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Differentiation of two *Bathyplectes* species, *B. anurus* and *B. curculionis*, parasitoids of the Alfalfa weevil (*Hypera postica*) in Spain

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Research Paper

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Abstract

The alfalfa weevil *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) is one of the most destructive alfalfa pests in the world, resulting in substantial economic losses. However, the amount of damage can be reduced by larval parasitoids of the genus *Bathyplectes* Förster (Hymenoptera: Ichneumonidae) as a conservation biological control strategy. Parasitoids are currently identified by morphological body characteristics, cocoon morphology, and/or DNA analysis, but geometric morphometrics (GM) applied to the wing vein arrangement may also reveal differences between specimens. We distinguished 61 *B. anurus* (Thomson) and 41 *B. curculionis* (Thomson) specimens, based on the appearance of the cocoon. GM revealed statistically significant differences in wing vein patterns and fore wing shapes between species, but not between sexes within the same species. The 1 M + 1R1 cell, also known as the horsehead cell, was revealed to be an easy and reliable morphological character for species differentiation. Despite the New World literature, this is the first European report providing a visual method to differentiate *B. anurus* from *B. curculionis*. This study highlights the importance of precise species identification methods, such as geometric morphometry. It can contribute to a better implementation of biological control strategies against the alfalfa weevil in Spain and other Mediterranean countries.

Introduction

The alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae), a pest of Eurasian origin, causes significant damage to alfalfa crops globally (Goosey, 2012; Harcourt and Guppy, 1984; Hoff *et al*, 2002; Pons and Nuñez, 2020; Saeidi and Moharramipour, 2017; Soroka *et al*, 2019). *Hypera postica* larvae are parasitised by solitary endoparasitoid wasps of several species from the genus *Bathyplectes* Förster (Hymenoptera: Ichneumonidae) (Kuhar *et al*, 1999; Yu *et al*, 2016). Despite their known association with *H. postica*, limited information exists regarding these parasitoids' biology, phenology, population dynamics and biocontrol potential in European and Mediterranean conditions.

Among the eight *Bathyplectes* species recorded in Spain (Ribes, 2012), only *B. anurus* (Thomson) and *B. curculionis* (Thomson) are associated with alfalfa. Recent studies in the Ebro Basin, Spain, revealed fluctuating abundance and parasitism rates for both species across years (Levi-Mourao *et al*, 2021b, 2021, 2022a, 2022b; Pons and Nuñez, 2020). However, it is noteworthy that other *Bathyplectes* species, such as *B. infernalis* (Gravenhorst) and *B. stenostigma* (Thomson), are also reported in the literature as parasitoids of *H. postica* in both Europe and North America (Chamberlin, 1926; Dysart and Coles, 1971; Horstmann, 1974; Kingsley *et al*, 1993; Radcliffe and Flanders, 1998; Ribes, 2012; Soroka *et al*, 2020).

Accurate identification of *Bathyplectes* parasitoids is essential for successful biological control programs but remains challenging, particularly for cryptic taxa (Levi-Mourao *et al*, 2022a; Pons and Nuñez, 2020). *Bathyplectes* species can be distinguished by ovipositor length, in the case of females, and following other morphological traits, such as the shape of the areolar area of the propodeum, have also been proposed as diagnostic (Horstmann, 1974; Soroka *et al*, 2020), although their applicability is often limited due to variability and interpretation difficulty. Cocoons provide diagnostic features: *B. anurus* cocoons

are dark brown, with a narrow raised yellowish band and exhibit jumping behaviour, unlike the light brown cocoons of *B. curculionis* with a flat, diffuse yellowish band (Brunson and Coles, 1968; Chamberlin, 1926; Day, 1970; Dysart and Day, 1976; Fisher *et al*, 1961). However, morphological variations in cocoons can lead to misidentification (Moore, 2014; Soroka *et al*, 2020).

