The Merodon planifacies subgroup (Diptera, Syrphidae) : Congruence of molecular and morphometric evidences reveal new taxa in Drakensberg mountains valleys (Republic of South Africa)

Djan, Mihajla

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The *Merodon planifacies* subgroup (Diptera, Syrphidae): congruence of molecular and morphometric evidence reveal new taxa in Drakensberg mountain valleys (Republic South Africa)

Djan, M., Ståhls, G., Veličković, N., Ačanski, J., Obreht Vidaković, D., Rojo, S., Perez-Banon, C., Radenković, S., Vujić, A.

Abstract

Genus *Merodon* Meigen, 1803 is very rarely found in the Afrotropical Region, contrary to Palaearctic (11 versus 160 known species). An ongoing study of the genus *Merodon* in Africa has revealed the existence of two new species into the taxon previously known as *Merodon planifacies* Bezzi, 1915. The *Merodon planifacies* subgroup belongs to the Afrotropical lineage of the *M. desuturinus* group. Morphological analysis of male genitalia has classified the available specimens of the *M. planifacies* taxon into two sets: the first one corresponded to *M. planifacies*, while the other, later named as *M. capi* complex was found exclusively at the Drakensberg mountains in Republic South Africa, specifically the Cathedral Peak National Park and the Royal Natal National Park. Further on molecular and morphometric evidences reveal within this complex two cryptic taxa: *M. capi* sp. nov. Vujić et Radenković and *M. roni* sp. nov. Radenković et Vujić.

Introduction

Hoverflies (Syrphidae) represent an insect group of great importance due to its pollinator role in ecosystems (Larson et al. 2001) and as indicators of ecosystem change (Vujić et al. 2016). The genus *Merodon* Meigen, 1803 (tribe Merodontini) is one of the species-richest hoverflies genera, distributed across the Palaearctic and Afrotropical regions (Ståhls et al. 2009; Vujić et al. 2012). It belongs to the tribe Merodontini together with genera *Azpeytia* Walker, 1865, *Eumerus* Meigen, 1822 and *Megatrigon* Johnson, 1898. *Azpeytia* is distributed in the Australasian region, while the latter genera occur in the Palaearctic and Afrotropical regions. In the Palaearctic region genera *Eumerus* and *Merodon* are mainly found in the Mediterranean basin. The genus *Merodon* is specioseus in the Palaearctic region with 160 formalized species (Ståhls et al. 2009; Vujić et al. *in prep*.). Contrary, in the Afrotropical region the number of

described species of genus *Merodon* is only 11 (The Biosystematic Database of World Diptera, accessed March 2018, Pape & Thompson, 2013; Radenković et al. 2018). The latter is unexpected, since according to the findings it is estimated that all *Merodon* species are phytophagous in geophyte (or bulbous) plants. Although the immature stages are described only for eight species and for additional eight host plants are assumed, it is supposed that *Merodon* larvae prefer underground storage organs of the families Hyacinthaceae (Ricarte et al. 2008, 2017; Andrić et al. 2014; Preradović et al. 2018). Considering these facts, one might expect to observe much higher species diversity within *Merodon* genus in the Afrotropical region, since the highest diversity of their so far known host plants is found in South Africa (Pfosser & Speta, 2014), and since the same diversity pattern is present in Mediterranean region, where the congruence in bulb plants diversity and *Merodon* species-richness is noted (Ricarte et al. 2017). Furthermore, it is worth to point out that within *Merodon* genus, certain number of species represents cryptic species, characterized by subtle morphological differences (Vujić et al. 2015; Popović et al. 2015; Šašić et al. 2016; Ačanski et al. 2016; Radenković et al. 2018a), and thus may be undetected so far. As the biology and development of the species of *Merodon* is dependent on particular species of bulbous plants, we argue that the diversity of bulbous plants in the study area could have influenced the species diversity of the *Merodon* taxa.

In the present study all available specimens of the *Merodon* genus from the Afrotropical region were identified in the collections of European, North American museums and Natal museum, representing 11 described species, *Merodon apimimus* Hull, 1944*, M. bombiformis* Hull, 1944*, M. cuthbertsoni* Curran, 1939*, M. melanocerus* Bezzi, 1915 subgroup (five species: *M. melanocerus, M. flavocerus, M. capensis*., *M. draconis* and *M. commutabilis*)*, M. multifasciatus* Curran, 1939*, M. planifacies* Bezzi, 1915 and *M. stevensoni* Curran, 1939. Total of 71 specimens were found in main Museums collections with Afrotropical material collected from 1902 until 2004. According to our first findings with more than 300 collected individuals during the on-going field research of the *Merodon* genus in South Africa (2011-2017) and above-mentioned fact, special attention has been drawn to the *M. planifacies*.

Merodon planifacies belongs to recently revised *M. desuturinus* group (Radenković et al. 2018b; Vujić et al. 2018). The *M desuturinus* group is characterized by the following characters: posterior side of mid coxa with pile; male genitalia: anterior lobe of surstylus with

curved distal prolongation. The main synapomorphic character which is found in all species from *desuturinus* group is a character of the male genitalia, the tip of the lateral sclerite of aedeagus is gradually tapering and curved downwards. The *M. desuturinus* group contains two lineages, which are geographically (and biogeographically) separated in the Palaearctic and Afrotropical regions, respectively (Radenković et al. 2018b). In addition to species *M. desuturinus* Vujić, Šimić et Radenković, 1995, the Palaearctic lineage includes three additional taxa, *M. cabanerensis* Marcos-Garcia, Vujić et Mengual, 2007 (Marcos-Garcia et al. 2007), *M. murorum* Fabricius, 1794 and *M. neolydicus* Vujić, 2018 (Vujić et al. 2018). The Afrotropical lineage consists of following taxa: *M. cuthbertsoni* and recently defined two subgroups, *M. melanocerus* sugroup with xy species (Radenković et al. 2018b) and *M. planifacies* subgroup with two taxa, *M. planifacies* and *M. stevensoni* (Vujić et al. 2018). *M. planifacies* and *M. stevensoni* was introduced as subgroup sharing absence of protruded oral margin which is covered by microtrichia (Vujić et al. 2018).

To our best knowledge, no genomic approaches have been developed yet in any of Syrphidae taxa. Moreover, a limited number of molecular markers have been developed so far. The gold standard among molecular markers for taxonomy of Syrphidae is the mitochondrial (mtDNA) cytochrome c oxidase I (COI) gene (Mengual et al. 2006; Masetti et al. 2006; Milankov et al. 2008; Ståhls et al. 2009; Marcos-García et al. 2011; Vujić et al. 2012, 2013; Popović et al. 2014; Nedeljković et al. 2015; Šašić et al. 2016; Chroni et al. 2017; Radenković et al. 2018a). Analyses of partial sequences of COI revealed cryptic taxa in the hoverfly genus *Merodon* (Vujić et al. 2012; Šašić et al. 2016; Radenković et al. 2018a). Contrary, in several studies, COI failed to resolve species boundaries within the same genus (Milankov et al. 2008; Ståhls et al. 2009), where the sequence divergence in COI was low and in several cases invariant. The latter may be the consequence of retained polymorphism or mitochondrial introgression between the taxa (Ståhls et al. 2009), or recent speciation event (Popović et al. 2014). Furthermore, there are cases of incongruence between morphological and molecular data (DNA barcoding) (Mengual et al. 2008). Additional nuclear DNA markers have been employed, namely rDNA (ITS2, 28S, 18S) sequences variability (Mengual et al. 2008; Nedeljkovic et al. 2013; Radenković et al. 2018a), used mainly in phylogenetic studies of different genera within Syrphidae family. This limited number of applicable genetic tools makes even more challenging the genetic characterisation of species of *Merodon* genus. As an additional approach in the integrative taxonomy of hoverflies, a landmark-based geometric

morphometry of wing trait variation has proven to be useful for resolving taxonomic uncertainness (Nedeljković et al. 2013; Vujić et al. 2013; Nedeljković et al. 2015; Ačanski et al. 2016). Method is powerful in detection of subtle wing shape variation and provides additional characters in cryptic species delimitation. Integrative taxonomy provides more rigorous delimitation and yields in reliable discovery of cryptic species (Schlick-Steiner et al. 2010), with prominent examples among hoverflies (Mengual et al. 2006; Masetti et al. 2006; Ståhls et al. 2009; Vujić et al. 2012, 2013; Popović et al. 2014; Nedeljković et al. 2015; Šašić et al. 2016; Ačanski et al. 2016; Chroni et al. 2017; Radenković et al. 2018a).

Morphological analysis of the available material from museum collections and new collected specimens identified a priori as *M. planifacies* reveals minor variability in male genitalia features and indicate possible existences of hidden diversity. Thus, we decided to use integrative taxonomy approach and to employ molecular and morphometric tools in order to discover complexity of this taxon.

Material and Methods

Material

Specimen sampling

During field researches conducted in Republic of South Africa in a period from 2011 to 2017, in total of 51 adult specimens identified as belonging to the *Merodon planifacies* taxon were collected from Drakensberg mountains (Fig. 1; Table S1). The specimens under study were collected by sweep net. Beside adult specimens, 6 larvae were found in following two valleys: the Cathedral Peak National Park and the Royal Natal National Park in Drakensberg mountains in bulbs of *Merwilla*. Holotype of *M. planifacies* was studied and specimen conspecific with holotype collected near Van Reenen used for molecular analysis.

(Fig.1 here)

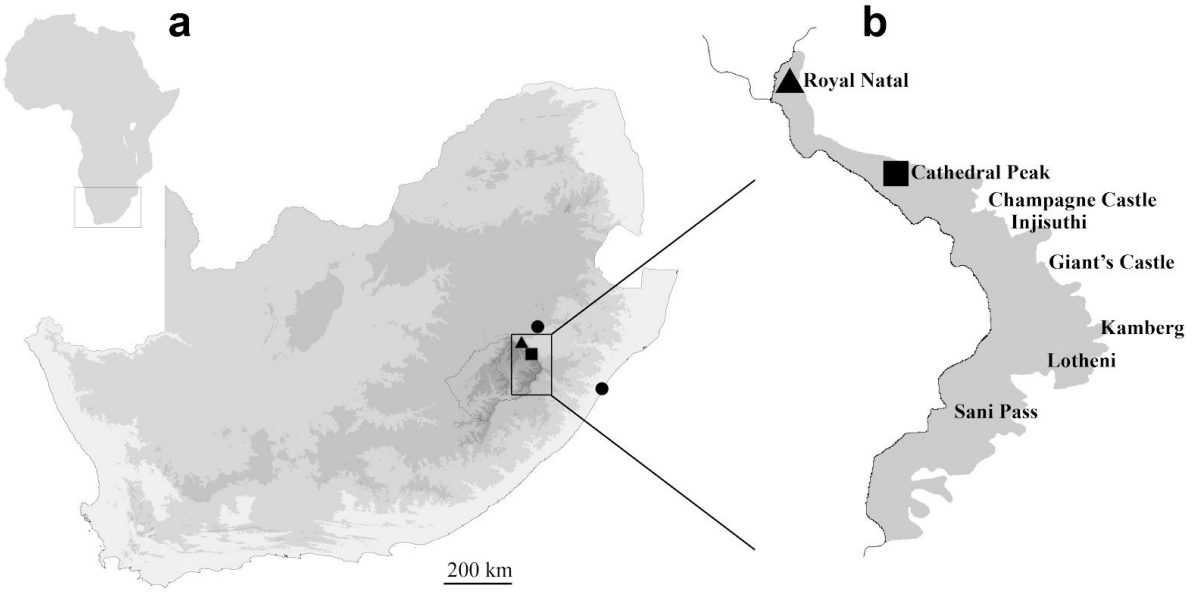

Figure 1. Map of population sampling locations of the *Merodon planifacies* subgroup. A. Map of South Africa showing the Drakensberg mountain range (box) B. Enlargement of Drakensberg mountain range. ● *Merodon planifacies*, ▲ *Merodon roni* **sp. nov.**, ■ *Merodon capi* **sp. nov.**

