and *Eumerus,* whose middle pairs are sub-divided. Ricarte et al. (2008) included the genus *Alipumilio* in the list of hoverfly taxa whose larvae posses four instead of the usual three pairs of lappets. Pérez-Bañón & Marcos-García (1998) showed that the number of lappets in *Eumerus* varies between species. The morphological characteristics of higher taxonomic categories of phytophagous syrphid larvae are based on generalizations from characters found in very few species. An increase in the number of species with described immature stages will make more data available and will improve the larval characterization of these higher taxonomic categories.

One of the relevant findings of the present study, based on the morphological analysis of reared adults, is the identification of *Merodon hurkmansi* specimens found in bulbs of *Muscari comosum* (L.) Miller commercially grown in Morocco. Ricarte et al. (2008) described these immature stages as *Merodon constans* (Rossi, 1794), a species distributed mainly in Central Europe with no records from the Mediterranean region (Speight 2012). Therefore, the description of its larva from Morocco would be unexpected.

Information on early stages offers an additional line of evidence which complements our present understanding of hoverfly biology (Rotheray & Gilbert 2011). In addition, much can be learned from species whose larval food plants are already known (Rotheray 1993). Life stages of many insect taxa are incompletely known (Miller et al. 2005), and larvae are often difficult (or impossible) to identify due to a lack of distinct morphological characteristics (Emery et al. 2009, Shin et al. 2013) or detailed species-level descriptions and illustrations (Zhou et al. 2007). Studies on the molecular identification of early stages and the association of adults using DNA sequences, particularly the mitochondrial gene COI, show that this method has promise in terms of reliability and speed (Caterino & Tishechkin 2006, Miller et al. 2005, Shin et al. 2013, Šípek & Ahrens 2011, Zhou et al. 2007). This approach, in conjunction with morphological data, has been used for taxonomic determination of early stages of insects of economical and agricultural importance, especially pests (Shin et al. 2013). It has been also used to identify plant parasites during morphologically indistinguishable but ecologically important life stages (Emery et al. 2009). In addition, this kind of studies may enhance our understanding of the diversity, ecology and evolution of plant-insect interactions (Emery et al. 2009). The discovery and description of larvae from additional hoverfly species will be very helpful in complementing existing information about this insect group, as well as improving our knowledge of plant-insect relationships.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Grant No. 173002 and Grant No. 43002, the Provincial Secretariat for Science and Technological Development, Grant No. 114-457-2173/2011-01 and by the project Status and Trends of European Pollinators, FP7-ENV-2009-1-244090 (STEP). This manuscript was edited for English language and content by Dr. Edward T. Petri (University of Novi Sad, Serbia). For editorial language revision we thank Duncan Sivell (Natural History Museum, London, United Kingdom).

References

BRADLEY R. D. & BAKER R. J. 2001: A test of the genetic species concept: Cytochrome-b sequences and mammals. *Journal of Mammalogy* **82**: 960–973.

CATERINO M. S. & TISHECHKIN A. K. 2006: DNA identification and morphological description of the first confirmed larvae of Hetaeriinae (Coleoptera: Histeridae). *Systematic Entomology* **31**: 405–418.

CHEN H., RANGASAMY M., TAN S. Y., WANG H. & SIEGFRIED B. D. 2010: Evaluation of five methods for total DNA extraction from Western corn rootworm beetles. *PLoS ONE* **5(8) (e11963)**: 1–6.

EKREM T., WILLASSEN E. & STUR E. 2007: A comprehensive DNA sequence library is essential for identification with DNA barcodes. *Molecular Phylogenetics and Evolution* **43**: 530–542.

EMERY V. J., LANDRY J. F. & ECKERT C. G. 2009: Barcoding arthropods: Combining DNA barcoding and morphological analysis to identify specialist floral parasites (Lepidoptera: Coleophoridae: Momphinae: Mompha). *Molecular Ecology Resources* **9 (Suppl. 1)**: 217–223.

FRANCUSKI Lj., LUDOŠKI J., VUJIĆ A. & MILANKOV V. 2011: Phenotypic evidence for hidden biodiversity in the Merodon aureus group (Diptera, Syrphidae) on the Balkan Peninsula: conservation implication. *Journal of Insect Conservation* **15**: 379–388.

HALL T. A. 1999: BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/ NT. *Nucleic Acids Symposia Series* **41**: 95–98.

HEBERT P. D. N., CYWINSKA A., BALL S. L. & DEWAARD J. R. 2003a: Biological identifications through DNA barcodes. *Proceedings of the Royal Society London B* **270**: 313–321.

HEBERT P. D. N., RATNASINGHAM S. & DEWAARD J. R. 2003b: Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society London B* **270** (**Suppl 1)**: S96–S99.

