

## Research article

[urn:lsid:zoobank.org:pub:4670E7D8-8F93-40E5-9FF7-8B8FE517C0AB](https://zoobank.org/pub:4670E7D8-8F93-40E5-9FF7-8B8FE517C0AB)**An assessment of new character in hoverfly species delimitation using linear and geometric morphometrics – genus *Merodon* Meigen, 1803 (Diptera: Syrphidae) as a case study**Jelena AČANSKI<sup>1,\*</sup>, Tamara TOT<sup>2</sup>, Ana GRKOVIĆ<sup>3</sup>, Marija MILIČIĆ<sup>4</sup>,  
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**Abstract.** Detection of morphologically indistinguishable cryptic species implies using an integrative taxonomic approach with a combination of molecular, contemporary morphological, ecological and other relevant analyses. Within a contemporary morphological analysis, two approaches are commonly used in hoverfly taxonomy: a geometric morphometric analysis of the wing and surstyle shape. Here, the importance of the R4+5 vein shape is tested in cryptic species delimitation within four *Merodon* species groups using linear and semilandmark geometric morphometric analyses. As expected, geometric morphometrics showed a stronger resolution compared to linear morphometrics. Linear morphometrics failed to detect differences related to sexual dimorphism or differences among the species *M. pruni* and *M. obscurus*. However, all cryptic species and sexes were separated with high significance based on the R4+5 vein shape. Moreover, obtained results concurred with the landmark-defined wing shape and molecular results published in previous studies. Additionally, combining two characters, the semilandmark R4+5 vein shape and the landmark-defined wing shape, provided more detailed and precise insights into the shape differences. Our results showed that the R4+5 vein shape stands out as an important character in species delimitation of hoverflies where the situation of this vein is present. Therefore, it can be beneficial as a single character or in combination with a landmark-based wing shape analysis.

**Keywords.** Cryptic species delimitation, hoverflies, R4+5 vein, taxonomy, wing venation.

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## Introduction

For centuries, species identification has focused on the morphology of various body parts as diagnostic characters. However, the development of new technologies leads to the usage of alternative characters in taxonomic research. Integrating different approaches, such as behavioural, molecular, morphometric, and biochemical, led to an exponential growth in the number of new species for science, especially cryptic and sibling species.

The family Syrphidae Latreille, 1802 has recently received much-deserved attention, considering they are the second most important pollinators after bees (Petanidou *et al.* 2011). Hence, discovering new hoverfly species is of great importance, especially if it concerns species with a limited distribution range. Furthermore, many newly discovered cryptic hoverfly species are endangered in the IUCN Red List of threatened species. Therefore, it is necessary to recognise different species in time to protect them, and any additional character contributing to their detection is beneficial. Specifically, within the genus *Merodon* Meigen, 1803, one of the largest hoverfly genera, more than 25 cryptic and sibling species have been described since 2016 (Ačanski *et al.* 2016; Šašić *et al.* 2016; Veselić *et al.* 2017; Kočiš Tubić *et al.* 2018; Radenković *et al.* 2018a, 2018b, 2020; Šašić Zorić *et al.* 2018, 2020; Vujić *et al.* 2018, 2019, 2020a, 2020b, 2020c, 2021a, 2021b, 2021c, 2022; Djan *et al.* 2020; Likov *et al.* 2020). Most of these studies used morphological, molecular and morphometric characters. The geometric morphometric analyses of the wing and surstyle shapes have become a popular element of hoverfly taxonomic research (Nedeljković *et al.* 2013, 2015; Vujić *et al.* 2013; Ačanski *et al.* 2016, 2022; Šašić *et al.* 2016; Kočiš Tubić *et al.* 2018; Radenković *et al.* 2018; Šašić Zorić *et al.* 2018, 2020; Vujić *et al.* 2021c). Using linear and geometric morphometric analyses allows for defining the wing shape or one of its parts, apart from a visual description. Linear morphometrics measures length and angles between defined structures, while geometric morphometrics is based on mathematical shape calculation using cartesian coordinates. An advantage of geometric morphometrics over linear morphometrics is primarily its high statistical sensitivity, which can detect differences in shape that are not visible to the human eye (Zelditch *et al.* 2004; Mutanen & Pretorius 2007). An additional advantage of this method is that it enables the drawing of intelligible pictures that depict the shape differences between species.

In Diptera Linnaeus, 1758, the radial vein (R) is the wing's main and largest vein. In Syrphidae, it is subdivided into three main branches: R1 (first longitudinal vein), R2+3 (second longitudinal vein) and R4+5 (third longitudinal vein). R4+5 vein is commonly referred to as a combination of branches 4 and 5 of the radial vein. Therefore, it is in common usage in taxonomic keys as R4+5. According to Speight (1987) and Speight & Sarthou (2017), this wing vein is more correctly recognised as branches 3 and 4 of the radial sector vein, together with a branch of the median vein, M1. Vein R4+5 in Syrphidae is an important taxonomic character, depending on whether it sinuates – arcuates into an underlying cell or straight. Sinuate R4+5 vein exists in the following European members of the subfamily Eristalinae Newman, 1834, genera *Anasimyia* Schiner, 1864, *Eristalis* Latreille, 1804, *Eristalinus* Rondani, 1845, *Helophilus* Fabricius, 1805, *Lejops* Rondani, 1857, *Mallota* Meigen, 1822, *Merodon*, *Mesembrius* Rondani, 1857, *Milesia* Latreille, 1804, *Myathropa* Rondani, 1845, *Parhelophilus* Girschner, 1897 and *Tropidia* Meigen, 1822, as well as some of the members of the subfamily Syrphinae Latreille, 1802, like genera *Didea* Macquart, 1834, *Lapposyrphus* Dušek & Láška, 1967, *Megasyrphus* Dušek & Láška, 1967 and *Scaeva* Fabricius, 1805.