Morphological analyses:

The type material of all described Afrotropical species of genus *Merodon* was examined from different Museums: BMNH - Natural History Museum, London, UK (*M. apimimus, M. melanocerus*, and *M. planifacies*); NMSA - Natal Museum, Pietermaritzburg, South Africa (*M. bombiformis*); NBC - Naturalis Biodiversity Centre, Leiden, The Netherlands (*M. cuthbertsoni, M. multifasciatus,* and *M. stevensoni*). Terminology follows McAlpine (1981) for non-genitalic morphology and Marcos-García et al. (2007) for morphology of the male terminalia. The male genitalia were extracted from dry specimens previously relaxed in a closed pot with a high level of humidity. Male genitalia were cleared by boiling in warm 10% potassium hydroxide (KOH) for 2–4 minutes. Acetic acid was then used to neutralize the KOH during 5 seconds. Genitalia were stored in microvials containing glycerol. Specimen measurements were taken in dorsal view with a micrometer and are presented as ranges. Drawings were made with a FSA 25 PE drawing tube attached to a binocular microscope Leica MZ16. Measurements were taken with an eyepiece graticule or micrometer. Specimen measurements were taken in dorsal view with a micrometer and are presented as ranges. Body length was measured from the lunula to the end of the abdomen, and wing length from the

base of the epaulet to the wing apex. Numbers of measured specimens are indicated by the notation "n". Photos were made with a Leica DFC320 camera connected to a personal computer. After photographing, CombineZ software (Hadley 2006) was used in order to create composite image with an extended depth of field, created from the in- focus areas of each image. Material is deposited in following Museum, institutions and private collections: NMSA - Natal Museum, Pietermaritzburg, South Africa

Molecular analyses

Total DNA was extracted from up to three legs of each specimen using the SDS Extraction Protocol (Chen et al. 2010) with slight modifications, or using the Nucleospin Tissue DNA extraction kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocols and then re-suspended in 50 µl of ultra-pure water. Genomic DNA vouchers are conserved at the Faculty of Sciences, Department of Biology and Ecology, University of Novi Sad (FSUNS), Serbia and at the Zoology unit of the Finnish Museum of Natural History, Helsinki, Finland.

Three different genomic regions were selected for species delimitation. The cytochrome c oxidase subunit I (COI) of mitochondrial DNA was amplified for two fragments. A 5'-fragment using forward primer LCO (5'-GCTCAACAAATCATAAAGATATTGG-3') and reverse primer HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (the fragment is recognized as the DNA barcode) (Folmer et al. 1994, Hebert et al. 2003) and a 3'- fragment using 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) were amplified. PCR amplifications of both COI gene fragments were carried out in 25 µl reaction volumes and the reaction mix consisted of 1xPCR buffer (Thermo Scientific), 2.5 mM $MgCl_2$, 0.1 mM of each nucleotide, 1.25 U Taq polymerase (Thermo Scientific), 2 pmol of each primer and 50 ng template DNA. Thermocycler conditions were: initial denaturation for 2 min at 95°C; 30 s denaturation at 94°, 30 s annealing at 49°C , 2 min extension at 72°C/30 cycles; and the final extension for 8 min at 72°C. Additionally, D2-3 expansion segment of the nuclear ribosomal 28S rRNA gene was PCR amplified using primers 28SF2 (5′-AGAGAGAGAGTTCAAGAGTACGTG-3′), and 28S3DR (5′-TAGTTCACCATCTTTCGGGTC-3′) (Belshaw et al. 2001), using the same PCR conditions as described for COI gene. The PCR products were purified using the Exo-Sap purification method, according to the manufacturer's protocol (Thermo Scientific).

All three fragments were sequenced and sequences were submitted to GenBank (for accession numbers see Table S1).

Molecular data analyses

All obtained sequences of three genomic regions were inspected, edited and assembled using the BioEdit software (Hall, 1999). Sequences alignment was done using the Clustal W (Thompson et al. 1994) algorithm implemented in BioEdit 7.0.9.0. (Hall, 1999). For the molecular data analyses all sequences were organized in two datasets. First dataset included 44 concatenated sequences of both COI fragments obtained from *Merodon planifacies* specimens (only specimens for which high quality sequences of both COI gene fragments were obtained were included), while the second dataset comprised 28S rRNA gene sequences of 35 *Merodon planifacies* specimens (poor quality sequences obtained for 9 specimens were excluded). The following species were also subjected to molecular analysis and included as outgroups for the both datasets: *Merodon luteihumerus* Marcos-García, Vujić et Mengual, 2007, *Megatrigon* aff. *argenteus* and *Eumerus niveitibia* Becker, 1921 (Table S1). In addition, COI gene and 28S rRNA gene sequences for two species (*Microdon bidens* (Fabricius, 1805) and *Platynochaetus setosus* Fabricius, 1794) were retrieved from GenBank (for accession numbers see Table S1). The concatenated COI gene dataset comprised in total 49 sequences, with a total length 1187bp. The second dataset included 40 28S rRNA gene sequences and the total length of the aligned second dataset was 591bp. *Microdon bidens* was used to root all phylogenetic trees. We constructed phylogenetic trees using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI) separately for both datasets. The parsimony (MP) analysis was done using NONA (Goloboff 1999) spawned with the aid of Winclada (Nixon 2002), using the heuristic search algorithm with 1000 random addition replicates (mult x 1000); holding 100 trees per round (hold／100), max trees set to 100 000; and applying TBR branch swapping. A Maximum Likelihood (ML) tree was constructed using RAxML 8.2.8 (Stamatakis 2014) using the CIPRES Science Gateway web portal (Miller et al. 2010) under the general time-reversible (GTR) evolutionary model with gamma distribution, and branch support was estimated with 1000 non-parametric bootstrap replicates. Bayesian phylogenetic analyses were carried out using GTR model of nucleotide substitution with Gamma correction for among-site variation in substitution rates (as estimated in MEGA version 6; Tamura et al. 2013) as priors in MrBayes ver.3.2 (Ronquist et al. 2012). Two

independent runs of four Markov chain Monte Carlo (MCMC) permutations were performed for 20,000,000 generations with sampling every 100 generations. Tracer v1.5 (Rambaut & Drummond, 2007, available from http://beast.bio.ed.ac.uk/Tracer) was used to summarise Bayesian analysis and to inspect the validity of the burn-in fraction applied. The first 25% of the sampled iterations/generations were discarded as burn-in, and 50% consensus trees were computed using FigTree v1.4.0 (Rambaut, 2009).

For further graphical representation of genetic relationships for the included specimens of the *Merodon planifacies* taxon, we constructed a Median-Joining (MJ) network (Bandelt et al. 1999) using Network v4.6.1.3 (available at http://www.fluxus-engineering.com/sharenet.htm) applying the default settings (ε = 0 and the variable sites weighted *equally* = 10), with additional post-processing with the maximum parsimony (MP) option. In order to further support the obtained results, we applied an automatic procedure that sorts the sequences into hypothetical species based on the barcode gap as implemented in ABGD software using default settings (Pmin=0.001, Pmax=0.1, Steps= 10, X (relative gap width) = 1.5, Nb bins= 20) and GTR model for pairwise distance calculation (Puillandre et al. 2011).

Analysis of molecular variance (AMOVA) among and within the species defined and pairwise Φst values were calculated using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010). We also calculated genetic distance between defined groups using software MEGA version 6 (Tamura et al. 2013), in order to estimate time of divergence between genetic clusters by uncorrected p-distance divided by the pairwise evolutionary rate/MYR as described in Prohl et al. (2010). We used the pairwise sequence divergence in the COI gene, relative to the mutational rate of 2.3% per million years as estimated for various arthropod taxa (Brower 1994).

Divergence time estimations were done assuming a 2.3% substitution rate per million years, which is commonly used for insects (Brower 1994), and using a linear regression method, according to the equation t = p/2r (t—divergence time, p—uncorrected p distance and r—rate of substitution per million years)

The correlation between genetic distances and geographical distances was performed using Mantel's non-parametric test on pairwise distance matrices (Mantel 1967), using the MANTEL procedure implemented in Arlequin.

Wing morphometry

Geometric morphometric analysis of wing shape was conducted on total 40 *Merodon planifacies* taxon specimens (Table S1). The right wing of each specimen was removed using micro-scissors and then mounted in Hoyer's medium on a microscopic slide. Wing slides are archived and labelled with a unique code in the FSUNS collection, together with other data relevant to the specimens. High-resolution photographs of the wings were made using a Leica DFC320 video camera attached to a Leica MZ16 stereomicroscope. Twelve homologous landmarks at vein intersections and terminations (Fig. 2) that could be reliably identified and representing wing shape were selected using the software TpsDig 2.05 (Rohlf 2006). In order to estimate measurement error each wing was digitized three times and average landmark coordinates were used. Due to the relatively small number of available female specimens, wing shape analyses were conducted on a two datasets, first with pooled sexes and second the male dataset separately.

(Fig.2 here)

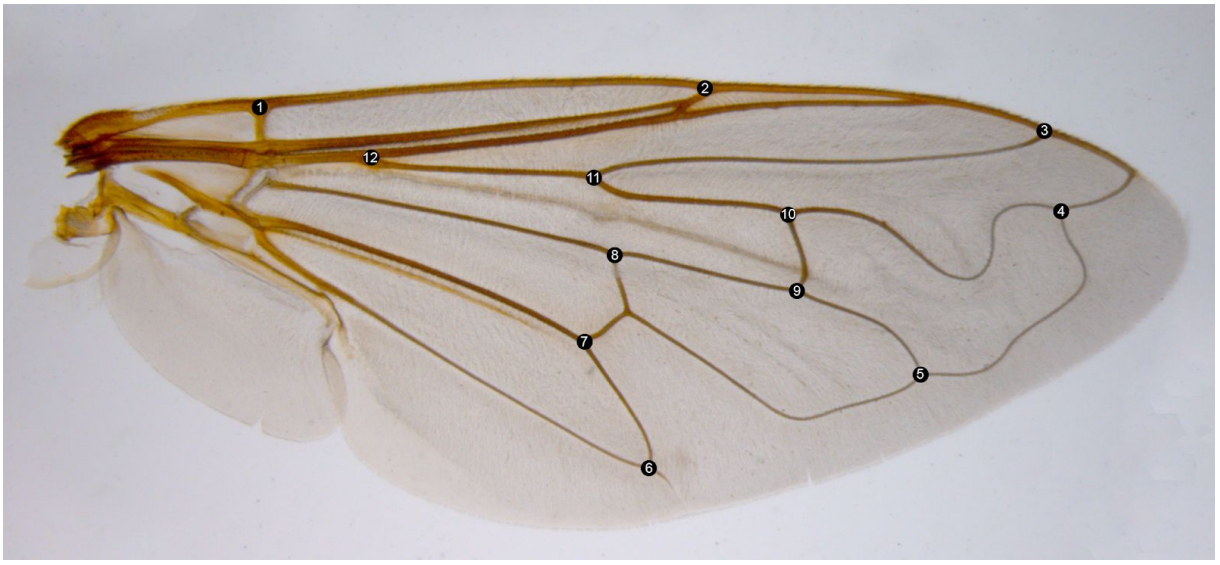

Figure 2. The location of twelve landmarks on a right wing selected for geometric morphometric analysis.