HEISS E. M. 1938: A classification of the larvae and puparia of the Syrphidae of Illinois exclusive of aquatic forms. *Illinois Biological Monographs* **16(4)**: 1–142.

HODSON W. E. H. 1932: The large narcissus fly, Merodon equestris, Fab. (Syrphidae). *Bulletin of Entomological Research* **23**: 429–448.

HURKMANS W. 1988: Ethology and ecology of Merodon in Turkey (Diptera: Syrphidae). *Entomologische Berichten* (Amsterdam) **48(7)**: 107–114.

HURKMANS W. 1993: A monograph of Merodon (Diptera: Syrphidae) Part 1. *Tijdschrift voor Entomologie* **136**: 147–234.

JOHNS G. C. & AVISE J. C. 1998: Tests for ancient species flocks based on molecular phylogenetic appraisals of Sebastes rockfishes and other marine fishes. *Evolution* **52**: 1135–1146.

MARCOS-GARCÍA M. A., VUJIĆ A. & MENGUAL X. 2007: Revision of Iberian species of the genus Merodon (Diptera: Syrphidae). *European Journal of Entomology* **104**: 531–572.

MARCOS-GARCÍA M. A., VUJIĆ A., RICARTE A. & STÅHLS G. 2011: Towards an integrated taxonomy of the Merodon equestris species complex (Diptera: Syrphidae) including description of a new species, with additional data on Iberian Merodon. *Canadian Entomologist* **143**: 332–348.

MENGUAL X., STÅHLS G., VUJIĆ A. & MARCOS-GARCÍA M. A. 2006: Integrative taxonomy of Iberian Merodon species (Diptera, Syrphidae). *Zootaxa* **1377**: 1–26.

MENGUAL X., STÅHLS G. & ROJO S. 2008: First phylogeny of predatory flower flies (Diptera, Syrphidae, Syrphinae) using mitochondrial COI and nuclear 28S rRNA genes: conflict and congruence with the current tribal classification. *Cladistics* **24**: 543–562.

MILANKOV V., VUJIĆ A. & LUDOŠKI J. 2001: Genetic divergence among cryptic taxa of Merodon avidus (Rossi, 1790) (Diptera: Syrphidae). *International Journal of Dipterological Research* **12(1)**: 15–24.

MILANKOV V., STÅHLS G. & VUJIĆ A. 2008a: Molecular diversity of populations of the Merodon ruficornis group on the Balkan Peninsula. *Journal of Zoological Systematics and Evolutionary Research* **46**: 143–152.

MILANKOV V., STÅHLS G. & VUJIĆ A. 2008b: Genetic diversity of populations of Merodon aureus and M. cinereus species complexes (Diptera, Syrphidae): integrative taxonomy and implications for conservation priorities on the Balkan Peninsula. *Conservation Genetics* **9**: 1125–1137.

MILANKOV V., LUDOŠKI J., STÅHLS G., STAMENKOVIĆ J. & VUJIĆ A. 2009: High molecular and phenotypic diversity in the Merodon avidus complex (Diptera, Syrphidae): cryptic speciation in a diverse insect taxon. *Zoological Journal of the Linnean Society* **155**: 819–833.

MILANKOV V., LUDOŠKI J., FRANCUSKI Lj., STÅHLS G. & VUJIĆ A. 2013: Genetic and phenotypic diversity patterns in Merodon albifrons Meigen, 1822 (Diptera: Syrphidae): evidence of intraspecific spatial and temporal structuring. *Biological Journal of the Linnean Society* **110**: 257–280.

MILLER K. B., ALARIE Y., WOLFE W. & WHITING M. F. 2005: Association of insect life stages using DNA sequences: the larvae of Pilodytes umbrinus (Motschulsky). *Systematic Entomology* **30**: 499–509.

PEHLİVAN E. & AKBULUT N. 1991: Some investigations on the syrphid species attacking on Narcissus in Karaburun (Izmir) and the biology and control measures of Merodon eques (F.) (Diptera). *Turkish Journal of Agriculture and Forestry* **15**: 47–81.

PÉREZ-BAÑÓN C. & MARCOS-GARCÍA A. 1998: Life history and description of the immature stages of Eumerus purpurariae (Diptera: Syrphidae) developing in Opuntia maxima. *European Journal of Entomology* **95**: 373–382.

PETANIDOU T. 1991: *Pollination ecology in a phryganic ecosystem.* PhD thesis, Aristotelian University, Thessaloniki, Greece.

POPOV G.V. 2001: What and where are Merodon feeding? Pp. 28–29. In: *First international workshop on the Syrphidae*. Staatliches Museum für Naturkunde, Stuttgart, Germany.

POPOV G.V. 2010: Merodon alexandri spec. nov. – a new species of hoverfly (Diptera: Syrphidae) from the northern Black Sea Region. *Studia Dipterologica* **16**: 133–151.