**Table 1.** The number of specimens used in the linear and geometric morphometrics analyses.

Species group	Species	Linear morphometric		Geometric morphometric	
		♂♂	♀♀	♂♂	♀♀
<i>aureus</i>	<i>Merodon aureus</i> Fabricius, 1805	20	19	37	19
	<i>Merodon calidus</i> Šašić, Ačanski & Vujić, 2020	20	20	37	28
	<i>Merodon ortus</i> Šašić, Ačanski & Vujić, 2020	9	–	9	–
<i>natans</i>	<i>Merodon calcaratus</i> (Fabricius, 1794)	13	21	13	21
	<i>Merodon natans</i> (Fabricius, 1794)	20	20	60	49
	<i>Merodon pulveris</i> Vujić & Radenković, 2011	20	20	63	70
<i>clavipes</i>	<i>Merodon clavipes</i> (Fabricius, 1781)	20	–	23	–
	<i>Merodon</i> aff. <i>clavipes</i>	10	–	10	–
<i>pruni</i>	<i>Merodon obscurus</i> Gil Collado, 1929	9	–	9	–
	<i>Merodon pruni</i> (Rossi, 1790)	20	–	45	–

To the best of our knowledge, the shape of the hoverfly R4+5 vein has not been quantified so far using either linear or geometric morphometrics. In previous publications, this character was used in keys to the level of genus or higher taxonomic categories, and a simple description of whether R4+5 is sinuate or not was used (Thompson & Skevington 2014; Thompson *et al.* 2017; Ramage *et al.* 2018; Speight 2020; Rego *et al.* 2022). However, its situation has never been used for species delimitation. Given that wings are highly heritable structures (Birdsall *et al.* 2000; Moraes *et al.* 2004) and their shape significantly contributes to species discrimination, we hypothesise that individual wing structures, such as the R4+5 vein, differ among species.

This paper aims to discover whether the R4+5 vein shape is useful in hoverfly species delimitation. To test our hypothesis, we selected valid cryptic species from four *Merodon* species groups for which already published wing shape and molecular results support their delimitation.

## Material and methods

A linear and geometric morphometric shape analysis of the R4+5 vein situation was carried out on cryptic and sibling species of four *Merodon* species groups from three lineages: *aureus* (*aureus* lineage), *clavipes* (*avidus-nigritarsis* lineage), *natans* (*natans* lineage) and *pruni* (*avidus-nigritarsis* lineage) (Table 1). We chose those species because their status was confirmed based on landmark-based wing shape and molecular analysis results (*aureus*: Vujić *et al.* 2020c; *natans*: Vujić *et al.* 2021c; *clavipes* and *pruni*: Vujić *et al.* in prep.). Hence, the semilandmark-based analysis was performed on the same dataset as the published wing landmark analysis for easier comparison.

## Wing preparation

The right wing of each specimen was dissected using microscissors under a Nikon SMZ18 stereo microscope, mounted on a microscopic slide using Hoyer's medium and covered with a cover glass. The

wings were photographed with a Nikon DS-Fi3 camera attached to a Nikon SMZ18 stereo microscope, labelled and archived with a unique code in the FSUNS database.

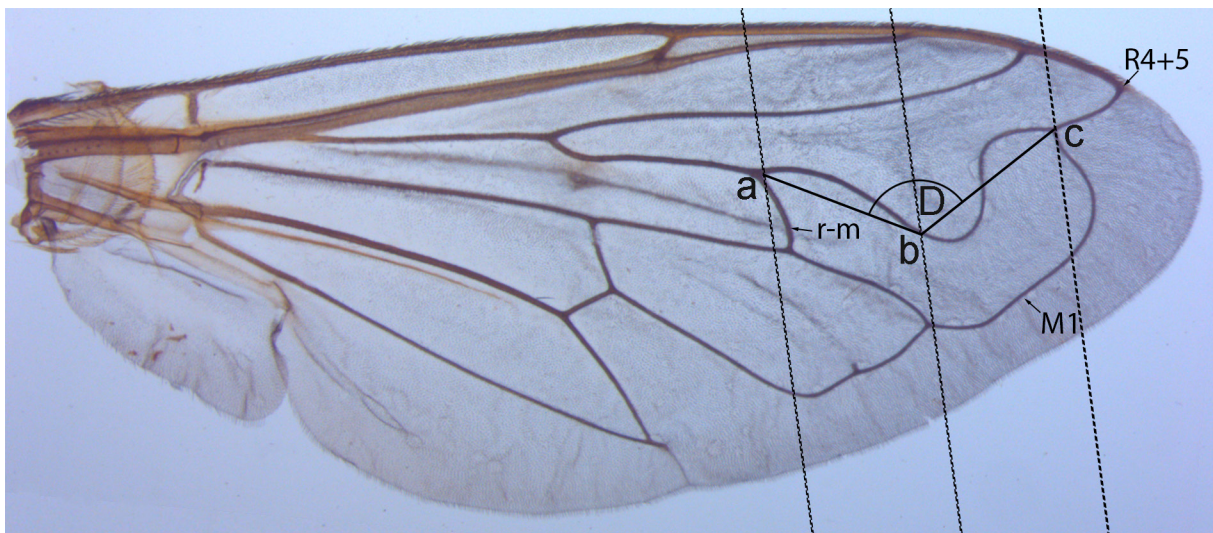
### Linear morphometrics

Up to 20 specimens of each species and sex were used for linear morphometric analysis (Table 1). Using IMP MakeFan8 software (Sheets 2012), a line (Fig. 1, marking b) was drawn in the middle between the intersections of R4+5 with r-m (Fig. 1, marking a) and R4+5 with M1 veins (Fig. 1, marking c), forming the angle named D. Angle D was measured using TpsDig ver. 2.05 software (Rohlf 2017). All data had a normal distribution (Kolmogorov-Smirnov test for normality). Differences among species were tested using ANOVA in Statistica for Windows ver. 14.

### Geometric morphometrics

For each species group, two separate analyses were conducted on the same dataset that was used for the wing shape analysis published in the following papers: *aureus*: Vujić *et al.* (2020c); *natans*: Vujić *et al.* (2021c); *clavipes* and *pruni*: Vujić *et al.* (in prep.). The first analysis included semilandmarks that quantify the shape of the R4+5 vein, while the second analysis represented a combination of semilandmarks and published landmark analysis. Both results were compared with published wing shape landmark results (Vujić *et al.* 2020c, 2021c, in prep.).

For the R4+5 vein shape quantification, 20 semilandmarks were digitised (from the intersection with r-m to the intersection with M1 veins) using the option “resample curve by length” in the TpsDig ver. 2.05 software (Fig. 2). The software CoordGen ver. 7.14 with an integrated Semiland module (Sheets 2012) was used for semilandmark superimposition using a distance-minimising protocol. This protocol minimised shape differences due to the arbitrary nature of semilandmark positions along the curve. In addition, a principal component analysis was carried out on the shape variables to reduce the dimensionality of the data set.