Generalized least-squares Procrustes superimposition was first applied to the landmark data to remove non-shape variation in terms of location, scale and orientation, and also to superimpose the objects in a common coordinate system (Rohlf & Slice 1990; Zelditch et al. 2004). Principal component analysis was carried out on the Procrustes shape variables to reduce the dimensionality of the data set. Then, principal components (PCs) that describe the

highest overall classification percentage were extracted using stepwise discriminant analysis (Baylaac & Frieß 2005). Canonical variate analysis (CVA), discriminant function analysis (DA) and Gaussian naïve Bayes classifier were employed to test differences in wing shape between taxa. Procrustes superimposition as well as visualization of differences in mean wing shape between taxa were obtained with MorphoJ v.2.0. software (Klingenberg 2011). Statistical analyses were made using Statistica® for Windows (Dell Statistica 2015).

Results

Merodon planifacies: more than one species

M. planifacies and *M. stevensoni* as members of the *planifacies* subgroup (Vujić et al. 2018) share a clear apomorphic character - reduction of oral margin (as on Fig. 3A, B, 4A, B). Additional diagnostic characters for these taxa are: oral margin and face covered by microtrichia (Fig. 3A, B) and thorax with orange coloured humerus and postalar calli (Fig. 5: h, pc). *M. stevensoni* was described based on one female from Southern Rhodesia. Further material of this taxon has not been found. Therefore, the taxonomic status remains unclear until discovery of additional material and male specimen.

(Fig. 3 here)

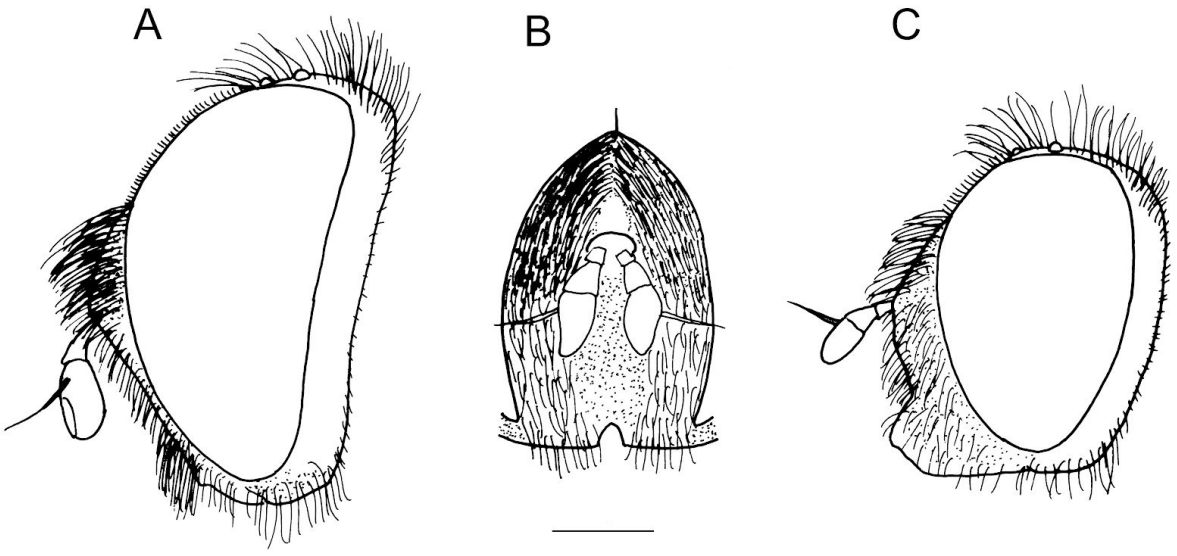

Figure 3. Head. A, B *Merodon planifacies* male (holotype); C *Merodon melanocerus* male (Royal Natal NP, RSA). A, C lateral view; B, anterior view. Scale = 1 mm.

Morphologically, in addition to morphological variability reported in the Taxonomy, all specimens identified as *Merodon planifacies* from RSA share very similar morphological features presented in General description. More detailed morphological analysis revealed that there are two groups of taxa clearly separated within *M. planifacies* taxon by structure of male genitalia: one (1) with folded ventral ridge of theca (Fig. 6B: tr) and second (2) with smooth ventral margin (Fig. 6A: tr). The first one corresponded to *M. planifacies*, while the other group was found exclusively at the Drakensberg mountains in RSA, specifically the Cathedral Peak National Park and the Royal Natal National Park.

(Fig. 4 here)

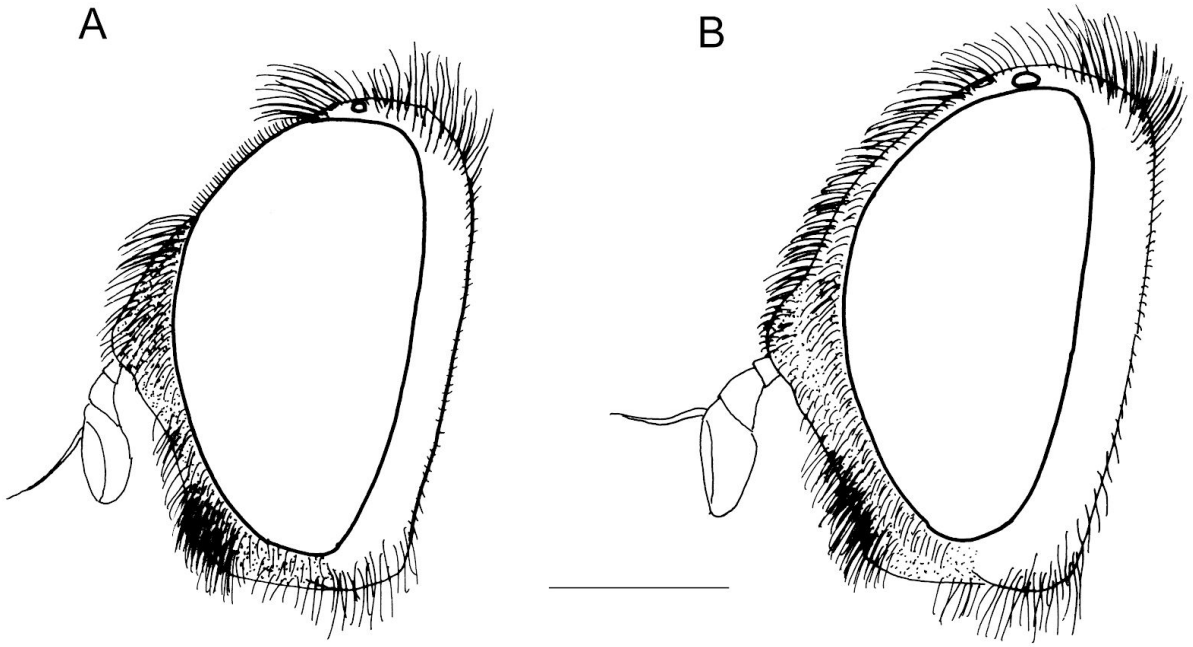

Figure 4. *Merodon roni* sp. n. (Royal Natal NP, RSA), head, lateral view; A male; B female. Scale = 1 mm.

Group 1 (folded theca)

Merodon planifacies was described from Durban (RSA) based on a single male. Revision of world collections added additional material from different part of Africa (from central until south). These specimens shared the apomorphic state of folded ventral ridge of theca with the male genitalia of holotype (Fig. 6B-D). But all these specimens from different localities shows subtle differences of morphological characters of male genitalia. The genitalia characters of these specimens also differ slightly when compared to the holotype. This indicates the possible existence of more species involved in this taxon. *M. planifacies* sensu stricto could be a group of geographically isolated species, which needs additional taxonomic

research based on integrative approach. Specimen collected in Van Reenen (RSA) for which we obtained molecular data is morphologically identical with holotype of *M. planifacies*. Both localities (Durban and Van Reenen) are related and we propose this specimen as genetic type.

(Fig. 5 here)

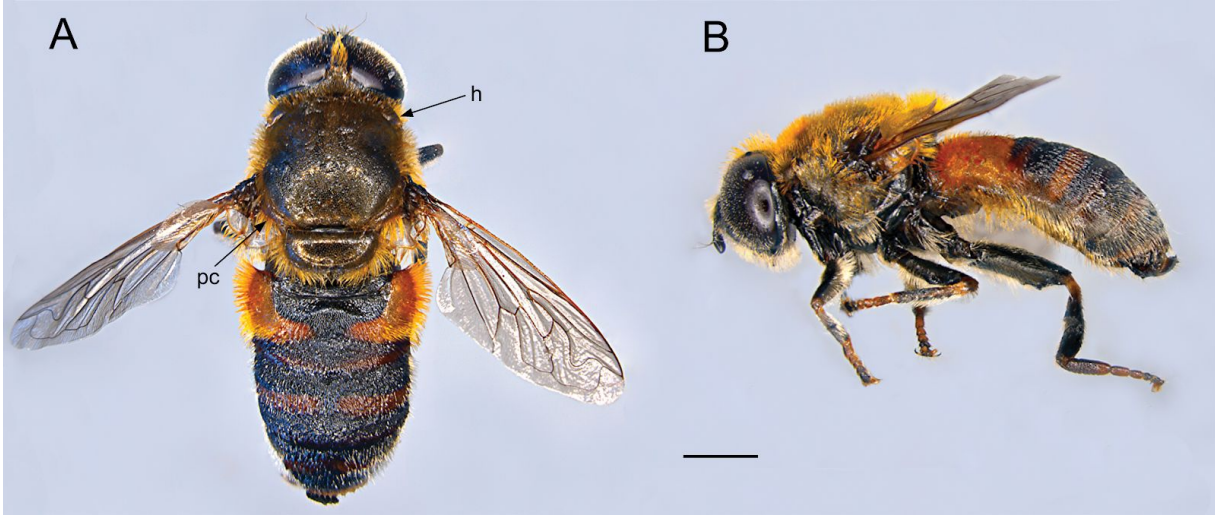

Figure 5. Habitus *Merodon planifacies*, male, A lateral view B dorsal view (RSA, Van Reenen). Abbreviation: h - humerus; pc - postalar calli. Scale = 2 mm.

Group 2 (smooth theca)

The two populations in two different valleys in Drakensberg mountains in (KwaZulu-Natal province, RSA) share identical morphological characters (see Taxonomy). We have proceeded with genetic and geomorphometric analyses in order to explore potential existence of cryptic species within these two populations, as was indicated in obtained data of first genetic screening .

