

## An integrated approach to delimiting species borders in the genus *Chrysotoxum* Meigen, 1803 (Diptera: Syrphidae), with description of two new species

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### Abstract

Integrative taxonomy tests the validity of taxa using methods additional to traditional morphology. The existence of two different morphotypes in specimens identified as *Chrysotoxum vernale* Loew (Diptera: Syrphidae) prompted their taxonomic study using an integrative approach that included morphology, wing and male-surstylus geometric morphometrics, genetic and ecological analyses. As a result, a new species is recognised, *Chrysotoxum montanum* Nedeljković & Vujić sp. nov., and *C. vernale* is re-defined. A lectotype and paralectotypes are designated for *C. vernale* to stabilize this concept. An additional species, *Chrysotoxum orthostylum* Vujić sp. nov., with distinctive male genitalia is also described. The three species share an antenna with the basoflagellomere shorter than the scape plus pedicel and terga with yellow fasciae not reaching the lateral margins. This study confirms the value of integrative approach for resolving species boundaries.

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### Introduction

Worldwide, syrphids (Diptera: Syrphidae) play important ecological roles in nature. Adults are usually conspicuous flies that feed on pollen and nectar from a wide range of plants in numerous habitats (Rotheray and Gilbert, 2011). In Europe, syrphids are one of the better studied Diptera families. However, the taxonomy of certain groups is still provisional and uncertain, such as the genus *Chrysotoxum* Meigen, 1803 (Speight, 2013).

*Chrysotoxum* species are present in all bio-geographic regions except for Australasia. In the Palaearctic region 71 species are recorded, 24 of which are present in Europe (Vockeroth, 1969; Violovitsh, 1974; Peck, 1988; Nedeljković *et al.*, 2013). These wasp-mimics can be separated from other syrphid genera by the yellow and black body colouration (yellow maculae in thoracic pleura and yellow fasciae in abdominal terga), the very long antenna and the convex, margined abdomen (Thompson and Rotheray, 1998). *Chrysotoxum* adults are found in many habitat types (Speight, 2013) and their poorly known larvae are associated with ants and/or root aphids (Rotheray, 1994).

The taxonomy of certain *Chrysotoxum* species has proven difficult due to subtle morphological differences (Nedeljković *et al.*, 2013) and high levels of intra-specific variability (Sang-Wook and Ho-Yeon, 2013). In these circumstances, integrative taxonomy is useful for testing species concepts. Integrative taxonomy is a multidisciplinary approach to taxonomy that aims to delimit the units of life's diversity from different and complementary perspectives (*e.g.* comparative

morphology, population genetics, ecology, behaviour) (Dayrat, 2005). Many species have been described among syrphids using an integrated approach (*e.g.* Marcos-García *et al.*, 2011; Radenković *et al.*, 2011; Skevington and Thompson, 2012; Locke and Skevington, 2013; Vujić *et al.*, 2013; Haarto and Ståhls, 2014). To find genetic differences between similar species, analyses of both mitochondrial (*e.g.* COI) and nuclear (*e.g.* ITS2) genes have been used (*e.g.* Masetti *et al.*, 2006; Vujić *et al.*, 2012, 2013). In other Diptera families such as Drosophilidae wing shape is highly dependent on genetic inheritance and therefore minimally affected by temperature (Birdsall *et al.*, 2000), altitude, and season (Bitner-Mathé and Klaczko, 1999). Wing morphometry has been used in integrative taxonomic studies of syrphids (*e.g.* Ludoški *et al.*, 2008; Francuski *et al.*, 2009, 2011; Vujić *et al.*, 2013; Nedeljković *et al.*, 2013). Male-genitalia morphometry has also been used (Nedeljković *et al.*, 2013). Ecology plays an important role in diversification processes and ecological analyses have proven useful in supporting species concepts (Bond and Stockman, 2008; Nosil, Harmon and Seehausen, 2009; Wooten and Gibbs, 2012; Zhu *et al.*, 2013). Funk *et al.* (2006) reported a highly significant positive association between ecological divergence and reproductive isolation across taxa. Although ecological analyses have not been used in integrative taxonomy of Syrphidae, unpublished data suggest they could be important (Ačanski J, Miličić M, Likov L, Radenković S, Vujić A, unpubl. data).

A preliminary examination of named specimens of *Chrysotoxum vernale* Loew, 1841 resulted in the identification of two different morphotypes according to the colour of long pile on thoracic scutum of males: yellow (morphotype A) and black (morphotype B) (Nedeljković, 2011). In addition, a new *Chrysotoxum* taxon sharing with morphotypes A and B an antenna with basoflagellomere shorter than scape and pedicel together and abdominal terga with yellow fasciae not reaching the lateral margins was recognized. The main objective of the present study is to test using an integrated approach, whether these morphotypes correspond to different species.

## Material and methods

### Material examined

A total of 400 *Chrysotoxum* specimens were examined. They were collected in Austria, Bosnia & Herze-

govina, Croatia, Finland, FYR Macedonia, Germany, Greece, Italy, Kyrgyzstan, Montenegro, Poland, Serbia and Turkey, by various collectors, from 1860 to 2013. The following abbreviations are used for where specimens are deposited: FSUNS, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Serbia; MZH, Zoological Museum, Finnish Museum of Natural History, Helsinki, Finland; NHMW, Naturhistorisches Museum, Wien, Austria; ZMHB, Zoologisches Museum of the Humboldt University, Berlin, Germany; RMNH, Naturalis Biodiversity Center, Leiden, The Netherlands; DD, Dieter Doczkal's and AS, Axel Ssymank's private collection, Germany. In addition, type material was also examined in the collection at Zoologisches Museum of the Humboldt University, Berlin (ZMHB) and Museo Zoologico La Specola, Firenze, Italy (LSF). Material examined is detailed in Table S1 (Supplementary information). Only type material is detailed in the text.

### Morphology

To describe and diagnose species, characters were studied using a Ceti® binocular microscope. Colour characters always refer to dry specimens. Body size was measured as the length ('L') from the tip of the frontal prominence (excluding antenna) to the tip of the abdomen. Proportional length of the antennal segments is given as a ratio ('r') of x: y: z ('x', scape; 'y', pedicel; 'z', basoflagellomere). Measurements were made using an eye-piece micrometer. Morphological terms follow Thompson (1999).

### Genetic analysis

Two or three legs of each specimen were used for total genomic DNA extraction from 25 *Chrysotoxum* dry specimens (Table S2), following Chen *et al.* (2010). Material analysed included 22 specimens of the *C. vernale* A, two specimens of the *C. vernale* B and one of third *Chrysotoxum* species. DNA voucher specimens are deposited in FSUNS.