**Fig. 1.** *Merodon aureus* Fabricius, 1805, ♂, right wing with the character used in linear morphometric: a = intersection of R4+5 with r-m vein; b = intersection of R4+5 vein with a line drawn in the middle between a and c; c = the intersection of R4+5 with M1 vein; D = the angle formed by the lines that connect a, b and c.

To explore the R4+5 vein shape, as well as a combination of semilandmark and landmark wing shape variation among the species, we employed canonical variate (CVA) and stepwise discriminant function analysis (DA). Superimposed outline drawings produced in MorphoJ ver. 2.0 (Klingenberg 2011) were used to visualise differences in mean R4+5 vein shape among species. All statistical analyses were performed in Statistica for Windows ver. 14 (TIBCO Software Inc).

## Results

### *Merodon aureus* group

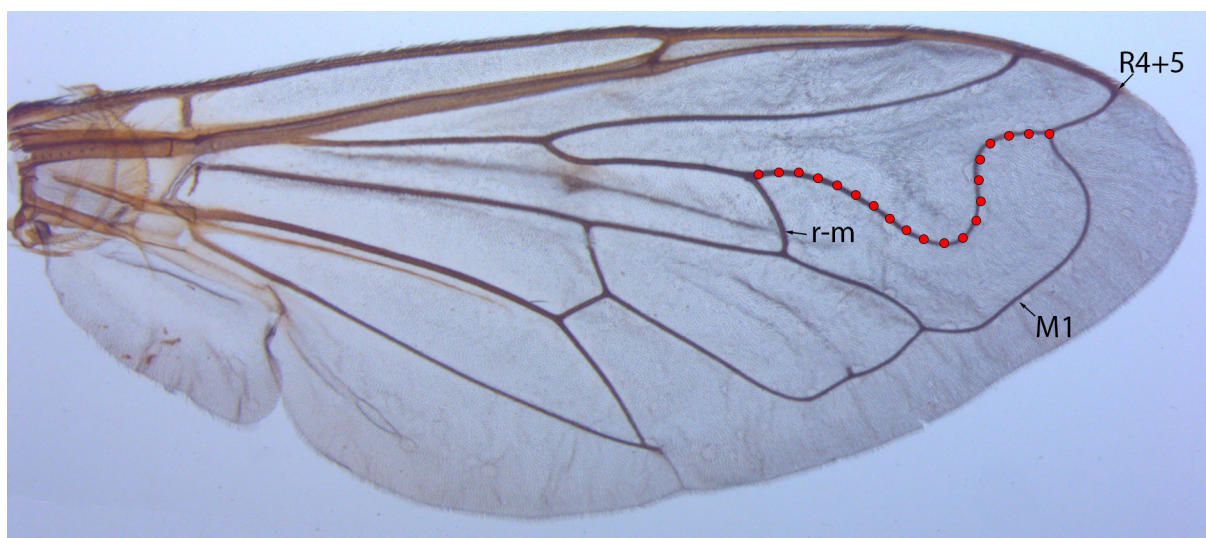
#### Linear morphometrics

ANOVA did not find significant differences between the sexes (ANOVA:  $F_{1,75} = 2.01$ ;  $P > 0.05$ ). However, highly significant differences between species were found (ANOVA:  $F_{1,75} = 38.09$ ;  $P < 0.01$ ). *Merodon ortus* Šašić Zorić, Ačanski & Vujić, 2020 had a larger angle at the intersection of the R4+5 vein and the middle line, indicating that *M. ortus* has a less sinuate R4+5 vein than *M. aureus* Fabricius, 1805 and *M. calidus* Vujić, Ačanski & Šašić, 2020 (Fig. 3).

#### Geometric morphometrics analysis of R4+5 vein shape

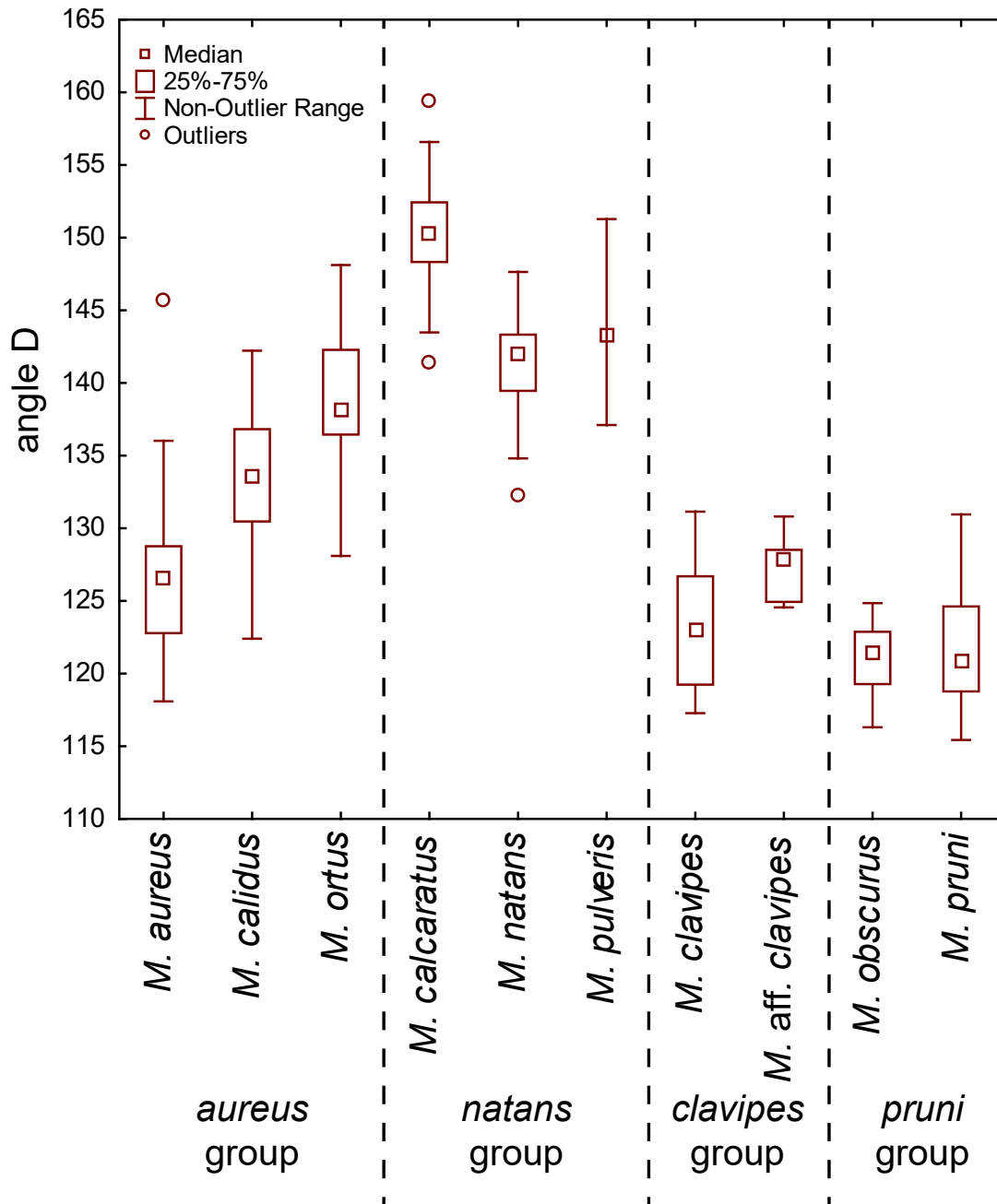
Due to sexual dimorphism, analyses were performed separately on males and females (ANOVA:  $F_{16,102} = 4.596$ ;  $P < 0.01$ ).