(Fig. 6 here)

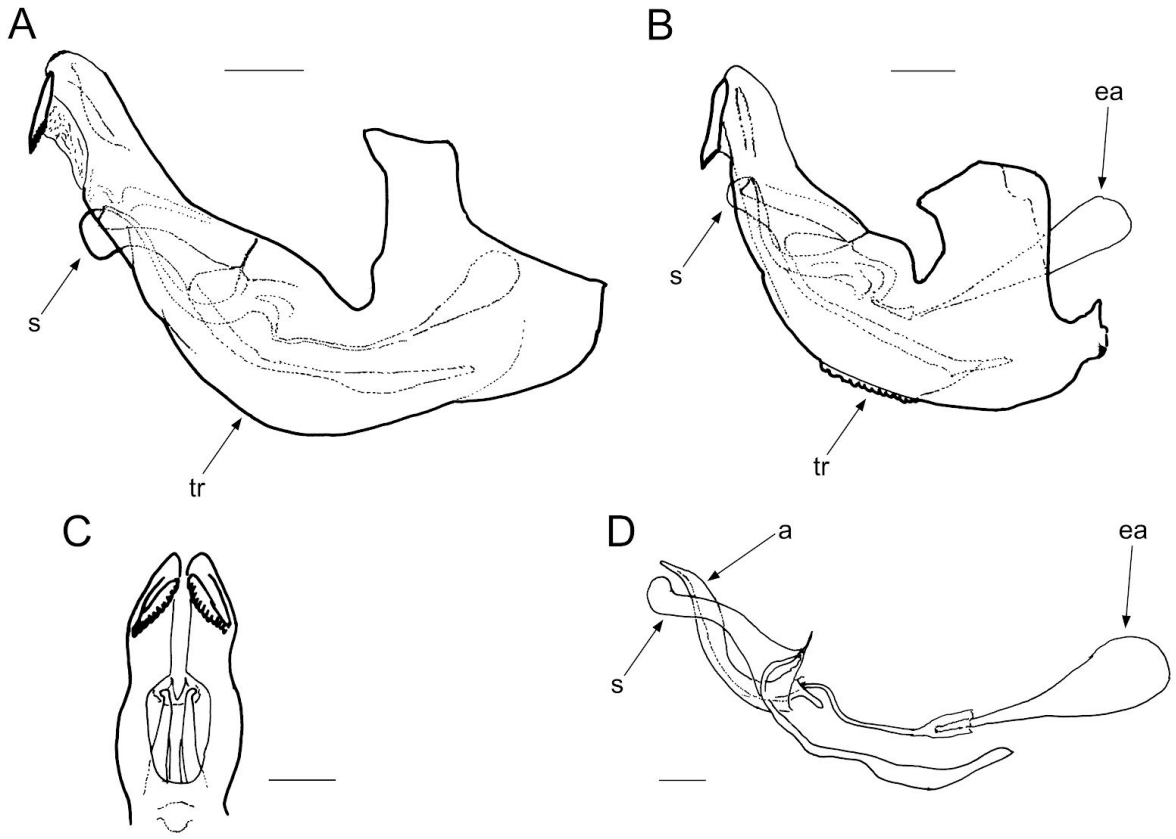

Figure 6. A, C, D *Merodon capi* sp. nov. (Cathedral Peak NP, RSA); B *M. planifacies* (holotype); A, B hypandrium, lateral view; C apical part of the hypandrium, anterior view; D aedeagus with accessory structures, lateral view. Abbreviation: a – lateral sclerite of aedeagus; m – ejaculatory apodeme; e – aedeagus; tr – ventral ridge of theca. Scale = 0.2 mm.

Molecular evidence

The MP analysis for the COI dataset resulted in one equally parsimonious tree, and the strict consensus tree is shown in Fig. 7. The *Merodon planifacies* specimens were resolved as a monophyletic clade in three separate clusters. The most divergent branch is the *M. planifacies* specimen from Van Reenen (one male), which also is distinct in its male genitalia characters (see above section), which corresponds with the holotype of *M. planifacies* Bezzi 1915. Unexpectedly, two additional clusters were present in the MP tree, showing remarkable genetic difference. The first cluster, described here as *M. capi* **sp. nov.**, comprised individuals sampled in the Cathedral Peak National Park in Drakensberg mountains (RSA). The second cluster consisted of specimens described here as *M. roni* **sp. nov.**, exclusively sampled in the

Royal Natal National Park in Drakensberg mountains. Both clusters also show some level of intraspecific divergence. Moreover, all larvae specimens collected (three in *Merwilla natalensis* bulbs in Cathedral Peak National Park and three in *Merwilla natalensis* bulbs in Royal Natal National Park) grouped in expected cluster according to COI haplotypes.

(Fig. 7 here)

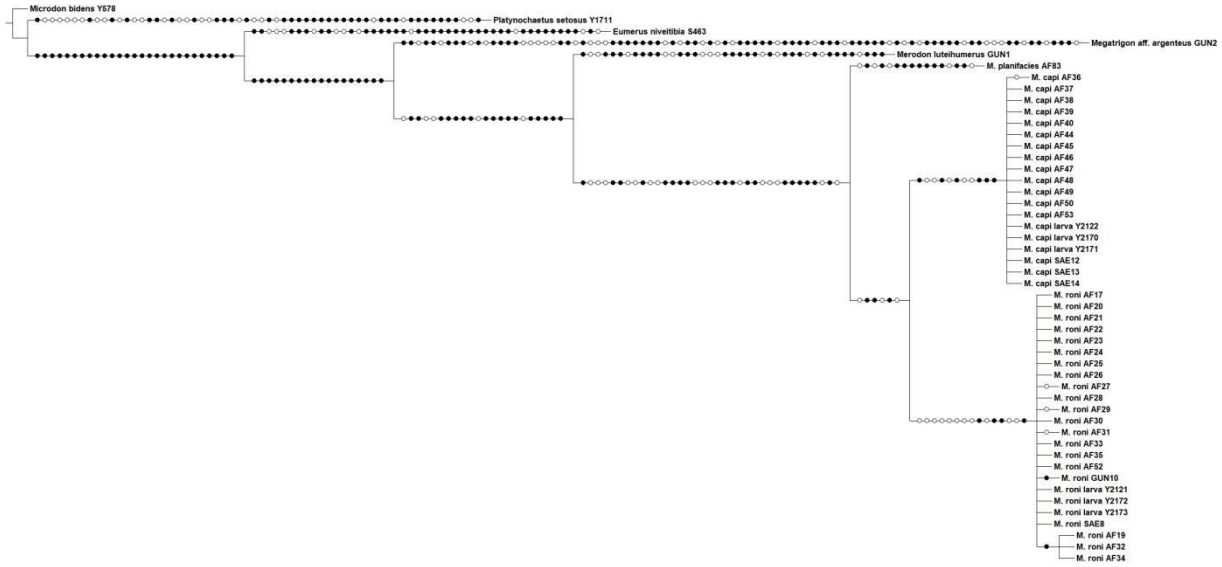

Figure 7. Maximum parsimony (MP) strict consensus tree of 1 equally parsimonious tree of *Merodon planifacies* complex based on COI 3' fragment and 5' fragment (the barcode fragment). Length 862 steps, Consistency index (CI) = 60, retention index (RI) = 75. Filled circles denote unique changes, open circles non-unique.

Since the MP approach depicts the simplest model and does not account for the model of evolutionary substitution, we were tempted to further analyse the COI dataset in order to provide unambiguous prove that there are actually three species within the *M. planifacies* taxon. A similar tree topology was obtained in the Maximum Likelihood and Bayesian phylogenetic trees (Fig. 8, Fig. 9). The existence of three separate clusters within *M. planifacies* taxon was supported with high bootstrap values (100, ML tree) and posterior probability values of 98-100% (BI tree).

(Fig. 8 here)

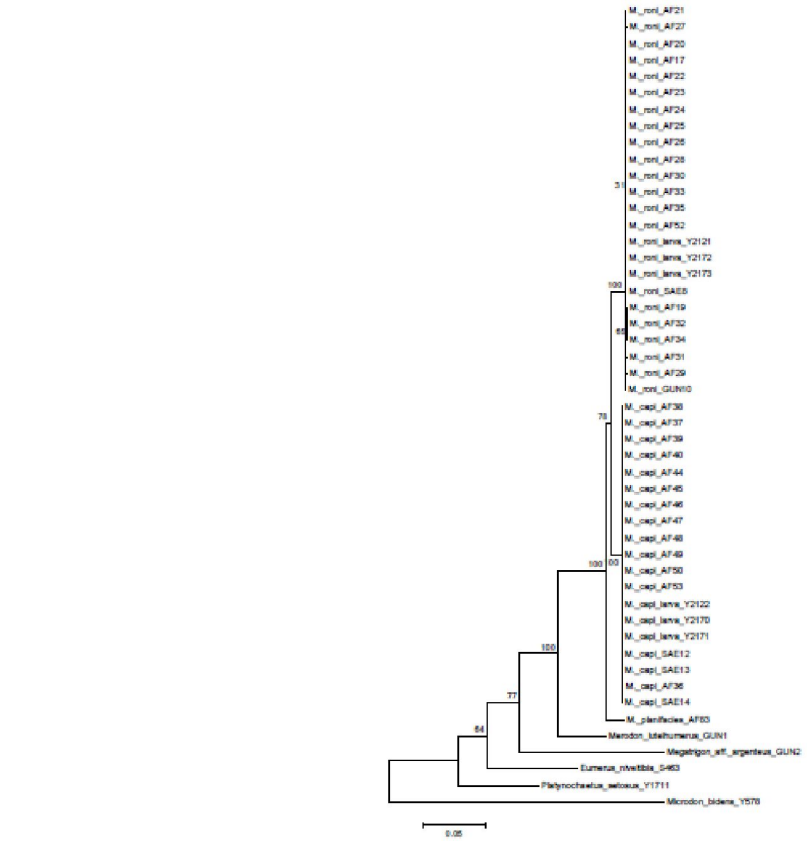

Figure 8. Maximum Likelihood tree of *Merodon planifacies* complex based on COI 3' fragment and 5' fragment (the barcode fragment). Branch support was estimated with 1000 non-parametric bootstrap replicates.