Specific molecular primers for the COI barcoding region have been developed for *B. anurus* and *B. curculionis*, enabling the identification of morphologically similar parasitoids within *H. postica* larvae (Levi-Mourao *et al*, 2022a). However, DNA-based methods are limited by their cost and the requirement of sequences existing in the DNA libraries. The landmark-based geometric morphometrics method (GM) offers an alternative, enabling species differentiation based on the shape and size of the morphological structure (Bookstein, 1991). GM is widely applied in resolving taxonomic and evolutionary issues (e.g., Mitrovski-Bogdanović *et al*, 2021; Žikić *et al*, 2017), and assessing morphological changes conditioned by external factors, for example, in toxicological tests using the insecticides, essential oils, or food colouring additives (Cvetković *et al*, 2020, 2024; Lazarević *et al*, 2019; Žikić *et al*, 2024).

This study evaluates the use of GM to distinguish *B. anurus* and *B. curculionis* based on fore wing morphology, complementing traditional methods, such as cocoon characteristics. We also assess fore wing size and shape for potential sexual dimorphism. An illustrated guide is provided to support the accurate and efficient identification of these parasitoids, enhancing their application in biological control programs.

Materials and methods

Collection and rearing of host larvae

Larvae of *H. postica* were collected by sweeping 180° with a 38-cm diameter net across several alfalfa fields in the Ebro Basin from March to May 2019 and 2020. Field-collected larvae were reared in 500 ml polyethylene cages (maximum 50 larvae per cage), covered with mesh for aeration. Fresh alfalfa shoots were provided daily. Cages were maintained in a climatic chamber set to 22°C, with an 8:16 (L:D) photoperiod and 50% relative humidity until pupation. Approximately 2,500 larvae were collected in 2019, 12% of which were parasitised by *Bathyplectes* species. In 2020, approximately 3,000 larvae were collected; however, a substantial proportion were killed by a field epizootic of the entomopathogenic fungus *Zoophthora phytonomi* (Arthur) (Zygomycetes: Entomophthorales).

Parasitoid identification

First, parasitoid adults were identified based on the morphological characteristics of their cocoons, following the descriptions by Day (1970), and Dysart and Day (1976). Female specimens were distinguished from males by the presence of a conspicuous ovipositor sheath (Soroka *et al*, 2020). Each specimen was labelled according to species and sex. Specimens of *B. anurus* and *B. curculionis* were frozen at –80 °C for subsequent wing excision.

Wing preparation

The right fore wing was carefully removed from each specimen, including both males and females, using fine entomological forceps under a Leica MZ125 stereoscopic microscope (Heinnenburg, Germany). The excised wings were mounted on microscope slides pre-coated with Beadle's medium. To ensure a flat, two-dimensional position, each slide was pressed with a microscope cover slip and allowed to air-dry for 15 days at room temperature. To maintain consistent pressure during the drying process, slides were placed between layers of polythene and cardboard, secured with two spring clips. This method ensured the wings were flattened evenly without causing damage. After drying, the wings were photographed using a Moticam Pro S5Plus digital camera (MoticEurope, Barcelona, Spain) connected to the stereoscopic microscope at 10× magnification. For reference, wing veins and cells are labelled in fig. 1 to clarify the comments regarding the fore wing venation.

Geometric morphometrics

The GM technique was used to examine potential differences in the shape of the fore wings of the two parasitoid species based on the wing venation pattern. To apply the GM method, 21 landmarks were precisely positioned on each fore wing. These landmarks were thoughtfully selected to accurately depict specific places on the wing (fig. 2), including the intersections of veins and the endpoints of veins that reach the wing margin. In addition to the landmarks, the 1 M + 1R1 cell, was outlined by a boundary defined by four curves. Landmarks and curves were digitised using StereoMorph software version 1.6.7 (Olsen and Westneat, 2015). The wing veins and cells nomenclature follows (Bennett *et al*, 2019).

Statistical analysis

To compare the wing shapes of the two parasitoid species, generalized Procrustes analysis (GPA) was performed to align the wing images, eliminating variation in scale, position, and orientation. This process resulted in a matrix of shape coordinates, known as Procrustes coordinates (Dryden and Mardia, 1998; Rohlf and Slice, 1990; Walker and Naylor, 2000; Zelditch *et al*, 2012). As a result of GPA analysis, the centroid size (CS), a geometric measure of size was also obtained. Shape variation was analysed and visualised through the principal component analysis (PCA). To assess statistical differences in fore wing size and shape the variate analysis of variance (ANOVA) and the multivariate analysis of variance (MANOVA) were used to examine differences between species, between sexes, and between sexes within each species. All statistical analyses were done in Geomorph version 4.0.5. (Adams *et al*, 2023) and gmShiny (Baken *et al*, 2021).