Regarding male specimens, DA showed that all species pairs differed highly significantly in R4+5 vein shape ( $P < 0.01$ ). Importantly, 89.16% of the male specimens were correctly classified into a priori defined groups. CVA produced two highly significant axes (CV1: Wilks' Lambda = 0.173;  $\chi^2 = 116.623$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.474;  $\chi^2 = 49.603$ ;  $P < 0.01$ ). CV1, with 61% of the total shape variation, separated *M. aureus* from *M. calidus*, whereas CV2, with 39% of the total shape variation, separated *M. ortus* from *M. aureus* and *M. calidus* (Fig. 4A). The superimposed outline drawings showed that *M. aureus* and *M. calidus* had the most similar R4+5 vein shape (Fig. 4D). *Merodon aureus* had the most sinuate R4+5 vein, whereas *M. ortus* had the least sinuate R4+5 vein (Fig. 4D).



**Fig. 2.** *Merodon aureus* Fabricius, 1805, ♂, right wing with the location of 20 semilandmarks selected for geometric morphometric analysis.

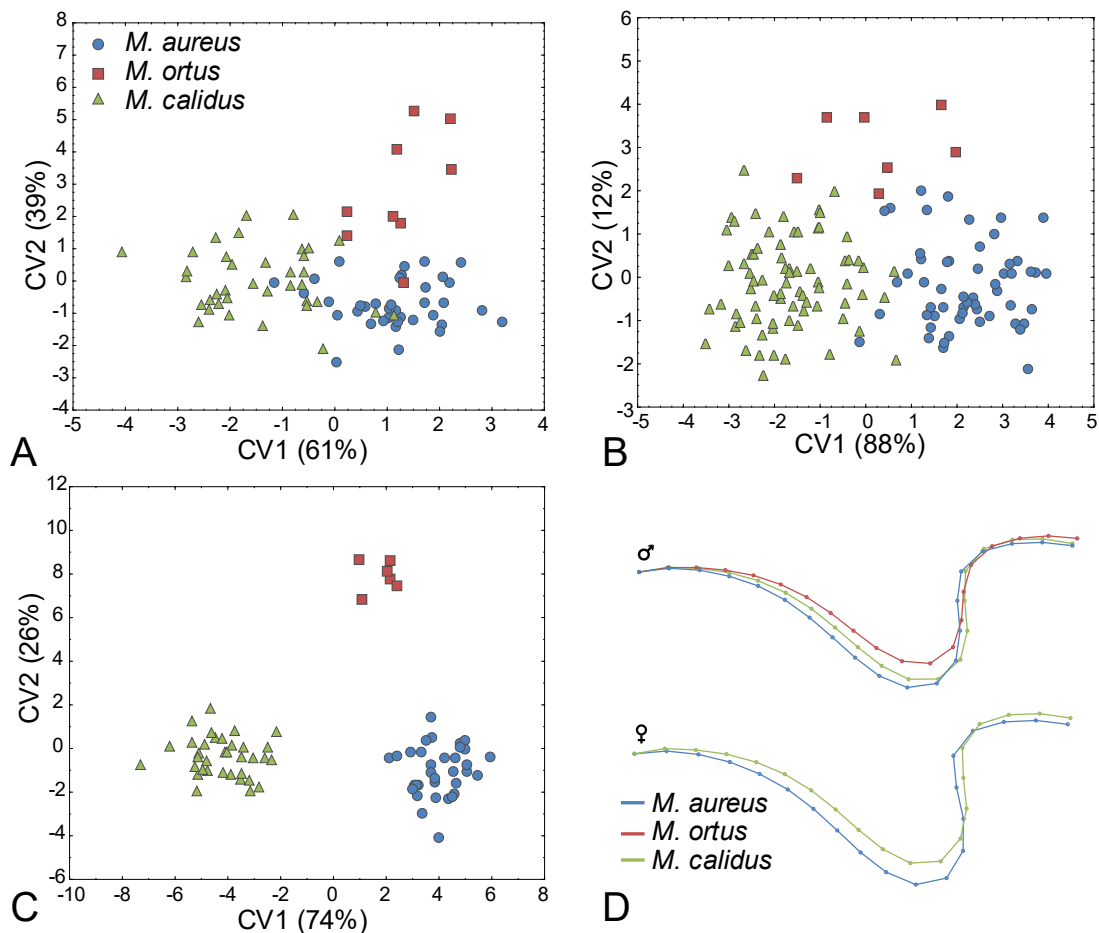
DA revealed that female specimens of *M. aureus* and *M. calidus* highly significantly differed in R4+5 vein shape ( $p < 0.01$ ). All female specimens were correctly classified into a priori defined groups based on the R4+5 vein shape. Additionally, CVA produced one highly significant axis (CV1: Wilks' Lambda = 0.101;  $\chi^2 = 71.195$ ;  $P < 0.01$ ). As in males, *M. aureus* had more sinuate R4+5 vein than *M. calidus* (Fig. 4D).



**Fig. 3.** Box plot showing a comparison of the angle at the intersection of the R4+5 vein and the middle line for all species used in the analysis.