The 5' region of cytochrome c oxidase I gene (COI) was amplified using forward primer LCO (5'-GCT-CAACAAATCATAAAGATATTGG-3') and reverse primer HCO (5'-TAAACTTCAGGGTGACCAAAA AATCA-3') (Folmer *et al.*, 1994). The universally conserved primers were used for amplifying and sequencing the 3' region of COI: forward primer C1-J-2183 (5'-CAACATTTATTTTGATTTTTTGG-3') (alias JERRY) and reverse primer TL2-N-3014 (5'-TC-

CAATGCACTAATCTGCCATATTA-3') (alias PAT) (Simon *et al.*, 1994).

PCR reactions were carried out in 25  $\mu$ l reactions containing: approx. 50 ng of DNA, 2 pmoles of each primer, 0.2  $\mu$ l of DNA polymerase (5U/  $\mu$ l), 2.5 mM MgCl<sub>2</sub>, 2.5  $\mu$ l 10 $\times$  Taq Buffer, 0.1 mM of each nucleotide and ultra-pure water. Thermocycler conditions were initial denaturing at 95°C for 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s annealing at 49°C, 2 min extension at 72°C, followed by a final extension of 8 min at 72°C.

Expected size of amplification products was confirmed with a standard 1.5% agarose gel electrophoresis. The remaining product was purified using Exonuclease I and Shrimp Alkaline Phosphatase enzymes following the manufacturer's instructions (Thermo Scientific, USA). All sequencing reactions were performed using the Big Dye Terminator Kit v3.1. (Applied Biosystems, USA) following the manufacturer's protocol; sequences were generated on ABI 3730xl DNA Analyzer and deposited in GenBank (accession numbers: KR019039- KR019063).

Sequences were aligned using the ClustalW algorithm (Thompson *et al.*, 1994) as implemented in BioEdit 7.0.9.0 (Hall, 1999), with final adjustments performed manually. The data set containing 5' region COI sequences had final length 542bp, and the final length of dataset containing 3' region COI sequences was 625bp. The final dataset with 25 mtDNA COI sequences was created by merging sequence information of 3' and 5' COI regions. Phylogenetic analysis for all species was performed using Maximum Likelihood (ML) method, and a tree was created in MEGA 6.0 (Tamura *et al.*, 2013). The Maximum Likelihood method applied is based on the Tamura-3 parameter model (T92+ $\gamma$ ;  $\gamma=0.05$ ) of nucleotide substitution as defined in MEGA 6.0 (Tamura *et al.*, 2013). Statistical support of internal nodes was calculated with 1000 bootstrap repetitions. *Eumerus flavitarsis* Zetterstedt, 1843 (AY212782) was used as an outgroup taxon. Median-joining (MJ) network (Bandelt *et al.*, 1999) was constructed using the software Network 4.6.1.1 (available at <http://www.fluxus-engineering.com/sharenet.htm>). The network was constructed using equal weights for all the mutations and setting parameter  $\epsilon$  to zero in order to restrict the choice of feasible links in final network.

### Geometric morphometry

Morphometric analyses were used to characterise all taxa. High-resolution pictures of wings and surstyli

were made using a Leica DFC320 video camera attached to a Leica MZ16 stereomicroscope. A video camera was connected to a PC computer in which the software to make pictures was installed. Landmarks (wings) and semi-landmarks (surstyli) were drawn on every picture using TpsDig 2.05 software (Rohlf, 2006). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to test differences in wing centroid size between sexes and taxa. Wing centroid size is the square root of the sum of the squared distances between the centre of the wing and each landmark (Zelditch *et al.*, 2004). Multivariate analysis of variance (MANOVA), canonical variate analysis (CVA) and discriminant function analysis (DA) were used to test differences in wing and surstylus shape between sexes and taxa. Statistical analyses were made using Statistica® for Windows (StatSoft, 2012). For males and females of morphotypes A and B analyses were carried out separately.

Variation of wing size and shape was studied in 249 specimens of all three taxa following Bookstein (1991). The right wing of each specimen was taken off by means of micro-scissors and then mounted in Hoyer's medium (Anderson, 1954) on a microscopic slide. Wings are archived and labelled using unique codes shown in Table S1 (Supplementary information). Sixteen homologous landmarks were chosen at vein intersections and terminations throughout the wing; landmarks were selected in positions of the wing that could be easily recognisable at any time (Fig. 1). To reduce the statistical errors due to the low number of specimens (Arnqvist and Mårtensson, 1998) wings of *Chrysotoxum orthostylum* Vujić sp. nov., were digitized 10 times. Generalised least squares Procrustes superimposition (GLS) was used to minimise non-shape variations in location, scale and orientation of wings, and also to superimpose the wings in a common coordinate system (Rohlf and Slice, 1990; Zelditch *et al.*, 2004). GLS, wing centroid size and partial-warp scores were computed using CoordGen7.14 and CVAgen7.14a, which are part of IMP package (Sheets, 2012). MorphoJ v2.0 was used to visualize the thin-plate spline deformation (Klingenberg, 2011).

For surstyli, outline shape was studied. Surstyli of 51 *Chrysotoxum* males were analyzed: 21 of morphotype A and 30 of morphotype B. The right surstylus was taken off using pins. Surstyli were mounted in Hoyer's medium on a microscopic slide and immobilized with a cover slip. In the absence of clearly-identifiable, homologous, anatomical loci, 30 semi-landmarks were generated (Fig. 2). For each specimen

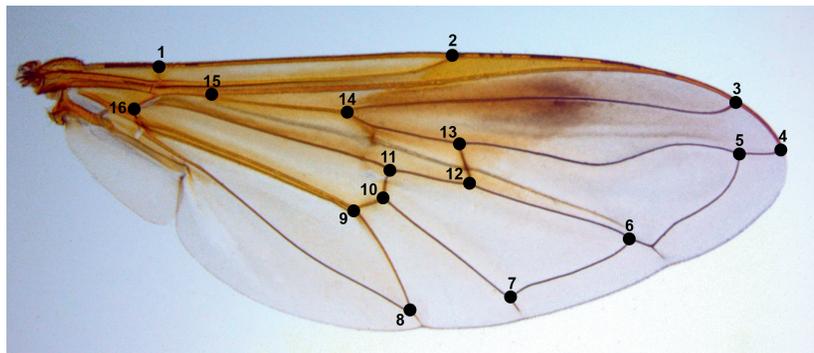


Fig. 1. Location of the 16 analysed landmarks on the right wing in the *Chrysotoxum vernale*.