RADENKOVIĆ S., VUJIĆ A., STÅHLS G., PÉREZ-BAÑÓN C., ROJO S., PETANIDOU T. & ŠIMIĆ S. 2011: Three new cryptic species of the genus Merodon Meigen (Diptera: Syrphidae) from the island of Lesvos (Greece). *Zootaxa* **2735**: 35–56.

REEMER M. & GOUDSMITS K. 2004: Oviposition observed in Chrysotoxum cautum, C. vernale and Merodon avidus (Diptera: Syrphidae). *Volucella* **7**: 217–218.

RICARTE A., MARCOS-GARCÍA M. A. & ROTHERAY G. E. 2008: The early stages and life histories of three Eumerus and two Merodon species (Diptera: Syrphidae) from the Mediterranean region. *Entomologica Fennica* **19**: 129–141.

ROTHERAY G. E. 1993: Colour guide to hoverfly larvae (Diptera, Syrphidae) in Britain and Europe. *Dipterists Digest* **9**: 1–156.

ROTHERAY G. E. & GILBERT F. 1999: Phylogeny of Paleartic Syrphidae (Diptera): evidence from larval stages. *Zoological Journal of the Linnean Society* **127**: 1–112.

ROTHERAY G. E. & GILBERT F. 2011: *The natural history of hoverflies*. Forrest text, UK, 334 pp.

SHIN S., JUNG S., LEE H. & LEE S. 2013: Molecular identification of dipteran pests (Diptera: Sciaroidea) from shiitake mushroom. *Molecular Ecology Resources* **13**: 200–209.

SIMON C., FRATI F., BECKENBACH A., CRESPI B., LIU H. & FLOOK P. 1994: Evolution, weighting and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. *Annals of the Entomological Society of America* **87**: 651–701.

SOMMAGGIO D. 1999: Syrphidae: can they be used as environmental bioindicators? *Agriculture, Ecosystems and Environment* **74**: 343–356.

SPEIGHT M. C. D. 2012: Species accounts of European Syrphidae (Diptera). In: *Syrph the Net, the database of European Syrphidae*. Syrph the Net publications, Vol. 69, Dublin, 296 pp.

STÅHLS G., VUJIĆ A., PÉREZ-BAÑON C., RADENKOVIĆ S., ROJO S. & PETANIDOU T. 2009: COI barcodes for identification of Merodon hoverflies (Diptera, Syrphidae) of Lesvos Island, Greece. *Molecular Ecology Resources* **9**: 1431–1438.

STEPANENKO O. V. & POPOV G. V. 1997: On the immature stages biology of Merodon nigritarsis Rondani, 1845 (Diptera: Syrphidae). *Kharkov Entomological Society Gazette* **5(2)**: 40–43.

STUCKENBERG B. R. 1956: The immature stages of Merodon bombiformis Hull, a potencial pest of bulbs in South Africa. *Journal of Entomological Society of South Africa* **19**: 219–224.

STUKE J. H. 2000: Phylogenetische Rekonstruktion der Verwandtschaftsbeziehungen innerhalb der Gattung Cheilosia Meigen, 1822 anhand der Larvenstadien (Diptera: Syrphidae). *Studia Dipterologica*, *Supplement* **8**: 1–118.

ŠÍPEK P. & AHRENS D. 2011: Inferring larval taxonomy and morphology in Maladera species (Coleoptera: Scarabaeidae: Sericini) using DNA taxonomy tools. *Systematic Entomology* **36**: 628–643.

TAMURA K., STECHER G., PETERSON D., FILIPSKI A., & KUMAR S. 2013: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

THOMPSON J. D., HIGGINS D. G. & GIBSON T. J. 1994: Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weigh matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

THOMPSON F. C. (ed.) 2010: *Syrphidae. Systema Dipterorum*. Available via http://www.diptera.org [accessed 28 January 2014].

VUJIĆ A., MARCOS-GARCÍA M. A., SARIBIYIK S. & RICARTE A. 2011: New data for the Merodon Meigen 1803 fauna (Diptera: Syrphidae) of Turkey including a new species description and status changes in several taxa. *Annales de la Société Entomologique de France (N. S.)* **47**: 78–88.

VUJIĆ A., RADENKOVIĆ S., STÅHLS G., AČANSKI J., STEFANOVIĆ A., VESELIĆ S., ANDRIĆ A. & HAYAT R. 2012: Systematics and taxonomy of the ruficornis group of genus Merodon Meigen (Diptera: Syrphidae). *Systematic Entomology* **37**: 578–602.

ZHOU X., KJER K. M. & MORSE J. C. 2007: Associating larvae and adults of Chinese Hydropsychidae caddisflies (Insecta:Trichoptera) using DNA sequences. *North American Benthological Society* **26**: 719–742.