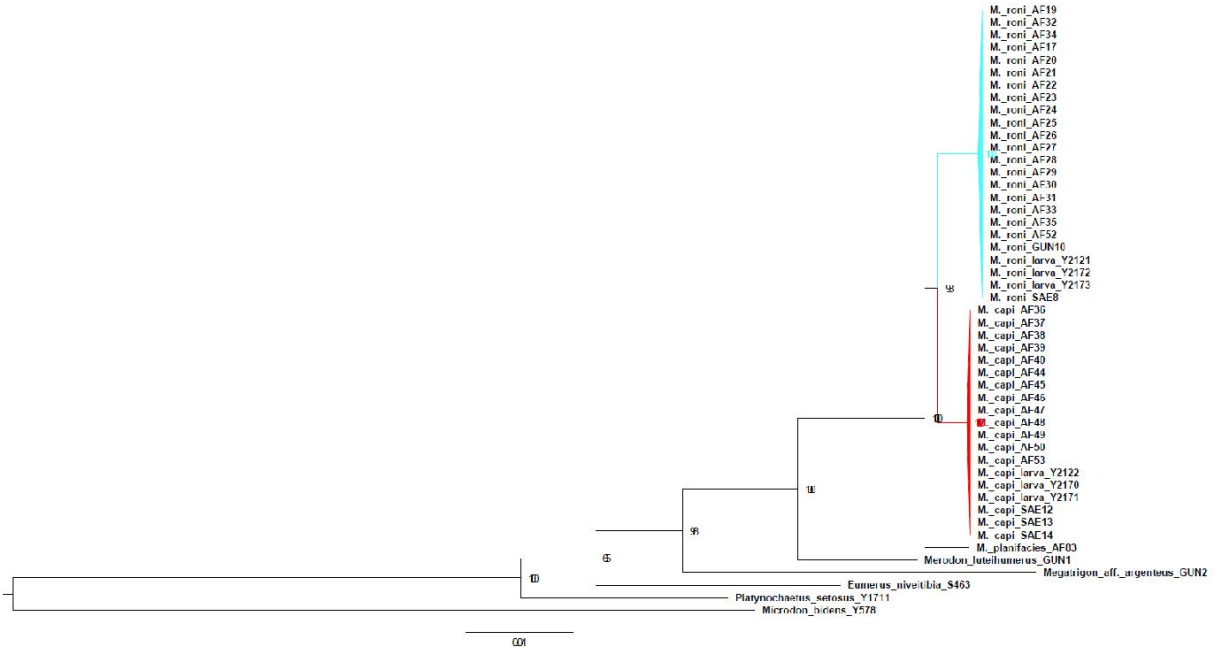

Figure 9. Bayesian phylogenetic tree of *Merodon planifacies* complex based on COI 3' fragment and 5' fragment (the barcode fragment).

The results of the phylogenetic analyses clearly support the presence of two new species here described as *M. capi* **sp. nov.** and *M. roni* **sp. nov.** (see Taxonomy for description). The MJ network for the three ingroup clusters of taxa revealed in total 44 mutational steps between *M. planifacies* haplotype and the *M. capi* **sp. nov.** group of haplotypes (2 COI mtDNA haplotypes revealed) (Fig. 10). The number of mutational steps between *M. planifacies* and *M. roni* **sp. nov.** is 49 (6 haplotypes for *M. roni* **sp. nov.** detected), while there are 30 mutational steps between *M. capi* **sp. nov.** and *M. roni* **sp. nov.**. Intraspecific haplotype differences for *M. roni* **sp. nov.** was one mutational step, as well as for *M. capi* **sp. nov.**.

(Fig. 5 here)

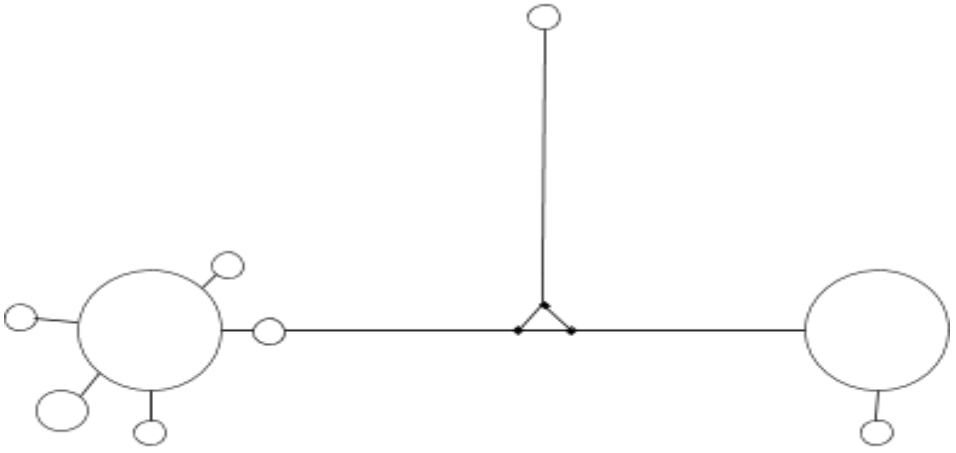

Figure 10. Median-joining network of the COI haplotypes of *Merodon planifacies, M. roni* **sp. nov.** and *M. capi* **sp. nov.**. Circle sizes are proportional to haplotype frequencies. Each branch represent one mutational step, if more than one mutation steps is present, it is denoted by the numbers. Black circles represent median vectors (unsampled or ancestral haplotypes).

AMOVA defined highly statistically significant percentage of variation among taxa of the *M. planifacies* taxon (Fst=0.9899; $p<0.05$). Significant genetic divergence was detected between *M. roni* **sp. nov.** and *M. capi* **sp. nov.** according to pairwise ϕst value (0.68221; $p<0.05$), while the significance of genetic differentiation was not detected in other species-pair comparisons, probably due to the fact that only one specimen of *M. planifacies* was included (Table 1).

Table 1. Genetic divergence among taxa of the *Merodon planifacies* taxon. Below diagonal - pairwise φst values; Above diagonal – significance level (+ - p<0.05; - - p>>0.05)

	M. planifacies	*M. roni* **sp. nov.**	*M. capi* **sp. nov.**
M. planifacies	/	-	-
M. roni **sp. nov.**	0.50362	/	+
M. capi **sp. nov.**	0.89474	0.68221	/

(Fig. 10 here)

In the further analysis conducted in order to assess divergence rates in the *M. planifacies* taxon we applied sorting of the sequences into hypothetical species as described in ABGD software (Puillandre et al. 2011). Initial partition with prior maximal distance P=1.67e-03, defined existence of three putative species in the dataset, corresponding to *M. planifacies*, *M. capi* **sp. nov.** and *M. roni* **sp. nov.**, at all significance levels.

Divergence time estimations based on COI mtDNA sequence variability among taxa of the *M. planifacies* taxon detected the most recent genetic separation between *M. capi* **sp. nov.** and *M. roni* **sp. nov.** and it is estimated to be ~1.150.000 years ago (p=2.6%).

Mantel's test in the Arlequin program did not indicate either a significant correlation between genetic distance and geographic distance (r = 0.439372) or a significant isolation by distance (p>0.05 for 10,000 randomizations).

The additional molecular marker 28S rRNA gene sequence failed to discriminate species *M. roni* **sp. nov.** and *M. capi* **sp. nov.**. Two genotypes were obtained for all included specimens, and we present only MP tree (Fig. 11). Due to the fact that this gene region is less powerful in discrimination of closely related species, this result was expected.

(Fig. 11 here)

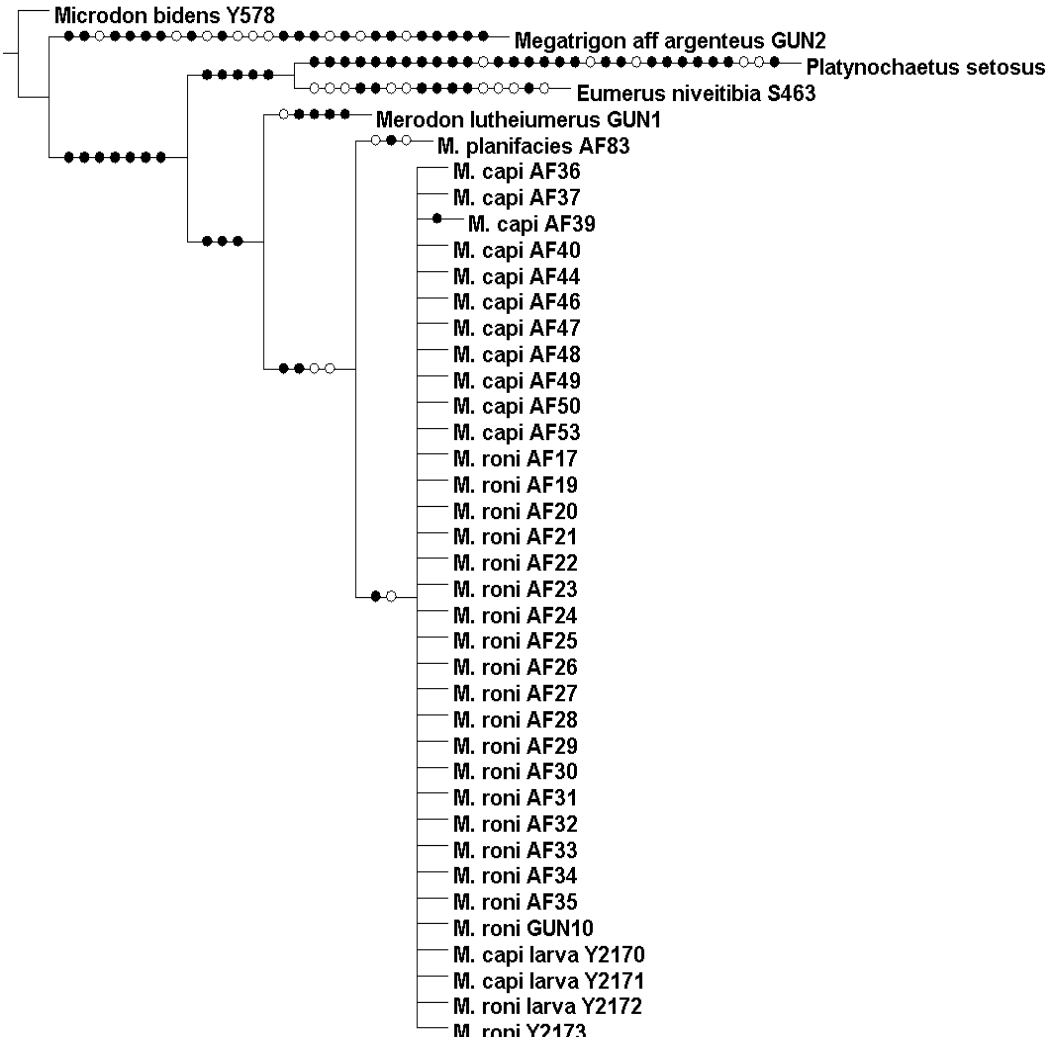

Figure 11. Strict consensus tree of 1 equally parsimonious trees based on 28S rRNA gene. Length 2017 steps, Consistency index (CI) = 88, retention index (RI) = 61. Filled circles denote unique changes, open circles non-unique.

Geometric morphometrics - wing shape analysis

In addition to the molecular data, support for the separation of two taxa *M. roni* **sp. nov.** and *M. capi* **sp. nov.** was evident from the geometric morphometric data.

Principal component analysis carried out on the Procrustes shape variables produced 20 PCs, from which 11 PCs for overall dataset, and 13 Pcs for males were selected using Stepwise

discriminant analysis. Differences in wing shape between *M. roni* **sp. nov.** and *M. capi* **sp. nov.** were highly significant using discriminant analysis (Males: $F_{13,17}$= 5.1978; p<0.01; Overall dataset: $F_{11,28}$= 4.306; p<0.01). Further, DA correctly classified species with very high overall classification success (Males: 96.97%; Overall dataset: 97.44%). In in both cases, only one specimen was misclassified. Moreover, congruent classification was obtained using Gaussian naive Bayes classifier with only two misclassified specimens of *M. capi* **sp. nov.** for entire dataset, and one misclassified *M. capi* **sp. nov.** among males.