Results

Specimen identification

Out of 5,500 *H. postica* larvae collected in the field (see M&M), 850 reached the pupal stage. From these, 102 *Bathyplectes* cocoons were collected, and all 102 adults successfully emerged from pupae. Based on the morphology of the cocoons, 61 individuals were identified as *B. anurus* and 41 as *B. curculionis*.

Discrimination of fore wing shape between the two bathyplectes species

A comprehensive set of 102 specimens, comprising 27 females and 28 males of *B. anurus*, along with 21 females and 26 males

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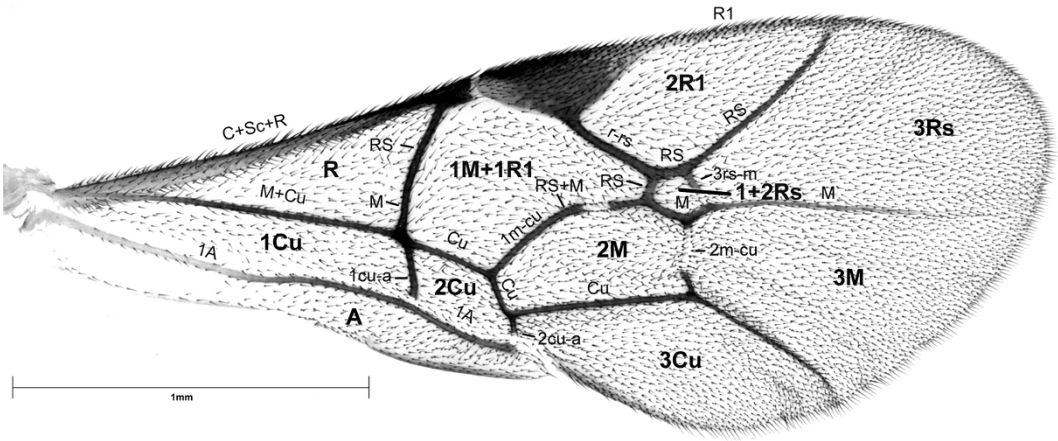

Figure 1. Nomenclature of fore wing venation and cells in female *Bathyplectes curculionis* (Bennett *et al.*, 2019).

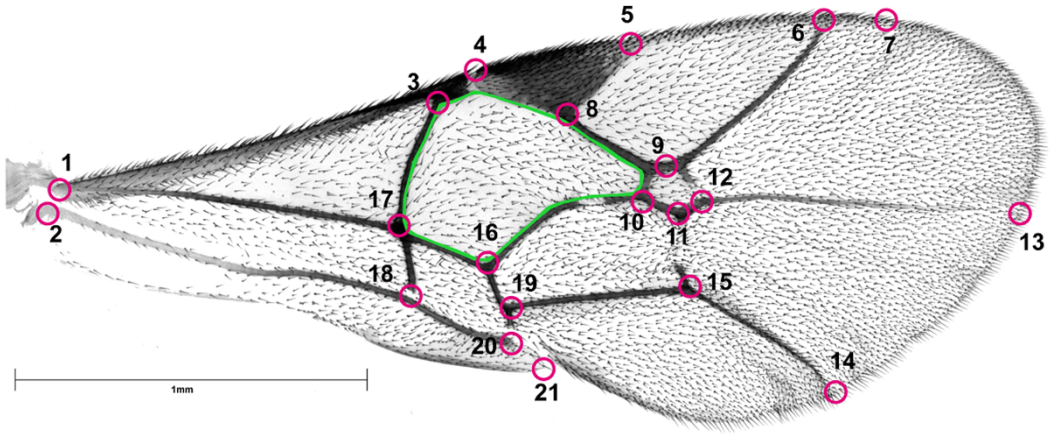

Figure 2. Position of landmarks and curves on the right fore wing of a female *Bathyplectes curculionis*. Landmark placement on the wings follows studies analysing wing venation in other hymenopterans (e.g., Mitrovski-Bogdanović *et al.*, 2021; Žikić *et al.*, 2017).