### Combined geometric morphometrics analysis of R4+5 vein shape and wing shape

All male specimens were correctly classified into a priori defined groups. Additionally, all species pairs differed highly significantly using both semilandmarks and landmarks (DA:  $P < 0.01$ ). CVA produced two highly significant exes, which clearly separated all species in the space defined by these two axes (CV1: Wilks' Lambda = 0.0085;  $\chi^2 = 264.718$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.148;  $\chi^2 = 106.119$ ;  $P < 0.01$ ) (Fig. 4C). Regarding females, *M. aureus* and *M. calidus* highly significantly differed in the combined semilandmark and landmark analysis (DA:  $p < 0.01$ ), and all female specimens were correctly classified into a priori defined groups. CVA produced one highly significant axis (CV1: Wilks' Lambda = 0.011;  $\chi^2 = 102.98$ ;  $P < 0.01$ ).



**Fig. 4.** Results of the geometric morphometric wing shape analysis of species of the *Merodon aureus* complex. **A.** Scatter plot of individual scores showing R4+5 vein shape variability. **B.** Scatter plot of individual scores showing wing shape variability from Vujić *et al.* (2020c). **C.** Scatter plot of individual scores showing semilandmark R4+5 vein shape and landmark wing shape variability **D.** Superimposed outline drawings showing R4+5 vein shape differences among investigated species.

***Merodon natans* group**

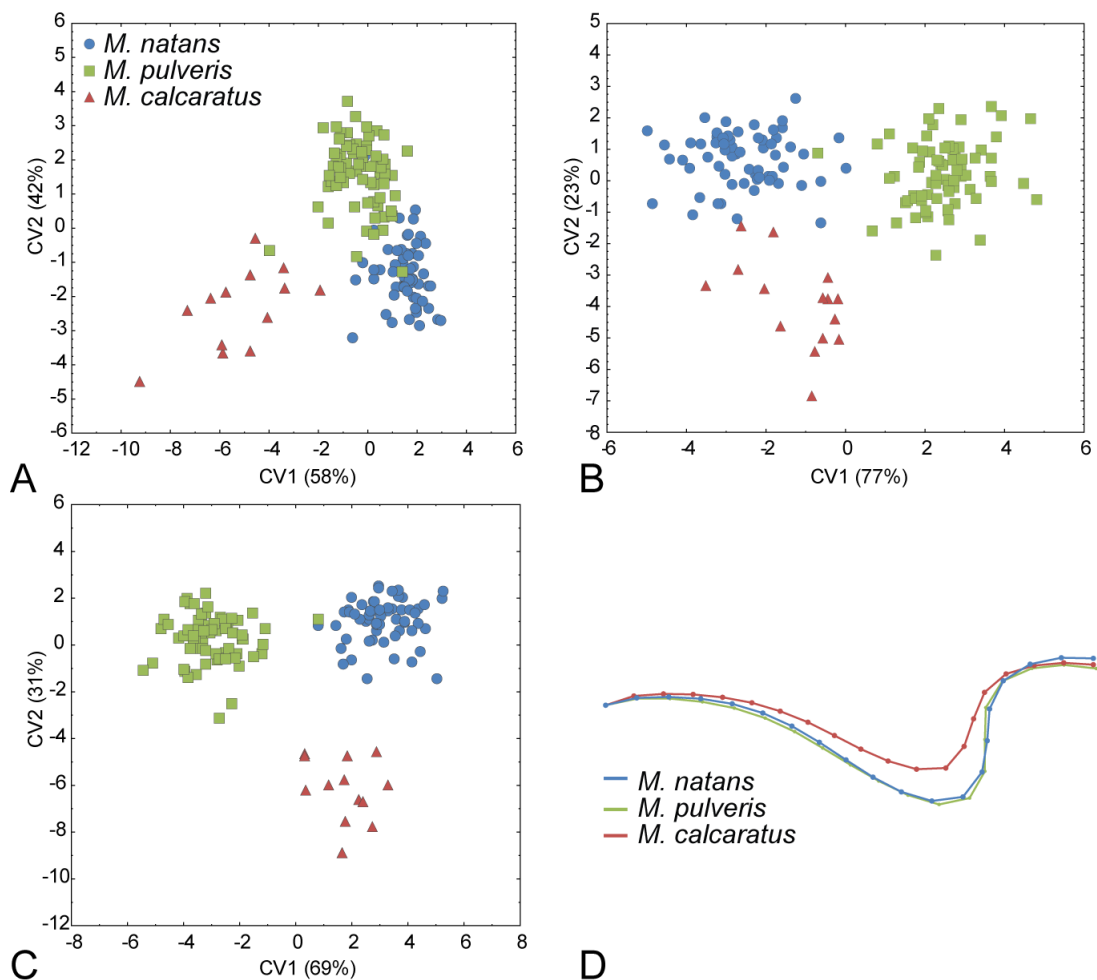
**Linear morphometrics**

ANOVA did not find significant differences between the sexes (ANOVA:  $F_{2,108} = 1.2$ ;  $P > 0.05$ ). However, highly significant differences between species were found (ANOVA:  $F_{2,111} = 72.1$ ;  $P < 0.01$ ). *Merodon calcaratus* (Fabricius, 1794) had a larger angle at the intersection of the R4+5 vein and the middle line, indicating that this species has a less sinuate R4+5 vein than other species of the *natans* group (Fig. 3).

**Geometric morphometrics analysis of R4+5 vein shape**

Due to sexual dimorphism, analyses were performed separately on males and females (ANOVA:  $F_{37,234} = 5.348$ ;  $P < 0.01$ ).

Regarding male specimens, DA clearly separated all species pairs based on R4+5 vein situation ( $P < 0.01$ ). 94.41% of all specimens were correctly classified into a priori defined groups. CVA