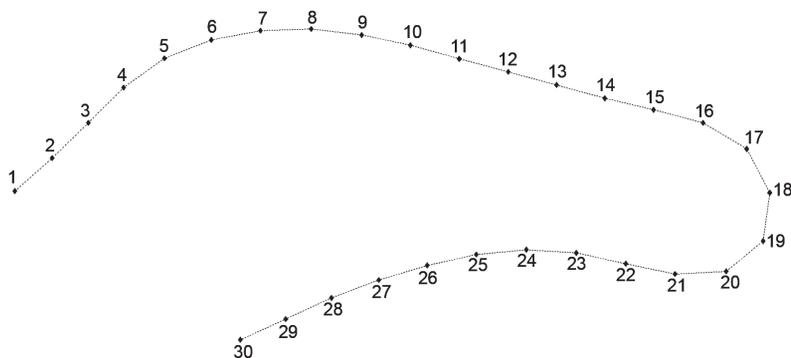


Fig. 2. Location of the 30 analysed semi-landmarks on the right surstylus of the male genitalia in the *Chrysotoxum vernale*.

semi-landmarks were drawn twice to reduce statistical error. To superimpose semi-landmarks, CoordGen7.14 and an integrated Semiland module were used following a distance-minimising protocol to minimize the shape differences due to the arbitrary nature of semi-landmark positions along the curve (Bookstein, 1997; Zelditch *et al.*, 2004).

#### Niche analysis

This analysis was carried out for 183 specimens of morphotype A and 121 of morphotype B from Croatia, Finland, FYR Macedonia, Germany, Greece, Montenegro, Poland and Serbia. Localities with geographic coordinates were used without modification. Records with only locality names were assigned coordinates using Google Earth (Google Inc, 2013), which was also used to confirm the validity of the localities with coordinates. All localities were represented in DivaGis (v7.5) (Hijmans *et al.*, 2012). A total of 19 Bioclim variables plus altitude (2.5 arc-minutes resolution) were generated for each locality on the basis of the WoldClim data set (Hijmans *et al.*, 2005). Climatic profiles obtained for the two morphotypes were analysed using Principal Component

Analysis (PCA). PCA was carried out applying a normal varimax rotation of factor loadings. Only factors with an eigenvalue greater than one were considered significant. Variables with a factor loading greater than 0.8 were interpreted as significantly correlated with the correspondent factor. ANOVA and Fisher LSD post hoc test were carried out to compare the derived factor scores between the two morphotypes. A scatter plot of PCA score values was used to graphically display position of these morphotypes in environmental space.

## Results

### Integrative taxonomy

Morphological differences between the two identified morphotypes of *C. vernale* (A and B *sensu* Nedeljković, 2011) were small and further analyses were required to test whether they were separate taxa. To find additional differences between morphotypes A (typical *C. vernale*) and B (*C. montanum* sp. nov.), DNA, the morphometry of wings and surstyli, and ecological niches were analysed.

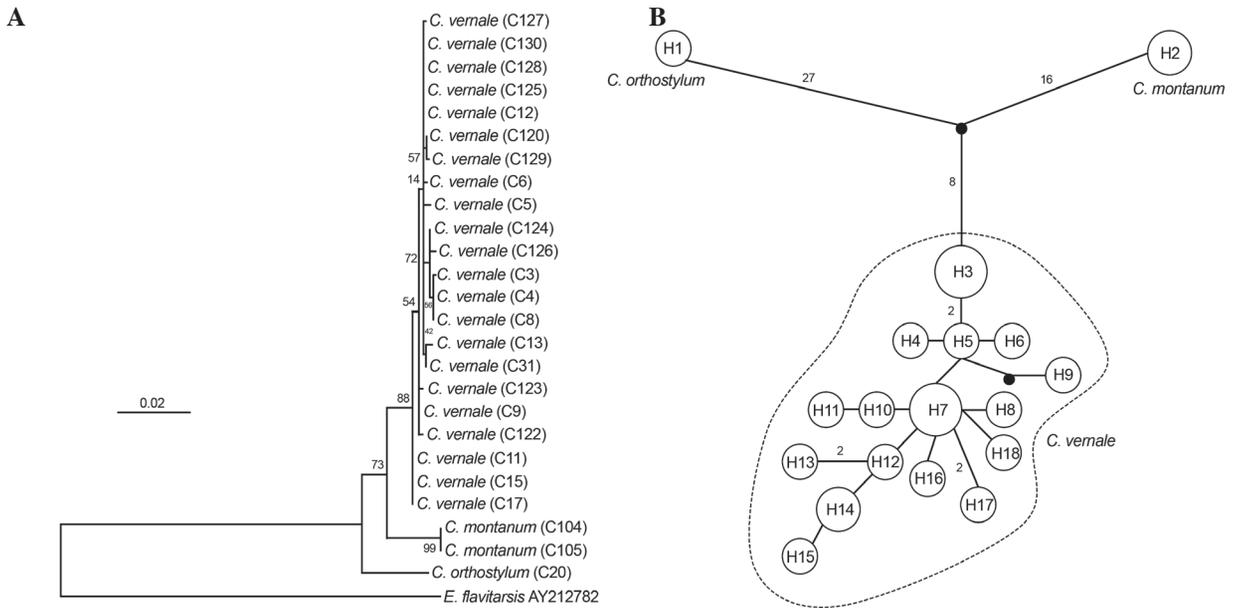


Fig. 3. Tree and network based on the merged 5' and 3' sequences of mtDNA COI. A, Maximum Likelihood (ML) tree [Bootstrap values are indicated at the nodes (1000 bootstrap replicates); outgroup, *Eumerus flavitarsis*]; B, Median Joining (MJ) network [Circle size corresponds to the haplotype frequency; numbers indicate the quantity of mutational steps].