Table 1. The MANOVA: the effects of categorical variables (species and sex) on fore wing shape. Abbreviations: df – degrees of freedom, SS – sums of squares, MS – mean square, rsq – the proportion of variation in shape explained by each categorical variable, f and z – effect size

	Df	SS	MS	Rsq	F	Z	*P*
Species	1	0.026288	0.026288	0.2179	27.7359	4.8712	0.001
Sex	1	0.000641	0.000641	0.00531	0.6762	−0.7667	0.772
Species × Sex	1	0.000829	0.000829	0.00687	0.8751	−0.0517	0.518
Residuals	99	0.092884	0.000948	0.76991			
Total	102	0.120642					

of *B. curculionis*, were employed for wing shape analysis through GM. The analysis of variance (ANOVA) revealed no significant differences in wing size either between the analysed species or between the sexes within each species. The statistical analysis conducted to assess wing shape (MANOVA) revealed significant differences between the two *Bathyplectes* species. However, no significant differences were observed between the sexes within each species (table 1). The results of geometric morphometrics of fore wings are shown in the morphospace defined by the first two principal components (PC1 × PC2). These axes cumulatively describe 46.53% of the total wing shape variability (fig. 3). Along with PC1, a clear separation between the two species is evident. Both females and males of *B. anurus* are positioned in the positive part of the PC1 axis (fig. 3). The fore wings of this species are slightly narrower and more elongated compared to those of *B. curculionis*. Notably, the 1 M + 1R1 cell, outlined by four curves (referring to fig. 1), appears more elongated. Also, the 2R1 cell is wider, particularly in the distal part; the 2 M cell is more elongated, and the 2Cu cell is slightly shorter and wider in *B. anurus*. Conversely, *B. curculionis* presents wider fore wings, with a distinct characteristic being the short and wide 1 M + 1R1 cell (in voluntary horse head cell). Additionally, all other features exhibit an opposite trend to those described for *B. anurus*. Notably, no discernible differences were identified between the

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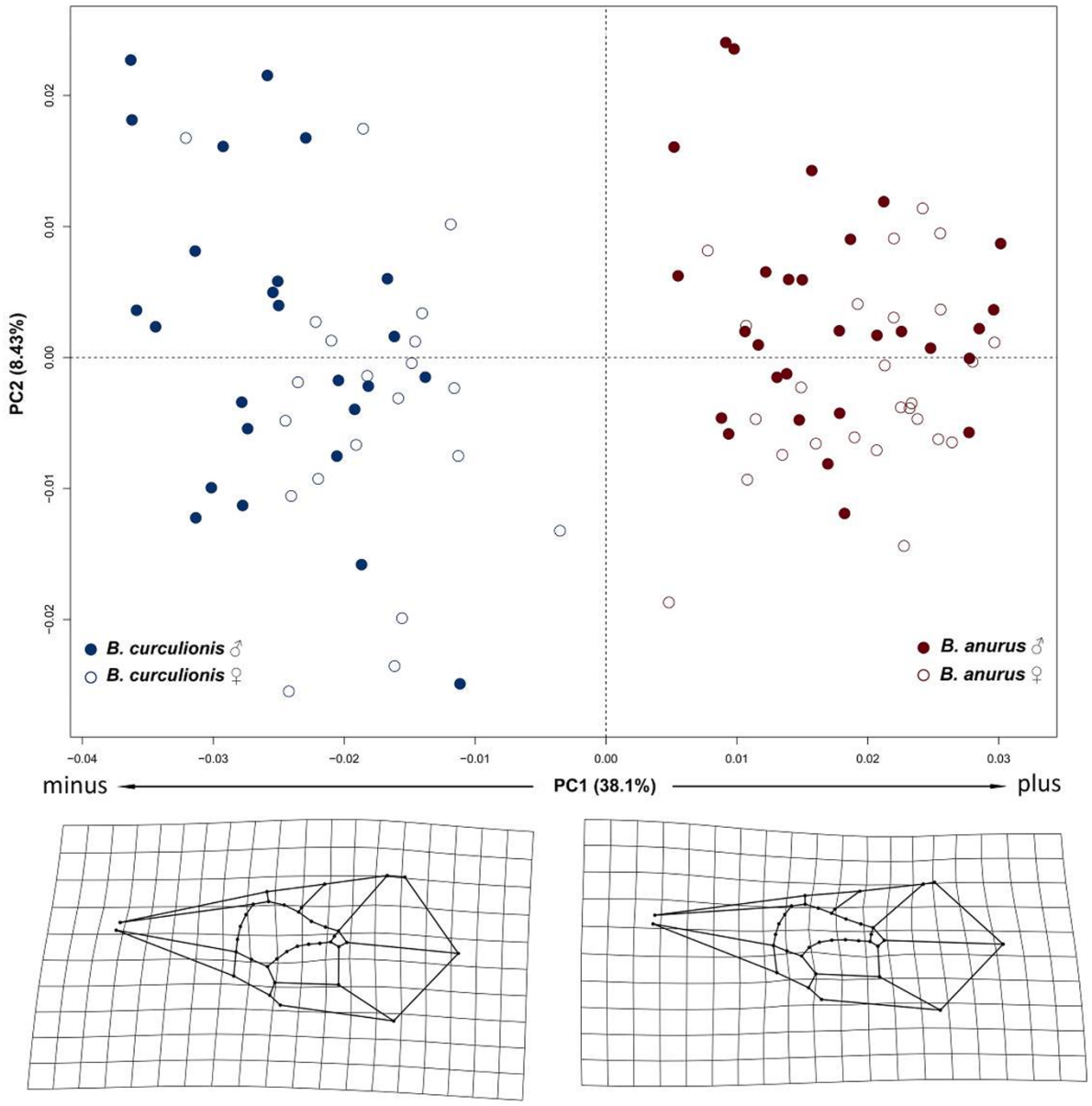