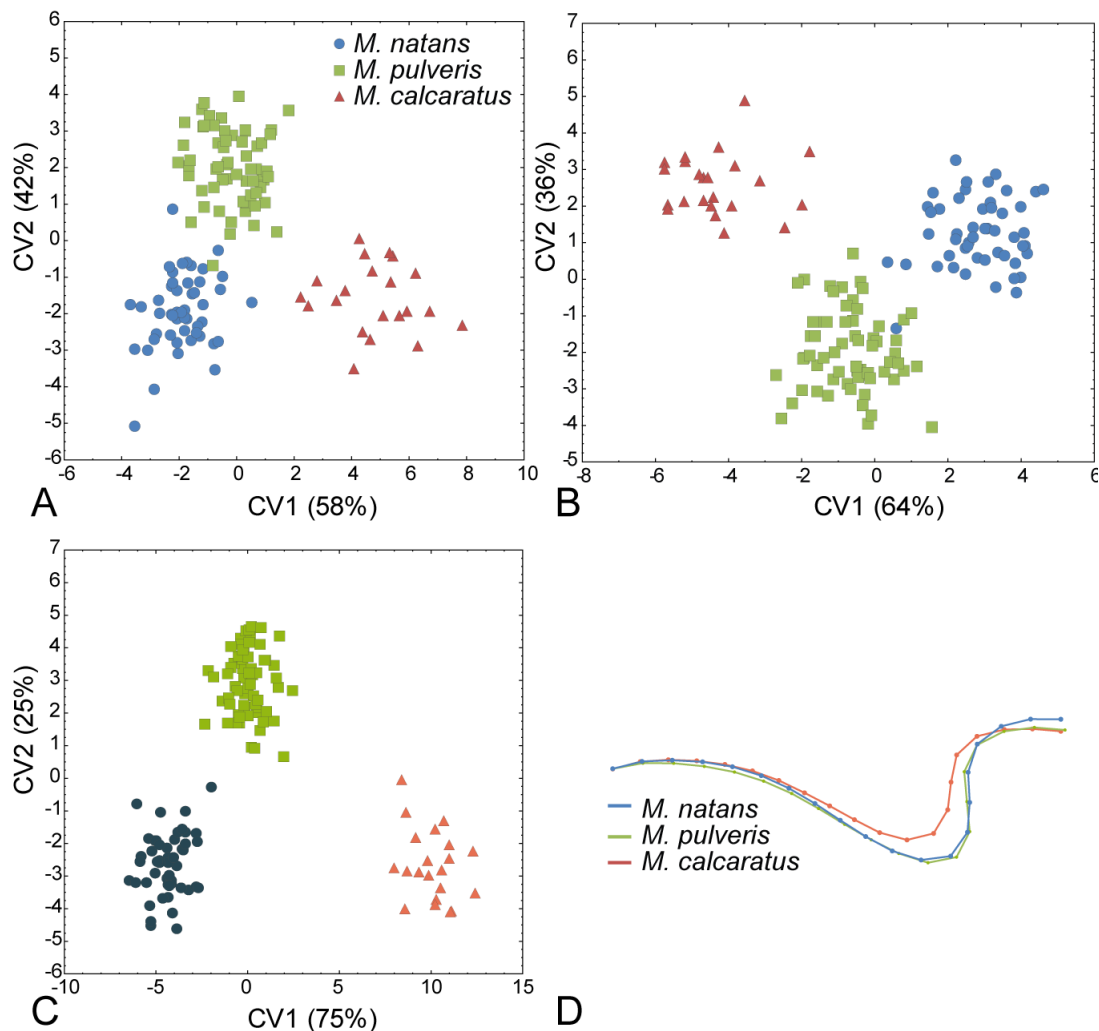


**Fig. 5.** Results of the geometric morphometric wing shape analysis of males of the *Merodon natans* group. **A.** Scatter plot of individual scores showing the R4+5 vein shape variability. **B.** Scatter plot of individual scores showing the wing shape variability from Vujić *et al.* (2021c). **C.** Scatter plot of individual scores showing the semilandmark R4+5 vein shape and landmark wing shape variability **D.** Superimposed outline drawings showing R4+5 vein shape differences among males of the investigated species.



produced two highly significant axes (CV1: Wilks' Lambda = 0.0625;  $\chi^2 = 338.211$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.282;  $\chi^2 = 154.55$ ;  $P < 0.01$ ). CV1, with 58% of the total shape variation, separated *M. calcaratus* from *M. natans* (Fabricius, 1794) and *M. pulveris* Vujić & Radenković, 2011, whereas CV2, with 42%, separated *M. natans* from *M. pulveris* (Fig 5A). Superimposed outline drawings indicated that *M. calcaratus* has the least sinuate R4+5 vein (Fig. 5D).

Females of all species pairs differed highly significantly by R4+5 vein shape (DA:  $P < 0.01$ ). Overall, classification success was high, 98.50% of all female specimens were correctly classified based on vein sinuation. All females of *M. calcaratus* were correctly classified. CVA produced two highly significant axes (CV1: Wilks' Lambda = 0.34;  $\chi^2 = 378.52$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.208;  $\chi^2 = 175.89$ ;  $P < 0.01$ ). As in males, the first axis clearly separated *M. calcaratus* from *M. natans* and *M. pulveris*, whereas CV2, with 42% of the total shape variation, separated *M. natans* from *M. pulveris* (Fig. 6A). As in males, females of *M. calcaratus* had the least sinuate R4+5 vein (Fig. 6 D).



**Fig. 6.** Results of the geometric morphometric wing shape analysis of females of the *Merodon natans* group. **A.** Scatter plot of individual scores showing the R4+5 vein shape variability. **B.** Scatter plot of individual scores showing wing shape variability from Vujić *et al.* (2021c). **C.** Scatter plot of individual scores showing the semilandmark R4+5 vein shape and landmark wing shape variability **D.** Superimposed outline drawings showing the R4+5 vein shape differences among females of the investigated species.

### Combined geometric morphometrics analysis of R4+5 vein shape and wing shape

Due to sexual dimorphism, analyses were performed separately on males and females (ANOVA:  $F_{60,211} = 7.93$ ;  $P < 0.01$ ).

Males of all species pairs differed highly significantly using both semilandmarks and landmarks (DA:  $P < 0.01$ ). Classification success was excellent, only one specimen out of 143 was misclassified (one *M. pulveris* as *M. natans*). CVA produced two highly significant axes (CV1: Wilks' Lambda = 0.019;  $\chi^2 = 505.313$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.199;  $\chi^2 = 208.199$ ;  $P < 0.01$ ). The first CV axis, with 69% of the total shape variation clearly separated *M. natans* from *M. pulveris*, whereas CV2, with the rest of the variability, clearly separated *M. calcaratus* from the other two species (Fig. 5C).

Females of all three species were correctly classified into a priori defined groups. All species pairs differed highly significantly (DA:  $P < 0.01$ ). CVA produced two highly significant axes (CV1: Wilks' Lambda = 0.0046;  $\chi^2 = 611.459$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.113;  $\chi^2 = 247.88$ ;  $P < 0.01$ ). Both CV axes clearly separated all species, the first CV1, with 75% of the total variation, separated *M. natans* from *M. calcaratus*, whereas CV2 separated *M. pulveris* from the other two species (Fig. 6C).