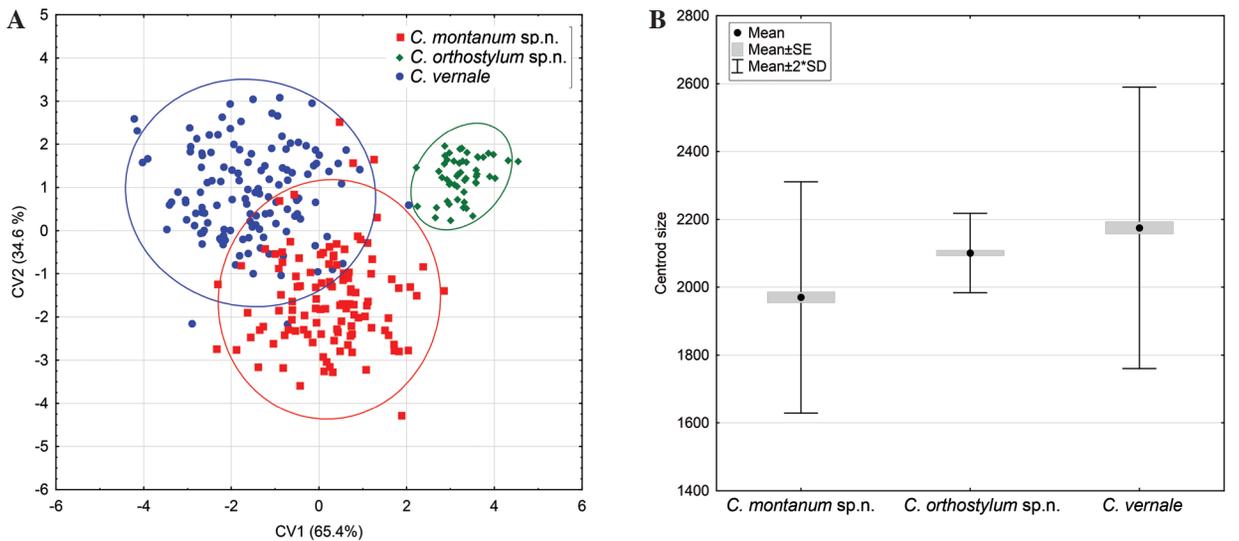


Fig. 4. Wing shape differences among analysed species. A, scatter plot of individual scores of the two first canonical axes; B, boxplot of mean centroid size.

Genetic evidence

Maximum-likelihood (ML) resolved relationships among three different *Chrysotoxum* species and a correspondent ML tree was obtained (Fig. 3A). ML tree based on mtDNA COI gene (Fig. 3A) revealed a clear

separation of *C. montanum* sp. nov. from the other species (bootstrap value 99). All specimens of *C. vernale* were part of a clearly differentiated cluster and certain level of intraspecific variability was noticed. Furthermore, one sequence of an additional taxon was detected in this morphologically well-defined group (antenna

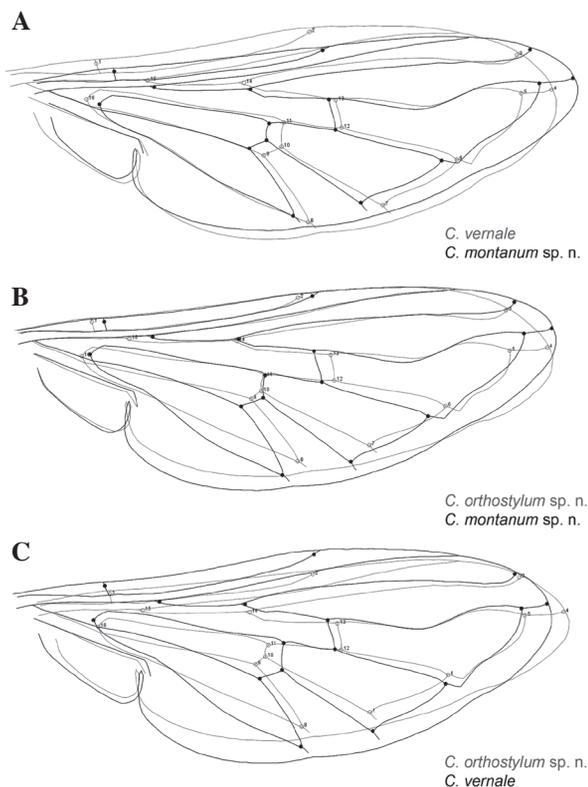


Fig. 5. Superimposed outline drawings showing wing shape differences between analysed species. A, *Chrysotoxum vernale* and *Chrysotoxum montanum* sp. nov.; B, *Chrysotoxum orthostylum* sp. nov. and *Chrysotoxum montanum* sp. nov.; C, *Chrysotoxum orthostylum* sp. nov. and *Chrysotoxum vernale*.

with the basoflagellomere shorter than the scape plus pedicel and terga with yellow fasciae not reaching the lateral margins), *C. orthostylum* sp. nov. was clearly separated as a third cluster. The MJ network suggests haplotype distribution consistent with a three cluster split in the ML tree (Fig. 3B). MtDNA COI haplotype of *C. orthostylum* is genetically the most divergent to *C. vernale* and *C. montanum*, with 35 and 44 mutational steps, respectively. Haplotype of mtDNA COI gene found in *C. montanum* shows 24 mutational steps to first haplotype in *C. vernale* haplogroup, and 16 steps to the first undetected haplotype in between (Fig. 3B).

#### Geometric morphometric evidence

##### Wing size and shape

The ANOVA of wing centroid size showed significant differences between *C. vernale* and *C. montanum* sp.

nov. (males:  $F_{1,117} = 8.99$ ;  $P < 0.00331$ , Tukey's post hoc test  $p < 0.01227$ ; females:  $F_{1,117} = 81.12$ ;  $p < 0.00000$ , Tukey's post hoc test  $p < 0.00010$ ). ANOVA test and Tukey's post hoc test showed significant differences in wing size between *C. orthostylum* sp. nov. and *C. vernale* ( $F_{1,182} = 6.69$ ;  $p < 0.01045$ , Tukey's post hoc test  $p < 0.00969$ ) and *C. orthostylum* sp. nov. and *C. montanum* sp. nov. ( $F_{1,166} = 30.12$ ;  $p < 0.00000$ , Tukey's post hoc test  $p < 0.00000$ ). *Chrysotoxum vernale* has larger wings than *C. montanum* sp. nov. and *C. orthostylum* sp. nov. (Fig. 4B).

Differences in wing shape between *C. vernale* and *C. montanum* sp. nov. were highly significant using MANOVA (males: Wilks' Lambda = 0.25314;  $F_{28,95} = 10.01020$ ;  $p < 0.00000$ ; females: Wilks' Lambda = 0.440454;  $F_{28,90} = 4.73120$ ;  $p < 0.00000$ ). Highly significant differences in wing shape were also shown between *C. orthostylum* sp. nov. and *C. vernale* (Wilks' Lambda = 0.15640;  $F_{28,155} = 29.85972$ ;  $p < 0.00000$ ), and *C. orthostylum* sp. nov. and *C. montanum* sp. nov. (Wilks' Lambda = 0.16331;  $F_{28,139} = 25.43285$ ;  $p < 0.00000$ ).