Figure 3. Differentiation of the two *Bathyplectes* species, within the morphospace, defined by PC1 and PC2. Red represents *B. anurus*, blue represents *B. curculionis*; filled circles indicate males, and outlined circles indicate females. The transformation grids illustrate the change in fore wing shape associated with the maximum and minimum values along the PC1 axis.

sexes or within each species when considering these morphometric parameters.

Morphological comparison of two Bathyplectes species

Upon observing the results derived from the GM analysis of the fore wings, there arose a necessity for a comparative presentation of selected morphological structures of *B. anurus* and *B. curculionis*. This was undertaken to streamline the identification process for these two species, primarily relying on conspicuous differences in wing venation and the arrangement of wing cells. A comparative illustrated guide to the identification of *B. anurus* and *B. curculionis* is given in figs. 4 and 5.

Focusing on body colouration (fig. 4), *B. anurus* individuals were observed to be black with light brown details on the legs and metasoma ventrally, while *B. curculionis* exhibited bright yellow details in those areas. Notably, the yellow longitudinal pattern on the hind tibia was particularly prominent in both sexes of *B. curculionis*. This colouration pattern is present in most of *B. anurus* individuals, although few individuals presented also black tibiae with an inconspicuous, pale brown pattern.

Identification of both female *Bathyplectes* species can be made with higher certainty when both are present in a sample, based on the length of the ovipositor and ovipositor sheath. The ovipositor of *B. curculionis* is longer than that of *B. anurus*. In cases where identification based on the ovipositor is not possible due to specimen deformation or improper mounting, a lateral view of the specimens reveals that the distal height of the metasoma in *B. curculionis* is almost twice as long as that in *B. anurus* (fig. 5A). In *B. anurus*, the ovipositor and sheath never extend beyond the tip of the metasoma. Additionally, another character for distinguishing these two species is the propodeal areolar (fig. 5B). When clearly visible,