(Fig. 12 here)

Additionally, canonical analysis produced one highly significant canonical axis related to wing shape differences (Males: CV1: Wilks' Lambda = 0.1915; $\chi 2$ = 35.5391; p<0.01; Overall dataset: CV1: Wilks' Lambda = 0.3465; $\chi 2$ = 32.8591; p<0.01). Differences in mean wing shape between males of *M. capi* **sp. nov.** and *M. roni* **sp. nov.** occur in basal and central part of wing which mostly results in the wing length differences (Fig. 12).

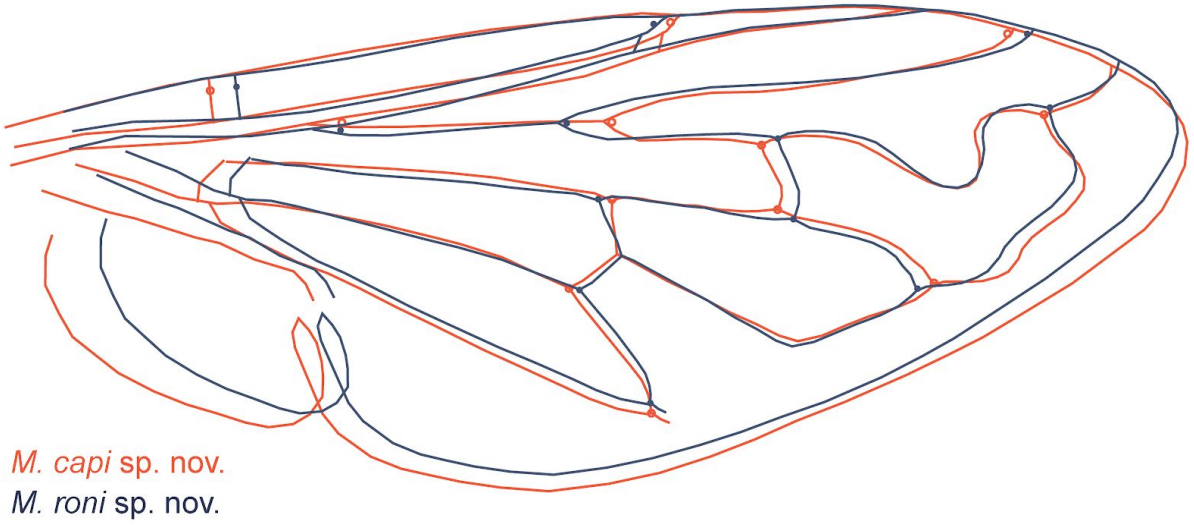

Figure 12. Superimposed outline drawings showing differences in average wing shape between and *M. capi* **sp. nov.** and *M. roni* **sp. nov.** Differences between the species have been exaggerated five-fold to make them more visible.

Taxonomy

Merodon planifacies subgroup

General description

Male (Fig. 5). Head (Fig. 3A, B, 4): Antenna (Fig. 14C, D) brown to reddish; first flagellomere elongated, about 1.5 times as long as broad. Face and frons covered with long whitish or yellow pile usually completely covered with microtrichia. Oral margin strongly reduced (Fig. 3A, B, 4A, B). Vertical triangle (Fig. 14A) equilateral, more or less microthichose, predominantly covered with long pale pile. Eye pile as long as pedicel. Occiput strongly microtrichose, covered with whitish or yellow pile.

(Fig. 13 here)

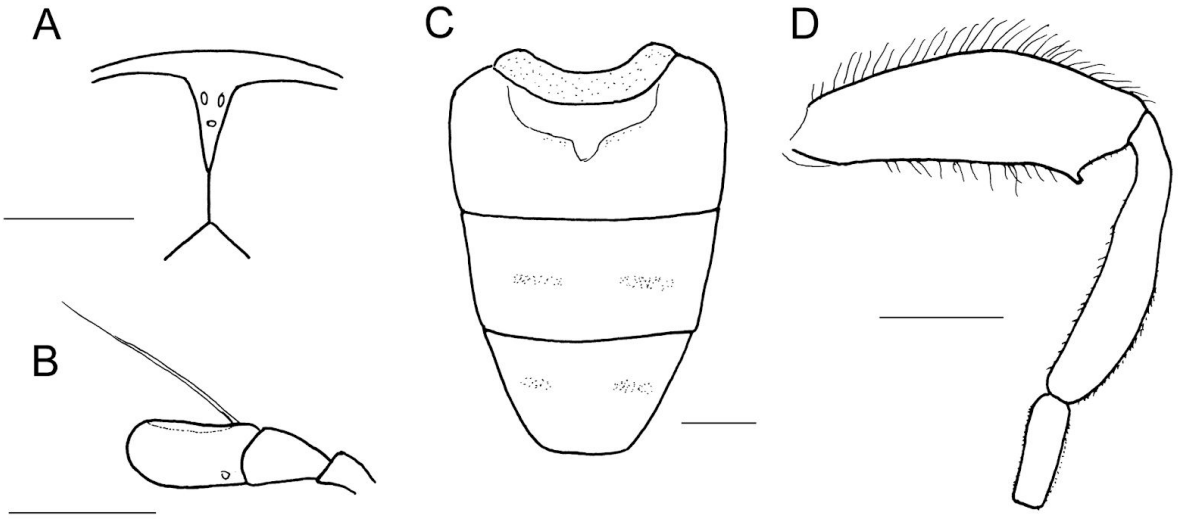

Figure 13. *Merodon planifacies* male (holotype); A frons, dorsal view; B hind leg, lateral view; C antenna, lateral view; D abdomen, dorsal view. Scale = 1 mm.

Thorax: Scutum and scutellum black with bronze lustre, mostly microtrichose, covered with relatively long, dense, erect yellowish pile, as long as first flagellomere. Pleurae covered with gray-green microtrichia and the following parts with long yellowish pile: anterior part of proepimeron, posterior part of anterior anepisternum, the most of posterior anepisternum except anterior end, antero-ventral and postero-dorsal part of katepisternum, anepimeron, metasternum; katatergit with dense, erect, short, yellowish pile. Wing hyaline, with dense microtrichia. Calypter yellow to brown. Halter with brown pedicel and yellow to brown capitulum. Legs (Fig. 15) usually dark brown. Femora dark, except usually paler apex; tibiae can be from completely dark to almost completely pale; color of tarsi varies. Metatrochanter without process. Metafemur moderately thickened and curved (Fig. 15A). Metatibia without spur. Pile on legs pale.

(Fig. 14 here)

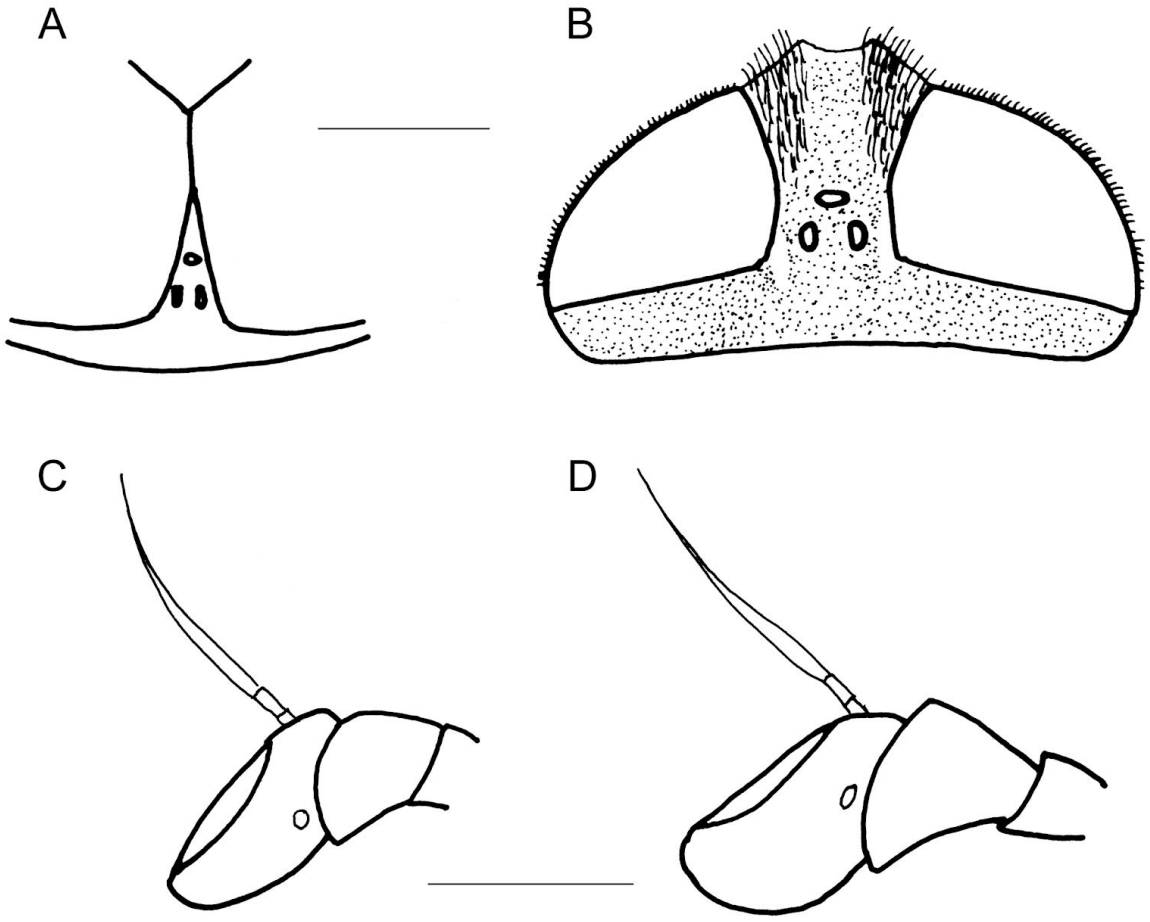

Figure 14. *Merodon roni* sp. n. (Royal Natal NP, RSA). A frons, male, dorsal view; B head, female, dorsal view; C-D antenna: C male; D female. Scale = 1 mm.

Abdomen from redish to black, as long as mesonotum. Tergites 2 – 4 with more or less distinct white transverse bands of microtrichia interrupted in the middle; tergite 2 with pair of orange antero-lateral spots; pile on tergites mainly erect and yellow; pile on central part adpressed and clearly shorter than on lateral margins. Black pile on tergites extend from posterior half of tergite 2, until anterior half of tergites 4; lateral sides of tergites and white microtrichose stripes covered with pale pile. Sternites blackish-brown, covered with very long pale yellow pile.