### *Merodon clavipes* and *pruni* groups

#### Linear morphometrics

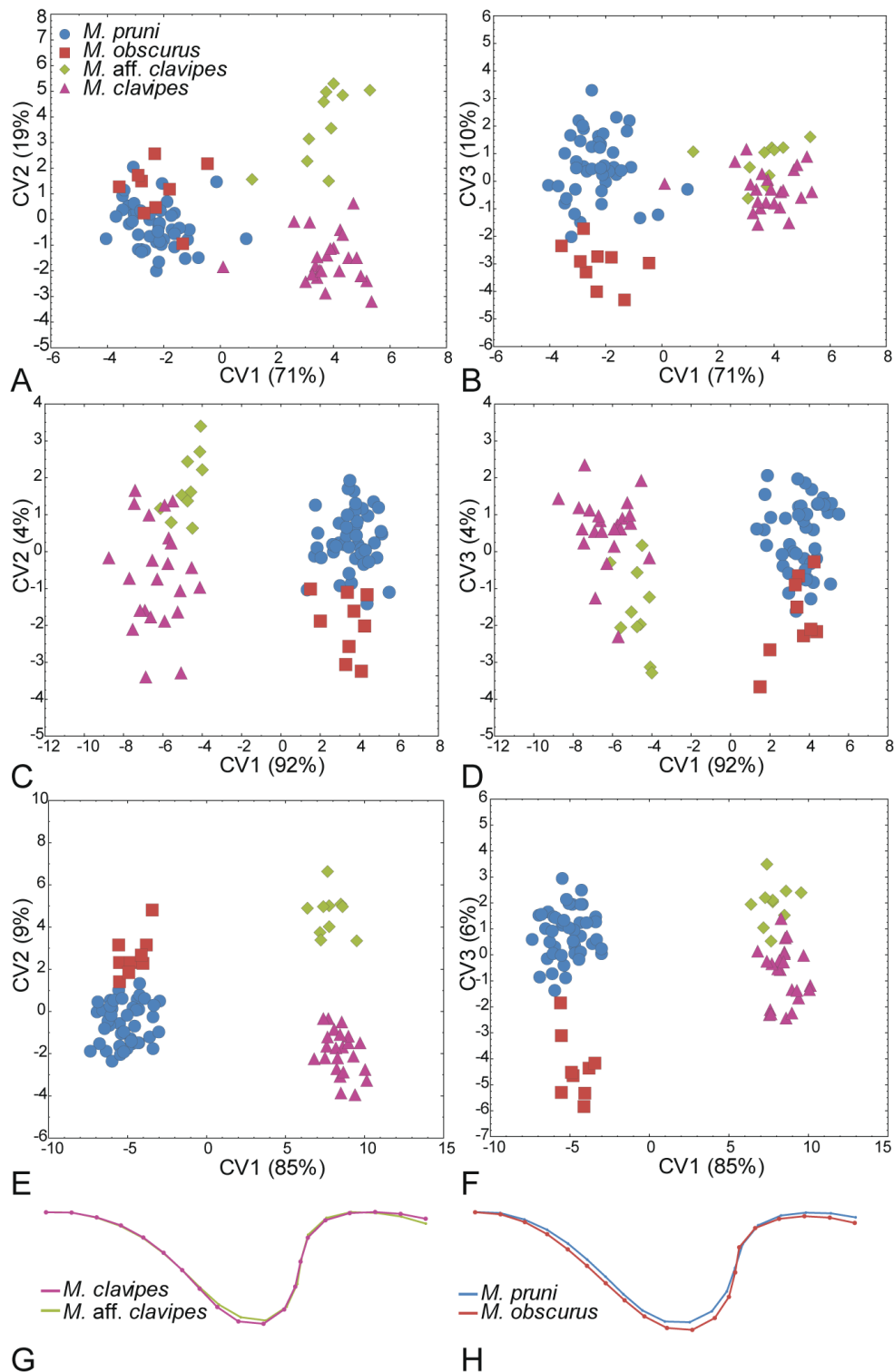
ANOVA did not find significant differences between *Merodon pruni* (Rossi, 1790) and *M. obscurus* Gil Collado, 1929 (ANOVA:  $F_{1,27} = 0.44$ ;  $P > 0.05$ ). However, there were highly significant differences between *M. clavipes* (Fabricius, 1781) and *M. aff. clavipes* (ANOVA:  $F_{1,29} = 8.40$ ;  $P < 0.01$ ). In addition, *Merodon aff. clavipes* had the largest angle at the intersection of the R4+5 vein and the middle line, indicating that *M. aff. clavipes* has the least sinuate R4+5 vein than other investigated species of these two groups (Fig. 3).

#### Geometric morphometrics analysis of R4+5 vein shape

All species pairs differed highly significantly based on R4+5 the vein shape (DA:  $P < 0.01$ ). Specimens were classified with overall a high classification success of 96.55% to a priori defined groups. CVA produced three highly significant axes (CV1: Wilks' Lambda = 0.0128;  $\chi^2 = 305.07$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.127;  $\chi^2 = 144.31$ ;  $P < 0.01$ ; CV3: Wilks' Lambda = 0.443;  $\chi^2 = 57.075$ ;  $P < 0.01$ ). CV1, with the majority of the shape variation (71%), separated the *clavipes* and *pruni* groups (Fig. 7A). CV2, with 19% of the total variation, separated *M. aff. clavipes* from *M. clavipes* (Fig. 7A), while CV3, with 10% of the total shape variation, separated *M. pruni* from *M. obscurus* (Fig. 7B). Differences in R4+5 vein sinuation between the *clavipes* and *pruni* groups are depicted on Fig. 7G–H. Although significant, differences between species inside species groups were subtle, especially in the *clavipes* group (Fig. 7G).

#### Combined geometric morphometrics analysis of R4+5 vein shape and wing shape

All male specimens were correctly classified to a priori defined groups, and all species pairs differed highly significantly using both semilandmarks and landmarks (DA:  $P < 0.01$ ). CVA produced three highly significant axes that describe the shape differences between species (CV1: Wilks' Lambda = 0.000995;  $\chi^2 = 463.155$ ;  $P < 0.01$ ; CV2: Wilks' Lambda = 0.0447;  $\chi^2 = 208.225$ ;  $P < 0.01$ ; CV3: Wilks' Lambda = 0.248;  $\chi^2 = 93.393$ ;  $P < 0.01$ ). CV1, with 85% of the total shape variation, clearly separated the *pruni* from the *clavipes* group (Fig. 7E). CV2, with 9% of the total shape variation, clearly separated *M. aff. clavipes* from *M. clavipes*, whereas CV3, with 6% of the total shape variation, clearly separated *M. pruni* from *M. obscurus* (Fig. 7F).