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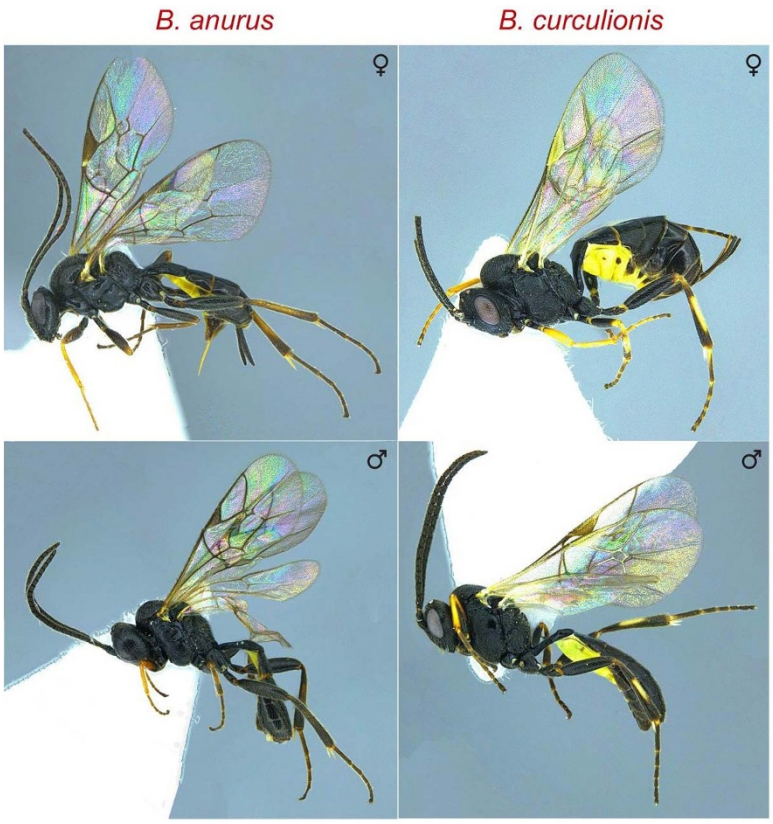

Figure 4. General habitus of males and females of two *Bathyplectes* species.

the area of this character in *B. anurus* is approximately twice as wide at its base – as measured by the distance between the origins of the dorsal propodeal carinae – as in *B. curculionis*, although this structure is sometimes poorly defined, making direct comparison difficult in certain specimens.

Although distinguishing the two *Bathyplectes* species based on the vein pattern on the fore wings can be challenging for the untrained eye, we have highlighted a few consistent differences, focusing on the most significant features. To facilitate following our observations, we have marked the selected structures in fig. 5C: two cells are labelled with Roman numerals, and three veins are labelled with Arabic numerals. For the nomenclature of wing veins and cells, refer to fig. 1. In *B. anurus*, the 1 M + 1R1 (I), also known as the horsehead cell, is narrower compared to *B. curculionis*. This narrowing is influenced by the lengthening of veins 2 (r-rs) and 3 (1 m-cu and Rs + M). Additionally, in *B. anurus*, vein 2 is smoothly curved, while in *B. curculionis*, it is slightly kinked in the middle. Moreover, in *B. anurus*, vein 1 (Rs and M) is more curved. The elongation of cell II (2 M) is also influenced by the elongation of vein 2.

The cocoons of *B. anurus* are relatively squat, with a height/width ratio of 1.4, whereas those of *B. curculionis* are more elongated, with a ratio of 1.9. Notably, the cocoons of *B. curculionis* exhibited significant variability in both size and proportions. Furthermore, although the width of the central belt of the cocoon is highly variable, it is consistently broader than that observed in *B. anurus* (fig. 5D).

Discussion

Accurate and easy species identification is essential for the success of biological control programs, particularly when working with parasitoids, as highlighted in previous studies (Huber *et al*, 2021; Moraes, 1987; Rosen, 1989). Female parasitoids are generally more informative than males due to their pronounced genital structures, especially the ovipositor, which serves as a key diagnostic feature. Consequently, many identification keys for Ichneumonoidea are based on female morphology. In most cases, species identification can be reliably achieved through adult morphological assessment or examination of cocoons.

However, identification becomes challenging when dealing with morphologically similar species or species complexes. In this study, we focused on distinguishing two *Bathyplectes* species parasitizing the same host, *H. postica*. Traditional identification methods, such as those based on cocoon characteristics and adult morphology, including the diagnostic key used by Soroka *et al* (2020), serve as a critical foundation. Nonetheless, in our case, initial identifications based on cocoon traits led to misclassification, as 61 specimens were initially identified as *B. anurus* and 41 as *B. curculionis*, whereas geometric morphometrics revealed 55 and 47, respectively. This highlights the need of complementary tools for accurate species identification.