(Fig. 15 here)

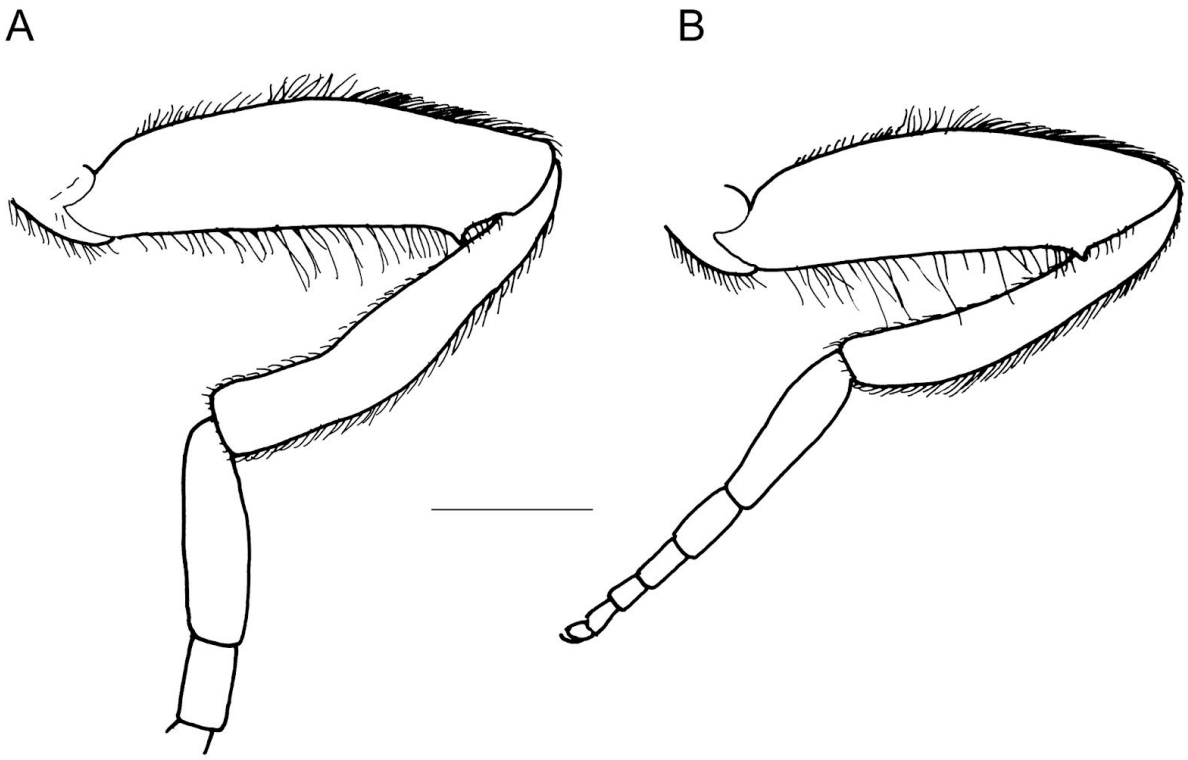

Figure 15. *Merodon roni* sp. n. (Royal Natal NP, RSA), hind leg, lateral view; a male; b female. Scale = 1 mm.

Male genitalia: Posterior surstyle lobe triangular, usually pointed anteriorly (Fig. 16A-C: pl); ventral margin of surstylus rounded (Fig. 16: d); anterior surstyle lobe (Fig. 16: al) with curved distal prolongation (Fig. 16DE: il) and two thorns on interior accessory lobe (Fig. 16DE: t); cercus elongated (Fig. 16: c). Hypandrium (Fig. 6) with broad theca. Lateral sclerite of aedeagus pointed and projected dorsally (Fig. 6D: s); aedeagus curved, relatively broad (Fig. 6D: a); ejaculatory apodeme narrow (Fig. 6D: ea).

Female. Similar to the male except normal sexual dimorphism (Fig. 4B, 14D, 15B).

Length: medium-sized species, body 10-12 mm, wing 6-8 mm.

Variability: This complex shown great variability in:

- color of tergites, from completely dark to orange-reddish;
- mesoscutum can be mostly pale or dark with at least orange humerus and postalar calli;
- microtricose on mesoscutum and tergites can be extremely expressed, with clear stripes and figures, but many specimens have reduced microtrichia.
- shape of antenna can be from usual (as on Fig. 3A) until specimens and populations with strong concave dorsal margin (as on Fig. 14D).

This variability is intra-specific and presumably depends on environmental conditions (e. g. time of adult flight season, temperature).

(Fig. 16 here)

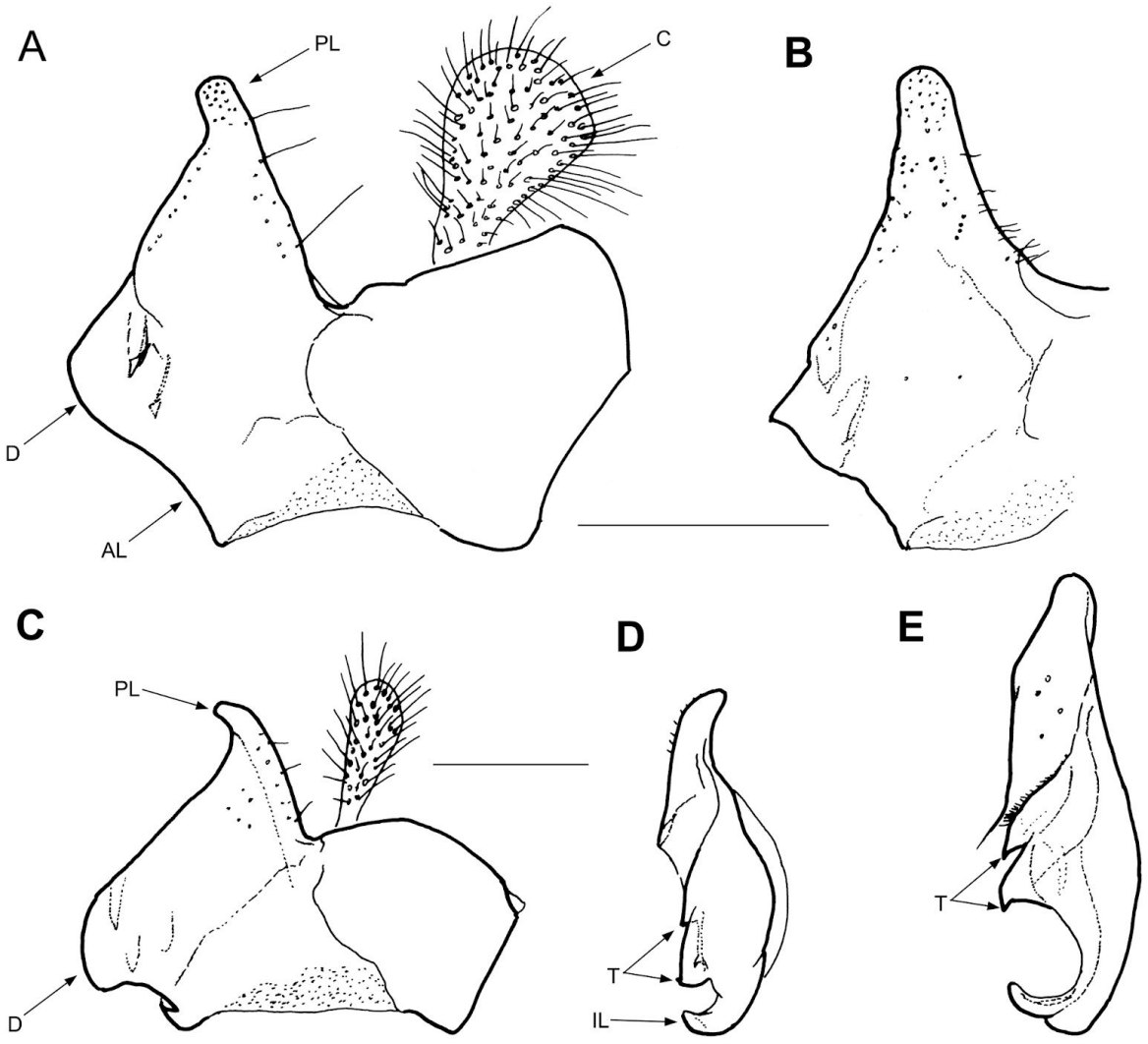

Figure 16. Male genitalia. (A, B, E) *Merodon capi* sp. nov. (Cathedral Peak NP, RSA); (C, D) *M. planifacies* (holotype); (A, B, C) epandrium, lateral view; (D, E) surstyle lobe, anterior view. Abbreviation: al – anterior surstyle lobe; d – ventral surstyle margin; il – distal prolongation of anterior lobe of surstylus; t – interior accessory lobe; pl – posterior surstyle lobe; c – cercus. Scale = 0.2 mm.

Taxa recognized

There are two group of taxa clearly separated by structure of male genitalia: one (1) *Merodon planifacies*, with folded ventral ridge of theca (Fig. 6B: tr), and second (2) *Merodon capi* complex, with smooth ventral margin (Fig. 6A: tr)

1. *Merodon planifacies* Bezzi 1915 (Fig.)

Diagnosis. In male genitalia thecal ridge folded (Fig. 6B: tr); posterior surstyle lobe strongly curved anteriorly (Fig. 16C: pl).

Material examined. Type material: Holotype: ♂, Republic of South Africa, Natal, Durban, 24.x.1902, leg. Muir F. (BMNH).

Additional material: ♂, Republic of South Africa, Kwa-Zulu Natal, Van Reenen, 23.ii.2016, leg. Vujić A., Radenković S., Veličković N., -28.379482S, 29.37828N (FSUNS, ZA2_149).

2. *Merodon capi* complex

Merodon capi **sp. nov.** Vujić et Radenković (Fig.)

Diagnosis. In male genitalia thecal ridge smooth (Fig. 6A: tr); posterior surstyle lobe slightly curved anteriorly (Fig. 16A: pl), or straight (Fig. 16B: pl). Differs from *M. planifacies* by male genitalia, from *M. planifacies* and *M. roni* **sp. nov.** by molecular data (xxx), and from *M. roni* **sp. nov.** by the wing morphometric characters (significant wing shape differences, Fig. 12). We not founded any classical morphological differences between *M. capi* **sp. nov.** and *M. roni* **sp. nov.**

Material examined. Type material: Holotype: ♂, Republic of South Africa, Drakensberg mountains, Cathedral Peak NP, 7.xii.2012, leg. Vujić A., -28.945872, 29.200756 (NMSA, 08879).

Paratypes: Republic of South Africa, Drakensberg mountains, Cathedral Peak NP: 6♂♂, 1♀, xi.2011, leg. Vujić A. (NMSA, FSUNS, G1476-81, G1483); 3♂♂, 1♀, Mlambonja River, xi.2011, leg. Vujić A., -28.956983, 29.173919 (FSUNS, G0884-87); 5♂♂, 1♀, blue pools, 7.xii.2012, leg. Vujić A., -28.945872, 29.200756 (NMSA, FSUNS, G2190-92, G2199, G2200).

Range. Endemic species for Drakensberg mountains in RSA. Until now recorded only in Cathedral Peak National Park (Fig. 17)

Etymology. The name *capi* is acronym based on the type area, Cathedral Peak National Park in Drakensberg mountains.

Merodon roni **sp. nov.** Radenković et Vujić (Fig. 4, 14, 15)

Diagnosis. In male genitalia thecal ridge smooth (as on Fig. 6A: tr); posterior surstyle lobe slightly curved anteriorly (as on Fig. 16A: pl), or straight (as on Fig. 16B: pl). Differs from *M. planifacies* by male genitalia, from *M. planifacies* and *M. capi* **sp. nov.** by molecular data (xxx), and from *M. capi* **sp. nov.** by the wing morphometric characters (significant wing shape differences, Fig. 12).

Wing morphometry and genetical data

Material examined. Type material: Holotype: ♂, Republic of South Africa, Drakensberg mountains, Royal Natal NP, Tiger fall, 2.xii.2012, leg. Vujić A., -28.688333, 28.930750 (NMSA, G2112).