DA correctly classified the material studied in accordance with the three defined species. The overall classification success of the DA was 92.95% which indicates wing shape is a reliable predictor of interspecific discrimination. Of the 249 digitalisations, only 21 were misclassified. Canonical variate analysis conducted on shape variables gave two highly significant axes. The first canonical axis (CV1), with 65.4 % of total variation, separated *C. vernale* from *C. orthostylum* sp. nov. (Wilks' Lambda = 0.09515;  $\chi^2 = 662.18$ ;  $p < 0.00000$ ). The second canonical axis (CV2), with 34.6% of total variation, separated *C. vernale* and *C. orthostylum* sp. nov. from *C. montanum* sp. nov. (Wilks' Lambda = 0.38388;  $\chi^2 = 269.52$ ;  $p < 0.00000$ ) (Fig. 4A). The superimposed outline drawings showing major wing deformations between *C. vernale* and *C. montanum* sp. nov. occur in the central and distal part of wing and are associated with landmarks 2, 4, 5 and 9-11 (Fig. 5A). Major differences in wing shape were found in the central part of the wing (landmarks 2, 7-11) of *C. orthostylum* sp. nov. and *C. vernale* (Fig. 5C), and in the central (landmarks 7, 8, 12 and 13) and apical parts in *C. orthostylum* sp. nov. and *C. montanum* sp. nov. (landmarks 4 and 5) (Fig. 5B). *Chrysotoxum vernale* has wider wings, while *C. orthostylum* sp. nov. longer wings.

##### Surstylus shape

Significant differences in surstylus shape between *C. vernale* and *C. montanum* sp. nov. were found with

Table 1. Principal component analysis (PCA) of 19 Bioclim variables plus altitude for *C. montanum* sp. nov. and *C. vernale*. Significant factor loadings are printed in bold.

Variables	Factor Loadings		
	PC1	PC2	PC3
Altitude	-0.54	<b>-0.81</b>	0.04
BIO1 = Annual Mean Temperature	0.24	<b>0.94</b>	0.17
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))	0.46	0.17	<b>0.84</b>
BIO3 = Isothermality (BIO2/BIO7) (*100)	0.02	0.05	<b>0.90</b>
BIO4 = Temperature Seasonality (standard deviation *100)	<b>0.83</b>	0.22	0.24
BIO5 = Max Temperature of Warmest Month	0.38	<b>0.85</b>	0.33
BIO6 = Min Temperature of Coldest Month	0.01	<b>0.98</b>	0.00
BIO7 = Temperature Annual Range (BIO5-BIO6)	0.69	0.19	0.60
BIO8 = Mean Temperature of Wettest Quarter	<b>0.77</b>	0.47	-0.18
BIO9 = Mean Temperature of Driest Quarter	-0.70	0.46	0.35
BIO10 = Mean Temperature of Warmest Quarter	0.35	<b>0.91</b>	0.18
BIO11 = Mean Temperature of Coldest Quarter	0.05	<b>0.98</b>	0.11
BIO12 = Annual Precipitation	<b>-0.89</b>	-0.35	-0.23
BIO13 = Precipitation of Wettest Month	<b>-0.84</b>	-0.14	-0.30
BIO14 = Precipitation of Driest Month	<b>-0.74</b>	-0.55	-0.25
BIO15 = Precipitation Seasonality (Coefficient of Variation)	0.51	0.59	0.04
BIO16 = Precipitation of Wettest Quarter	<b>-0.84</b>	-0.17	-0.29
BIO17 = Precipitation of Driest Quarter	<b>-0.80</b>	-0.49	-0.25
BIO18 = Precipitation of Warmest Quarter	-0.38	-0.61	-0.51
BIO19 = Precipitation of Coldest Quarter	<b>-0.94</b>	-0.21	-0.08
Eigenvalue	12.21	3.48	1.96
Variance explained (%)	61.0	17.4	9.8

MANOVA ( $F_{1,91} = 32.3$ ;  $p < 0.00099$ ) and canonical analyses (axis 1: Wilks' Lambda = 0.0425;  $\chi^2 = 199.02$ ;  $p < 0.00099$ ). Discriminant analysis of surstylus shape classified correctly *C. vernale* and *C. montanum* sp. nov. with 100% overall classification success. This high rate of overall classification success indicates that surstylus shape is a reliable predictor of interspecific discrimination. Representation of the thin-plate spline deformation of surstyli showed differences in surstylus shape between *C. vernale* and *C. montanum* sp. nov. (Fig. 6).

#### Ecological evidence

The PCA reduces the Bioclim variables (Table 1) to three PCs with eigenvalue  $\geq 1.88\%$  of the variation. Separation among species were statistically significant along PC1 and PC2 (ANOVA, PC1:  $F_{1,302} = 141.26$ ;  $p < 0.00000$ , Fisher LSD post hoc test  $p < 0.00000$ ; PC2:  $F_{1,302} = 155.27$ ;  $p < 0.00000$ , Fisher LSD post hoc test  $p < 0.00000$ ), but not along PC3 ( $F_{1,302} = 1.90$ ;  $p < 0.168397$ ). Because of this, PC3 was excluded in the interpretation of results. The precipitation-related PC1 explained 61% of the total variation, while the tem-

perature- and altitude-related PC2 explained 17% of the total variation (Table 1). The scatter plot of PC1 against PC2 illustrated a clear niche separation between *C. vernale* and *C. montanum* sp. nov. *Chrysotoxum vernale* occurs across a wide temperature range, precipitation and altitude (Fig. 7), while *C. montanum* sp. nov. occurs in a much narrower range of temperatures. *Chrysotoxum montanum* sp. nov. occurs only at high altitudes with high precipitation, and its distribution is strongly influenced by the precipitation in the coldest and wettest quarters of the year (BIO19, BIO12, BIO16 and BIO13).

#### Discussion

In this study two new species of the *C. vernale* species complex are described, *C. montanum* sp. nov. and *C. orthostylum* sp. nov., and *C. vernale* is re-defined. *C. vernale* and *C. montanum* sp. nov. are similar species, while *C. orthostylum* sp. nov. can be clearly separated from *C. vernale* and *C. montanum* sp. nov. based on morphology. An integrative approach (traditional

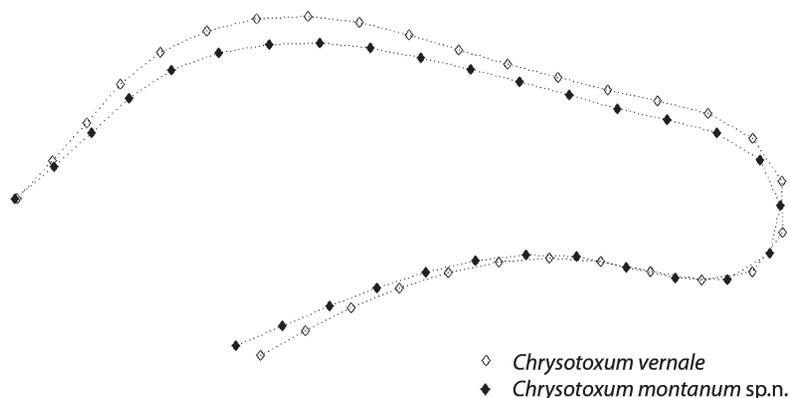


Fig. 6. Superimposed outline drawings showing surstylus shape differences between *Chrysotoxum montanum* sp. nov. and *Chrysotoxum vernale*.