An additional trait used in *Bathyplectes* spp. identification is pupae jumping behaviour, a characteristic typical of the Campopleginae subfamily. However, only *B. anurus* exhibits this behaviour. Day (1970) showed that this trait can sometimes be absent if the parasitoid larvae are dead or in the pre-pupal stage, which can lead to errors in identification.

While the original descriptions by Thomson (1883) are available, there is limited information on the adult morphology of *Bathyplectes* species, which are known to closely resemble one another (Horstmann, 1974; Moore, 2014; Soroka *et al*, 2020). The ovipositor sheath length is the primary distinguished trait for females of *B. anurus* and *B. curculionis* species discrimination

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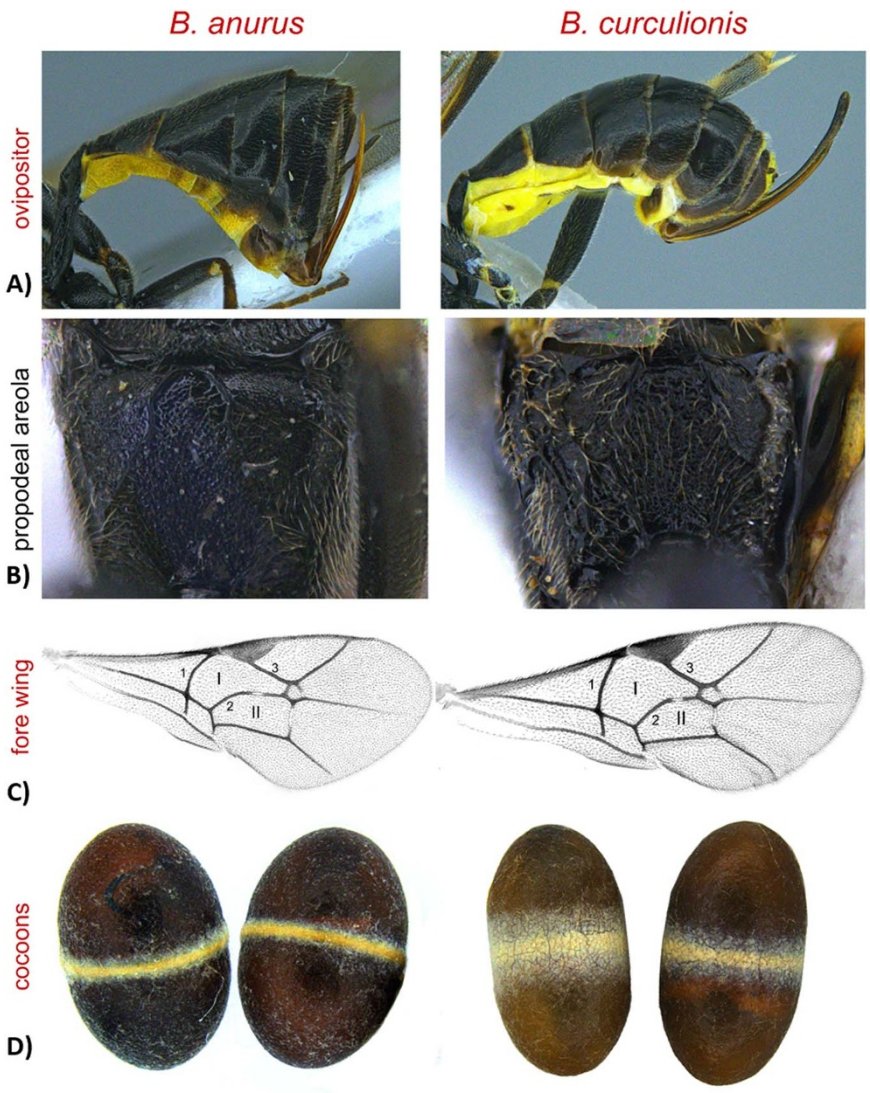

Figure 5. The most important morphological characters of *bathyplectes* species for quick identification: (A) ovipositor length, (B) shape of propodeal areolar area, (C) fore wing shape, and (D) cocoon morphology.