Paratypes: Republic of South Africa, Drakensberg mountains, Royal Natal NP: 1♂, xi.2011, leg. Vujić A., (FSUNS, G1482); 4♂♂, Thukela River, xi.2011, leg. Vujić A., -28.718083, 28.933972 (NMSA, FSUNS, G0872-75); 8♂♂, 2♀♀, Tiger fall, 2.xii.2012, leg. Vujić A., -28.688333, 28.930750 (NMSA, FSUNS, G2111, G2113, G2116, G2118-24); 1♂, way to Thukela, 3.xii.2012, leg. Vujić A., -28.718083, 28.933972 (FSUNS, G2147); 1♀, Gorge car park, 3.xii.2012, leg. Ssymank A., -28.713472, 28.935000 (Axel Ssymank collection, 08889); 1♀, Gorge , 4.xii.2012, leg. Banon C. P., -28.688333, 28.930750 (CIBIO, G2181); 4♂♂, 3♀♀, trail to Tiger fall, 4.xii.2012, leg. Ssymank A., -28.688333, 28.930750 (Axel Ssymank collection, 08882-88); 2♂♂, Crack, 8.xii.2012, leg. Rojo S., -28.684528, 28.437417 (CIBIO, G2210); The Crack path: 2♂♂, 1♀, 4.xii.2012, leg. Stahls G., -28.684444, 28.933611 (LUOMUS, 08890-92), 1♂, 8.xii.2012, leg. Stahls G., -28.684444, 28.933611 (LUOMUS, 08880); 2♂♂, trail to the Crack, 9.xii.2012, leg. Ssymank A., -28.684528, 28.937417 (Axel Ssymank collection, 08881).

Range. Endemic species for Drakensberg mountains in RSA. Until now recorded only in Royal Natal National Park

Etymology. The name *roni* is acronym based on the type area, Royal Natal National Park in Drakensberg mountains.

(Fig. 17 here)

Figure 17. Cathedral Peak National Park, typical habitats of *Merodon capi* **sp. nov.**

Field observations

Our fieldwork was obtained during seven years (2011-2017) of study. We spent In Royal Natal NP 18 days and 14 days in Cathedral Peak NP with average of 6 hours of work per day. During these days we collected studied individuals (51) and observed double more specimens (about 100) in the field. All specimens fly low, settle in the sun, usually on foliage of the leaves or bulbs of host plant (*Merwilla natalensis*) or near by, not more than 20 meters from plant population. There were no any observed flower visiting by any of adult specimens during all these 32 days. Comparing with most of other species of genus *Merodon*, adults of *M. capi* **sp. nov.** and *M. roni* **sp. nov.** do not fly fast and show very weak dispersal ability. Apparently they are very localized at the infested area with host plant population. Bulbs of *Merwilla natalensis* usually appear at least half of size out of ground (Fig. 18) and observation of adult oviposition is quite usual comparing with other *Merodon* species. Both valleys contain large populations of *Merwilla natalensis* predominantly distributed at steep sides usually on the rocks (Fig. 17).

(Fig. 18 here)

Figure 18. *Merwilla natalensis*, host plant of *Merodon roni* **sp. nov.** and *M. capi* **sp. nov.** Abbreviation: la - third instar of larva of *M. roni* **sp.nov.**

Discussion

The *M planifacies* subgroup belongs to the *Merodon desuturinus* monophyletic group within genus *Merodon* (Mengual et al. 2006). According to morphological and molecular data, the

Afrotropical lineage of *Merodon desuturinus* group includes three taxa, *M. cuthbertsoni*, *M. melanocerus* subgroup, and *M. planifacies* subgroup. The *Merodon melanocerus* recently was described as a subgroup of species, which comprises five species, both morphologically and genetically separated: *M. melanocerus, M. flavocerus, M. capensis*., *M. draconis* and *M. commutabilis* (Radenkovic et al. 2018b). *M. stevensoni* and *M. planifacies* taxa as subgroup within group are characterized by distinct apomorphic character - reduced oral margin covered by microtrichia. Because of lack of material *M. stevensoni* was excluded from this analysis. While all taxa comprising the *desuturinus* group can be distinguished by clear diagnostic adult morphological features, revealed species within *Merodon planifacies* taxon can be delimited only based on integrative taxonomy approach.

Molecular analyses in our study supported monophyly of *M. planifacies* taxon, resolved in three clusters. According to all performed analyses and based on genetic and morphometric data, three species are found within *M. planifacies* taxon: *M. planifacies*, and two species placed to *M. capi* complex: *M. capi* **sp. nov.** and *M. roni* **sp. nov.** Although *M. planifaces* was represented in our sample with only two male specimens, it is clearly separated based on morphological data (structure of male genitalia) and molecular data (applying both COI and 28S rRNA gene sequences), according to which in phylogenetic trees it represents the most divergent branch within *M. planifacies* taxon. Due to the fact that only two specimens has been included; no morphometric analyses could be done for this species. The latter two species, *M. roni* **sp. nov.** and *M. capi* **sp. nov.**, are morphologically indistinguishable, and thus it was a surprising unexpected finding. Even though, molecular and morphometric data gave a strong confirmation for separation of these species within the *M. capi* complex. Both species represent endemic species for Drakensberg mountains in RSA. It is not uncommon for *Merodon* genus to comprise species with extraordinary morphological similarities (Speight 2014), but to best of our knowledge this is a rare example that no other characters commonly used in integrative taxonomy could not reveal species within complex (except molecular data and wing morphometrics) and very the first proof in the Afrotropical lineage of *desuturinus* group. In the better studied lineage of *desuturinus* group, the Palaearctic lineage, three endemo-relicts are found: *M. cabanerensis* from restricted area in central Spain, *M. desuturinus* localized on Durmitor and Kopaonik mountains on the Balkans and *M. neolydicus* present in several countries of Eastern Mediterranean (Turkey, Syria, Lebanon, Israel) until Iran (Vujić et al. 1995; Mengual et al. 2006; Marcos-Garcia et al. 2007; Vujić et al.

20112018). Molecular data for these species showed, for example, uncorrected pairwise COI divergence of 7.32% between *M. desuturinus* species and *M.cabanerensis* from Spain (Mengual et al. 2006; Milankov et al. 2008). Here we document a lower but highly significant pairwise difference between *M. roni* **sp. nov.** and *M. capi* **sp. nov.** of 2.6% based on COI sequence, which actually represent two species inhabiting two valleys in the same mountain range, with a geographic distance of XXX, with proven no significant correlation between genetic distance and geographic distance. Similar pattern was not observed in *M. desuturinus* species, inhabiting two high mountains on the Balkans, where both populations represent same species sharing morphological characters and molecular signatures. Intraspecific genetic variability was low for this species (Milankov et al. 2008), assuming due to small population size of the spatially isolated populations and narrow ecological niche associated with possible past bottlenecks. Giving the above facts, it is expected to found endemic species within *Merodon desuturinus* group, but never the less, *M. roni* **sp. nov.** and *M. capi* **sp. nov.** represent a unique example of genetic endemism in hoverflies, which show large genetic distance between highly related species as compared to other examples (Ståhls et al. 2009; Šašić et al. 2016; Ačanski et al. 2016; Radenković et al. 2018a). Further support for the separation of two new taxa *M. roni* **sp. nov**. and *M. capi* **sp. nov.** was obtained from the geometric morphometric data for differences in wing shape, which were highly significant and using discriminant analysis an overall classification success was 97.47%. Integration of molecular and morphometric data provided strong support for revealing and defining *capi* complex with two species within *M. planifacies* subgroup. However, in our study 28S rRNA genotypes failed to discriminate between *M. roni* **sp. nov.** and *M. capi* **sp. nov.**, which was expected for the cryptic species belonging to the same species complex. The 28S rRNA gene is composed of highly conservative and variable domains, and represents a useful molecular marker for taxonomical studies, usually above species level (Hwang et al. 1999; Raupach et al. 2010). However, 28S was successfully applied in phylogenetic studies of hoverflies (Pérez-Bañón et al. 2003; Ståhls et al. 2004; Vujić et al. 2012; Mengual et al. 2015). Due to the lower mutational rate as compared to protein-coding genes, the main application of 28S rRNA gene sequence is in the analyses of distantly related species.

The presence of species complexes is not uncommon in hoverfiles (Šašić et al. 2016; Ačanski et al. 2016; Radenković et al. 2018a). However, according to our findings, *M. planifacies* taxon consists of species that apparently have been separated for a long time period

(~1.150.000 years) without evolving any clear morphological differences. The main shortcoming in our conclusions may be that those are based on evidence of single gene, even though accompanied by morphometric evidence, and that there is still no developed method for testing the reproductive isolation between species, due to the lack of appropriate rearing methods. On the other hand, COI mtDNA sequence (specifically DNA barcoding) is widely recognized as a tool in integrative taxonomy, especially in Syrphidae (Mengual et al. 2006; Masetti et al. 200; Marcos-García et al. 2011; Vujić et al. 2012, 2013; Popović et al. 2014; Nedeljković et al. 2015; Šašić et al. 2016; Chroni et al. 2017), with a high reliability for species delimitation. Furthermore, considering the modern species concept, a unified species concept according to de Queiroz (2007), we may conclude that species defined in the *Merodon planifacies* taxon verily represent separately evolving metapopulation lineages, which is according to the concept the only necessary property. Our molecular and morphometric data clearly point to the separately evolving lineages, and the open question is which force was/is responsible for preventing the evolvement of other properties that might be expected (morphology, male genitalia structure, etc.).

The genetic evidences for existence of three species within the *planifacies* taxon are also valid in the alternative species concepts. We have found exclusive coalescence of haplotypes which points to genealogical species and we have defined clear genetic clusters with no shared haplotypes, based on COI mtDNA. The geographical distribution of those genetic clusters points to endemism. Furthermore, the occupation of different ecological niche by defined species might represent a secondary species criterion congruent with ecological species concept. If we adopt all these properties we may unequivocally consider presence of three species.

Focusing on *M. capi* complex, species *M. roni* **sp. nov.** and *M. capi* **sp. nov.**, we observe that species delimitation is based on reciprocial monophyly of the alleles at mtDNA COI locus, which is not found for 28S rDNA locus. This fact might weaken the genetic proof and species delimitation. Moreover, since mtDNA is maternally inherited, it might be argued that the reciprocial monophyly is a result of low dispersal of females, and that actually defined species represent same population of a single species. This is especially valid in the case where no other evidences are available. We have no approach yet to observe whether or not we have similar pattern for several nuclear DNA loci, but additional evidence from morphometric analyses of wing shape encouraged delimitation of two morphologically indistinguishable

species. Furthermore, single-locus data continues to be main tool in molecular taxonomy and represents common approach for species delimitation (Fujisawa & Barraclough 2013; Ratnasingham & Hebert 2013). Larvae of both species were collected in the bulbs of same plant species *Merwilla natalensis*. Moreover, in this particular complex, we believe that low dispersal of females is not only force which driven to observed genetic exclusivity. Rather, it may be a consequence of speciation driven by developmental ecological preferences and very long time of isolation ~1.150.000 years. In favour to this hypothesis, we observed no isolation by distance pattern, since geographic information is crucial to distinguish whether we have true genetic separation or not.

Figures

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