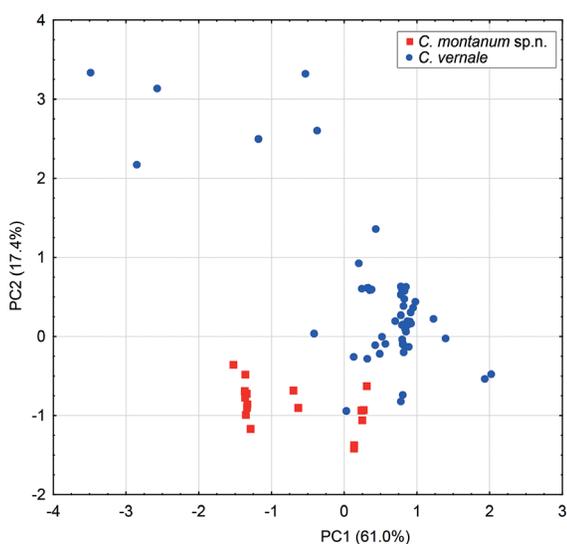


Fig. 7. Scatter plot of factor loadings of two PC scores showing the positions of *Chrysotoxum montanum* sp. nov. and *Chrysotoxum vernale* in the environmental space.

morphology, genetics, geometric morphometry, ecology) was used to distinguish *C. montanum* sp. nov. from the similar *C. vernale*.

The main morphological character distinguishing *C. montanum* sp. nov. from *C. vernale* is the colour of pile on face, occiput, scutum and scutellum in males which are black in *C. montanum* sp. nov. (Fig. 8B) and yellow in *C. vernale* (Fig. 8A). In many syrphid species such as *Episyrphus balteatus* (De Geer, 1776) and *Eupeodes corollae* (Fabricius, 1794) colour differences are not a fixed character and depend on the period of adult emergence and on the temperature experienced by the pupa (Dušek and Láska, 1974). It is also known that within certain genera, spring emerging

species have overall body colours darker than those of the lighter summer-emerging species (Rotheray and Gilbert, 2011). Genetic, morphometric and ecological evidence obtained here is congruent and indicates that differences between morphotypes A and B of *C. vernale* (*sensu* Nedeljković, 2011) are explained by their belonging to different taxa, *C. montanum* sp. nov. (B) and *C. vernale* (A).

The European *Chrysotoxum* species can be grouped according to the proportional length of the basoflagellomere: species with basoflagellomere shorter than scape and pedicel together, *Chrysotoxum bicinctum* (Linnaeus, 1758), *Chrysotoxum elegans* Loew, 1841, *Chrysotoxum festivum* (Linnaeus, 1758), *Chrysotoxum gracile* Becker, 1921, *Chrysotoxum lineare* (Zetterstedt, 1819), *C. montanum* sp. nov., *Chrysotoxum octomaculatum* Curtis, 1837, *C. orthostylum* sp. nov., *Chrysotoxum parmense* Rondani, 1845, *Chrysotoxum tomentosum* Giglio-Tos, 1890, *C. vernale* and *Chrysotoxum verralli* Collin, 1940; species with basoflagellomere longer than scape and pedicel together, *Chrysotoxum arcuatum* (Linnaeus, 1758), *Chrysotoxum cisalpinum* Rondani, 1845, *Chrysotoxum cautum* (Harris, 1776), *Chrysotoxum fasciolatum* (De Geer, 1776) and *Chrysotoxum intermedium* Meigen, 1822. Within the group with a relatively short basoflagellomere, Masseti *et al.* (2006) defined the morphological *festivum* group, *C. bicinctum*, *C. festivum*, *C. elegans*, *C. octomaculatum*, *C. parmense* and *C. vernale*, excluding characters such as the colour of femur and lateral margin of terga 3 and 4. Based on morphology, *C. tomentosum*, *C. montanum* sp. nov., and *C. orthostylum* sp. nov. are likely to belong to the *festivum* group (*sensu* Masseti *et al.*, 2006). These three species have the yellow fasciae on terga 3 and 4 not reaching the lateral margin of the tergum, but the *festivum* group (*sensu* Masseti *et al.*,

2006) includes some species with yellow fasciae reaching the lateral margin. Further morphological and molecular analyses are required to access the concept of a *festivum* group.

Regarding the genetic results of the present study *C. montanum* sp. nov. was grouped in a separate cluster of the ML tree (Fig. 3A). Analysis showed that COI sequences of mtDNA separated *C. montanum* sp. nov. and *C. vernale*. The species *C. montanum* sp. nov. clustered in a clearly independent position both in the ML tree (Fig. 3A) and MJ network (Fig. 3B). The species *C. orthostylum* sp. nov. also clustered in an independent position in the ML tree (Fig. 3A), and showed the greatest genetic divergence from *C. vernale* and *C. montanum* in the MJ network (Fig. 3B). Only one fresh specimen of *C. orthostylum* sp. nov. was available for molecular study and further specimens must be analysed to determine the phylogenetic position of *C. orthostylum* sp. nov.