(Horstmann, 1974; Soroka *et al*, 2020). This character is associated with host larval instar preference, as it facilitates the parasitism of early instars hidden in unfolded leaves and buds, a behaviour previously described for *B. curculionis* in several studies (Barney et al., 1978; Bartell and Pass, 1980; Dowell and Horn, 1977; Duodu and Davis, 1974; Levi-Mourao *et al*, 2022a). Differences in host-stage preferences and search patterns create partial temporal and spatial refuges that allow multiple parasitoid species to utilise a single host species (Dowell and Horn, 1977; Harcourt and Guppy, 1991). The temporal separation between *Bathyplectes* species can be attributed to the timing of adult flight. In the Ebro Basin, where *B. anurus* and *B. curculionis* occur sympatrically, the peak flight activity of *B. anurus* occurs earlier in the season than that of *B. curculionis* (Levi-Mourao *et al*, 2021; Pons and Nuñez, 2020).

Distinguishing the shape of the areolar area on the propodeum can sometimes be challenging. Although it is a highly reliable character, the propodeal carina is weakly developed in some individuals and high variability was found between individuals; therefore, if the specimen is a male, other morphological characters must be examined. Additionally, the presence of pits on the clypeus can aid in species discrimination (Horstmann, 1974; Soroka *et al*, 2020). However, these traits require some expertise and a trained eye.

Conventional molecular barcoding methods, such as COI sequence analysis, are commonly used for species identification, but they are costly, labour-intensive and occasionally encounter challenges during DNA isolation (King et al., 2008; Levi-Mourao *et al*, 2022a). Molecular analyses previously conducted on individuals from the same population using COI primers showed high specificity (Levi-Mourao *et al*, 2022a), which supports, though indirectly, the reliability of the species identification in this study.

In contrast, alternative methods, such as GM, have not been previously explored for *Bathyplectes* species. In this study, we demonstrated that the analysis of the fore wing morphology via GM offers a reliable and cost-effective alternative for adult identification, especially when both species are available for comparison. By integrating GM with other methods, as in previous studies (Petrović *et al*, 2015, 2019), we successfully differentiated between *B. anurus* and *B. curculionis* based on fore wing shape. Our results show that wing shape differed significantly between the two species, with *B. curculionis* having wider fore wings. A key characteristic distinguishing the two species is the 1 M + 1R1 cell, also known as the 'horsehead' cell, which is narrower in *B. anurus* than

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in *B. curculionis*. This finding aligns with previous reports, which noted that *B. curculionis* has wider wings than *B. anurus* (Jervis *et al*, 2003), although without specifying the distinct appearance of the horsehead cell. Differences in wing morphology between species may reflect niche specialisation and host selection strategies that minimise interspecific competition and interspecific mating (Dowell and Horn, 1977; Harcourt, 1990). Studies on host selection and handling have shown that *B. anurus* is faster in host handling, while *B. curculionis* achieves greater dispersal rates (Dowell and Horn, 1977; Harcourt, 1990). It should be noted that in many individuals, the difference in the horsehead cell can be observed even without preparing the wings or applying GM.

Sexual dimorphism is common among insects and other animals (Hopkins and Kopp, 2021). Koinobiont endoparasitoids such as *Bathyplectes* spp. show less pronounced sexual dimorphism (Gross, 1993; Quicke, 2014), with males and females often being similar in size, shape, and colour (Jervis *et al*, 2003; Soroka *et al*, 2020). Our findings corroborate this, as we observed intraspecific overlap in wing morphology between the sexes in both species, suggesting similar allometry, a characteristics shared by some ichneumonid parasitoids (Gauld and Fitton, 1987).

In the analysed, randomly selected sample, cell 1 M + 1R1 was identified as the most reliable morphological character for distinguishing the species, applicable to both sexes. In conclusion, this study underscores the importance of precise species identification methods, such as GM, in distinguishing closely related parasitoid species. This is vital for effective biological control strategies and could contribute to the sustainable production of alfalfa in Spain and potentially other Mediterranean countries.

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Competing interests. The authors have no relevant financial or non-financial interests to disclose.

Data availability. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request. Specimens have been deposited in the entomological collection of the Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona).

Ethical approval. This article does not contain any studies involving human participants or animals other than insects.

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