**Fig. 7.** Results of the geometric morphometric wing shape analysis of males of the *Merodon clavipes* and *pruni* groups. **A–B.** Scatter plot of individual scores showing the R4+5 vein shape variability. **C–D.** Scatter plot of individual scores showing the wing shape variability from Vujić *et al.* (in prep.). **E–F.** Scatter plot of individual scores showing the semilandmark R4+5 vein shape and landmark wing shape variability. **G–H.** Superimposed outline drawings showing the R4+5 vein shape differences between the males of the investigated species.

## Discussion

The wings of insects are the body parts most commonly subjected to geometric morphometric analyses (Tatsuta *et al.* 2018). They are suitable for geometric morphometric studies for several reasons. First, the wing shape is a highly heritable structure, meaning that its shape is mainly controlled by species-specific DNA (Birdsall *et al.* 2000; Moraes *et al.* 2004). They are two-dimensional structures with stable venation that allow us to choose a uniform position of landmarks on each wing unambiguously. Additionally, wing shape usage in taxonomy in various insect groups has confirmed its effectiveness in species separation (Hemiptera (Li *et al.* 2017; Gushki *et al.* 2018), Diptera (Šašić *et al.* 2016; de Souza *et al.* 2020; Sauer *et al.* 2020; Vujić *et al.* 2020a), Hymenoptera (Baracchi *et al.* 2011; Quezada-Euan *et al.* 2015).

In integrative hoverfly taxonomy, three characters have been used in geometric morphometrics: wing shape, surstyle shape (male genitalia) and larva posterior respiratory process shape (e.g., Ačanski *et al.* 2016; Radenković *et al.* 2018; Aracil *et al.* 2022). However, the majority of geometric morphometric hoverfly studies rely on wing shape. Several genera were studied, and the wing shape proved a reliable character for species delimitation in all cases (*Merodon*: Ačanski *et al.* 2016, 2022; Radenković *et al.* 2018; *Chrysotoxum* Meigen, 1803: Nedeljković *et al.* 2013, 2015; *Pipiza* Fallén, 1810: Vujić *et al.* 2013; *Eumerus* Meigen, 1822: Chroni *et al.* 2018; *Paragus* Latreille, 1804: Tot 2021). It is also important to emphasise that wing shape results were concordant with molecular results in all mentioned studies. All those studies were based on the entire wing shape, and it is an open question of whether the single-wing structure can contribute to hoverfly species delimitation.

Here, we demonstrate the importance of the R4+5 vein in hoverfly species delimitation. The main characteristic of the R4+5 vein is whether it sinuates – arcuates into an underlying cell or is straight. This feature mainly discriminates several genera of the subfamilies Eristalinae and Syrphinae. However, to the best of our knowledge, the R4+5 vein situation has not been used in species delimitation of Syrphidae.

Both linear and geometric morphometrics analyses showed that this character is valuable in species delimitation. As expected, linear morphometrics showed a weaker resolution compared to geometric morphometrics. That approach has failed to detect differences related to sexual dimorphism, as well as to detect differences between species *M. pruni* and *M. obscurus*. The latter can be related to a small sample size, or the differences between these two species are so discrete that linear morphometrics cannot detect them. However, all other species of the examined species groups were successfully separated based on angle D. Considering that this character succeeded in separating morphologically inseparable species, we are confident of its future usefulness in hoverfly taxonomy.

Contrary to linear morphometrics, geometric morphometric analysis detected differences in the R4+5 vein situation between all cryptic species, as well as between sexes, even between species with a smaller sample size like in the *pruni* and *clavipes* groups. These species groups consist of two species, nominal species and second one with redefined status or new to science. In the case of the *pruni* group, *M. obscurus* was redefined as a valid species, while in the case of the *clavipes* group, a new species, *Merodon* aff. *clavipes*, was recognized, and its description as a new species will be provided elsewhere (Vujić *et al.* in prep). A comparison of published results of landmark shape analysis for each analysed species group (Vujić *et al.* 2020c, 2021c, in prep.) and the results provided here show that the results are consistent. One vein shape is expected to have a much weaker resolution than the entire wing shape. However, results follow the resolution obtained based on the landmark-based wing shape. This is also supported by the classification rate, which is high in both the R4+5 vein and wing shape. For example, for the *M. aureus* group the overall percentage of correct classification for the R4+5 vein was slightly lower than in the wing shape analyses of males and higher in females than in Vujić *et al.* (2020c). Thus,

as with landmark wing shape results, R4+5 results were also in accordance with previously obtained molecular results (Vujić *et al.* 2020c, 2021c, in prep.).

The additional advantage of geometric morphometrics is that it provides superimposed outline drawings that depict the R4+5 vein shape differences between defined groups. We can see that the R4+5 vein situation differs between cryptic species, species groups and sexes. According to the analysed groups, we can conclude that within the genus *Merodon*, females have a more sinuated R4+5 vein than males.

Besides the R4+5 vein situation as a single morphometric character, we tested the significance and resolution of species separation using a combination of two characters, the R4+5 vein and the landmark-defined wing shape. As expected, the combined R4+5 vein and wing shape provided a more detailed insight into the shape differences. Here, all species were clearly separated in CVA space, and the overall classification rates were higher than in separate character analyses. All specimens, except one male of *M. pulveris* classified as *M. natans*, were correctly classified. These results follow the conclusion of Chaiphongpachara & Laojun (2019), where combining landmarks with semilandmarks identifies species of *Culex* Linnaeus, 1758 better than just landmarks.

The disadvantage of combined landmarks and semilandmarks can be a higher number of variables, especially in a smaller sample size, as in the case of some species of the *pruni* and *clavipes* groups. However, this can be addressed using dimensionality shape reduction. One of the solutions is the usage of a subset of independent principal components that describe the highest overall classification percentage calculated in a backward stepwise discriminant analysis. Here, all variables are included in the model, and at each step, the variable that contributes the least to the prediction of group members will be eliminated. Thus, only variables in the model that contribute the most to the discrimination between groups will be included in the analysis (TIBCO Statistica).

In conclusion, the results of this work highlight the R4+5 vein as a valuable character in cryptic hoverfly species delimitation. It can be beneficial as a single character or in combination with a landmark-based wing shape analysis. Besides the genus *Merodon*, this character can also be used in other genera where the situation of this vein is present. Additionally, the finding that R4+5 vein situation is distinct even in cryptic species raises new questions about what other veins may be useful in species separation.

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