Prior to the present study, the COI region had already been shown as a useful taxonomic tool; for instance, it proved informative for separating *C. cautum* from *C. bicinctum*, and also *C. parmense* and *C. fasciolatum*, but failed in delimiting species borders between *C. festivum* and *C. elegans* (Masetti *et al.*, 2006). Our preliminary investigation on 3' fragment of COI gene variation in the genus *Chrysotoxum* (Veličković *et al.*, 2012) showed that the implementation of COI as a molecular marker was found insufficiently precise to identify the differences within *vernale* complex (morphotypes *C. vernale* A, *C. vernale* B) and *festivum* complex (morphotypes *C. festivum* A and *C. festivum* B, and *C. elegans*). Following these conclusions Nedeljković *et al.* (2013) have used ITS2 as a marker of choice differences have been found between *C. festivum* and *C. elegans*. In the present study we have tested our second idea that the combination of both 5' and 3' sequences of COI gene will generate sufficient level of divergence for delimiting putative cryptic species. We have shown that COI was sufficient to separate *C. montanum* sp. nov. from the similar *C. vernale*. One of the conclusions could be that there is no *a priori* chosen single marker that could successfully solve taxonomic problems in Syrphidae. Results of this study show that the COI gene has justified its reputation of a stable barcoding marker, but there have been cases within the genus *Chrysotoxum* (Masetti *et al.*, 2006), and even in other syrphid genera when the mtDNA COI failed to work well and the sequence divergence was relatively low, and even invariant (Milankov *et al.*, 2008, 2009; Francuski *et al.*,

2011, Haarto and Ståhls, 2014). Therefore an integrative taxonomy approach provides a better method to delimit closely related hoverfly species.

Significant differences in wing size and shape between *C. montanum* sp. nov., *C. vernale* and *C. orthostylum* were detected (Fig. 5A). Inconspicuous but statistically significant differences in surstylar shape between *C. montanum* sp. nov. and *C. vernale* were also revealed with a geometric morphometric analysis; this is the second example after that of Nedeljković *et al.* (2013) in which geometric morphometry of genitalia has successfully contributed to the separation of cryptic syrphid species. In addition, niche divergence analysis reveals that *C. montanum* sp. nov. is more specialised than *C. vernale*. *Chrysotoxum montanum* sp. nov. occurs only at high altitudes with high precipitation, while *C. vernale* is able to thrive in a wider range of environmental conditions of temperature, precipitation and altitude. The sympatric (*e.g.* in FYR Macedonia, Golema poljana) and synchronic (early May to mid-August) distributions of *C. montanum* sp. nov. and *C. vernale* also support their taxonomic separation.

Animal diversity in the Balkan Peninsula is exceptionally rich and includes many endemic and relict species (Savić, 2008). In this region, insects have experienced an extraordinary adaptive radiation as a result of glacial and inter-glacial periods, particularly in Coleoptera (Mesarosh, 1996), Lepidoptera (Jakšić, 1998) and Diptera, Syrphidae (Vujić *et al.*, 2001). In the present study, integrative taxonomy is confirmed as a useful tool to better understand the entomological diversity in the Balkans and consequently improve the basic knowledge required for species conservation in this exceptional place.

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## Online supplementary information

S1. List of material examined.

S2. List of specimens used for molecular analyses.

## Appendix

### Descriptions of new species

Two new species of the *vernale* complex are described in the present study, and the species *C. vernale* is re-defined. The three studied species shared an antenna with the basoflagellomere shorter than scape and pedicel together and abdominal terga with yellow fasciae not reaching the lateral margins.

Family Syrphidae

Genus *Chrysotoxum* Meigen, 1803

*Chrysotoxum montanum* Nedeljković & Vujić sp. nov.

(= morphotype B *sensu* Nedeljković, 2011)

(Figs 8b, 11a, 13a)

**Holotype.** male, SERBIA, Kopaonik, Klisura Samokovske reke-mešano, 16.vi.2012, leg. A. Vujić (G1530, C104).

**Paratypes.** AUSTRIA: 1m, Frankenfels, 6/78, Bgst, (S11479), (NHMW); BOSNIA & HERZEGOVINA: 2m, Igman-Bjelašnica, 26.VI.1989. (S11301, S11302); 1m, Jahorina, 26.VI.1989. (S11300); MONTENEGRO: Durmitor, 1m, Crepulj poljana, 4.VII.1991. (S11361), 1m, Crno jezero, 21.VI.1983. (S11324), 3m, do Male Karlice, 13.VII.1981. (S11358, S11359, S11360), 3m, do Zminjeg jezera, 3.VII.1981. (S11352, S11353, S11354), 3f, Ivan Do, 16.VIII.1984. (S11328), 27.VI.1993. (S11329, S11330); 1m, 1f, Jablan jezero, 20.VII.1995. (S11346, S11347); 4m, ka Jablan jezeru, 7.VII.1991. (S11342, S11343, S11344, S11345); 10m, ka Savinom kuku, 11.VII.1981. (S11305, S11306, S11307, S11308, S11311, S11313, S11309, S11310, S11312, S11314), 4m, Kanjon Sušice, 6.VII.1994. (S11337, S11341), 9. VII.1991. (S11338, S11327), 8m, Mlinski potok, 3. VII.1991. (S11321, S11322, S11323), 6.VII.1983. (S11315, S11316), 22.VI.1985. (S11317, S11318, S11319), 2m, 2f, Motički gaj, 9-10.VII.1983. (S11320, S11355, S11356, S11357), 1m, 1f, Otoka Crnog jezera, 24. VI.1983. (S11325, S11326) 1f, Pirlitor-Tara, 25.VI.1983. (S11363), 1m, Pitomine, 1.VII.1983. (S11362), 1f, Razvršje, 21.VIII.1984. (S11364), 2m, Sušica, 28. VI.1985. (S11334, S11335), 2m, 1f, Sušica-Skakala, 25-26.VI.1997. (S11366), 29.VI.1993. (S11339, S11340), 4m, Sušičko jezero, 25.VI.1985. (S11331, S11332, S11333), 25-27.VI.1986. (S11336), 5m, 2f, Sušičko jezero-Sastavci, 2.VI.1998. (S11365, S11370), 20.VI.1998. (S11367, S11368, S11369, S11371, S11372), 1m, Tepca, 6.VII.1983. (S11348), 3m, Tepca-kanjon Tare, 21.VI. 1983. (S11349, S11350, S11351); SERBIA: Kopaonik,

1m, 1f, Bačište, 4.VII.1986. (S06423, S06321), 2m, do Jablanove ravni, 23.VI.1991. (S06416, S06417), 2m, Duboka reka, 6.VII.1986. (S06420), 18.VII.1986. (S06421), 1f, Jablanova ravan, 20.V.1986. (S06390), 2m, Jasle-Jablanova ravan, 14.VI.1986. (S11444, S06418), 1m, 1f, Jasle-Čukara, 20.VI.1996. (S06415), 23.V.1993. (S06419), 1m, Kadijevac, 21.VI.1991. (S06422), 27m, 1f, Samokovska reka, 5.VII.1986. (S06402), 14.VIII.1997. (S06389), 16.VI.1986. (S06395, S06396, S06409, S06406, S06388, S06410, S06397, S06398, S06399, S06401, S06400, S06394), 19.VI.1996. (S06392, S06393), 21.VI.1991. (S06403), 22.V.1986. (S06407), 22.VI.1991. (S06404, S06405, S06386, S06411, S06412), 24.V.1992. (S06413, S06387), 24.V.1993. (S06391), 27.V.1994. (S06406, S06414), 1f, Treska - bukva, 17.VII.1985. (S06425), 1f, Velika reka, 14.VI.1986. (S06424), 1m, Klisura Samokovske reke-mešano, 16.VI.2012. (G1531/C105); Prokletije, 1m, ka Volušnici, 29.VII.1994. (S06385); Šar planina, 1m, Muržica, 12.VII.1996. (S06426); 3m, Ošljak, 29.VI.1988. (S06427, S06428, S11445).

**Diagnosis.** Medium to large species (15-17 mm, n = 10); r = 1:0.8:1.8; vertical and frontal triangles with black pile; scutum with black pile (Fig. 8B); katepisternum completely black (Fig. 9C) or with a very small yellow macula; scutellum with long black pile; male genitalia very similar to that in *C. vernale*; surstylus 1.3 times longer than hypandrium (excluding superior lobes and aedeagus); epandrium plus surstylus 1.2-1.3 times longer than hypandrium (excluding superior lobes and aedeagus); in female frons black, with two triangular pollinose maculae. *Chrysotoxum montanum* has longer wings than *C. vernale*. *Chrysotoxum montanum* occurs only at high altitude. ML tree based on mtDNA COI gene revealed a clear separation of *C. montanum* from *C. vernale*.

**Description.** Male (holotype). L = 15 mm.

**Head.** Eye pile straight and yellow, denser and longer on the dorsal surface; vertical triangle black, with long black pile and white pollinosity on the posterior corners; frontal triangle black, shiny except for the white pollinosity posteriorly, black haired; antenna black; scape and pedicel with black pile; arista bare, dark brown; r = 1:0.8:1.8; face yellow, yellow haired on the lower part and black haired on the upper part; face with a central black vitta extending from antennal bases to mouth edge (vitta as wide as a fourth of the face

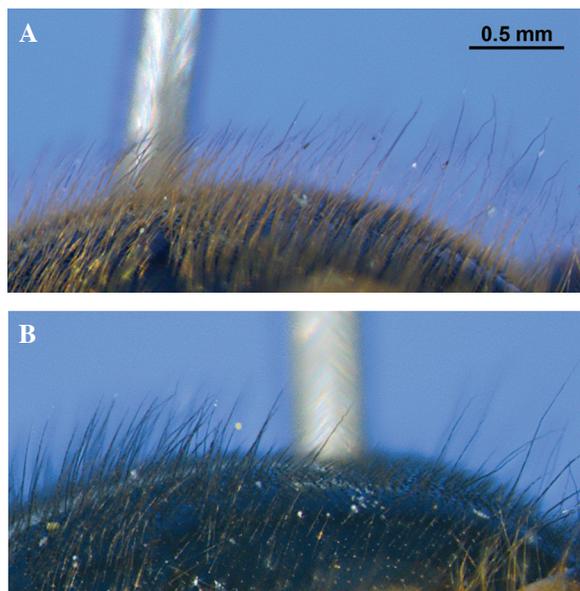


Fig. 8. Thoracic scutum of males, lateral view. A, *Chrysotoxum vernale*; B, *Chrysotoxum montanum* sp. nov.

width); mouth edge narrowly black; gena shiny yellow and yellow-haired; occiput white pollinose, with long black pile.

**Thorax.** Scutum black with two central, silvery-pollinose vittae extending for two thirds of the scutum length from its anterior margin; scutum with both long and short black pile intermixed (Fig. 8B); katapisternum completely black (Fig. 9C); scutellum yellow except

for a black central disc and lateral corners, with long black pile; legs yellow except for the black basal third of femora; wing membrane extensively microtrichose; wing cells  $R_1$  and  $R_{2+3}$  brown pigmented.

**Abdomen.** Shiny black, with short black pile, except for the yellow-haired tergum 1 and anterior part of tergum 2; all terga with two yellow fasciae not reaching the lateral margins (Fig. 11A); all terga with black pile except for the yellow pile on the fasciae; all sterna shiny black and with yellow pile; anterior margin of sternum 3 with two interconnected yellow fasciae reaching the lateral margins; anterior margin of sternum 4 with two yellow maculae reaching the lateral margins.

**Genitalia.** Surstylus 2.5× longer than wide (Fig. 13A); triangular-shaped hypandrium, 1.5× longer than its basal width; surstylus 1.27-1.33 times longer than hypandrium (excluding superior lobes and aedeagus).

**Female.** Similar to the male except for the following characters: frons with two triangular maculae of silvery pollinosity; frons and scutum with short black pile.

**Geographic distribution.** Austria, Bosnia & Herzegovina, FYR Macedonia, Montenegro, Serbia (Fig. 14A).

**Etymology.** The epithet *montanum* means ‘belonging to a mountain’ and refers to the fact that this species is associated with mountain habitats.

**Flight period.** Early May to late August.

**Habitat and flowers visited.** This species has been found with other *Chrysotoxum* species in the grassy edges of conifer forests at high altitudes. Flowers visited: no data.

Table 2. Diagnostic comparison between *C. montanum* sp. nov. and *C. vernale* based on traditional morphology.

Character	<i>C. vernale</i>	<i>C. montanum</i> sp. nov.
<i>Male and female</i>		
Colour of face pile	yellow	black on the upper part of face
Colour of occiput pile	yellow	black
Colour of scutellum pile	yellow	black
Colour of scutum pile	yellow (Fig. 8A)	black (Fig. 8B)
Katapisternum	with a yellow macula (Fig. 9B)	black (Fig. 9C) or with a small yellow macula
<i>Male</i>		
Colour of vertical triangle pile	yellow except for some black pile posteriorly	black
Colour of frontal triangle pile	yellow	black
Ratio length/width of surstylus (lateral view)	2.7-3.2 (Fig. 13C)	2.5-3 (Fig. 13A)
<i>Female</i>		
Shape of frontal pollinose maculae	rectangular	triangular

**Taxonomic notes.** In this new species, the katepisternum colour ranges from completely black (Fig. 9C) to black with a small yellow macula anteriorly. *Chrysotoxum montanum* sp. nov. can be separated

from *C. orthostylum* sp. nov. and *C. vernale* by the colour of the long pile on the scutum, which are black in *C. montanum* sp. nov. (Fig. 8B) and yellow in the other species. For further morphological differences between *C. montanum* sp. nov. and *C. vernale* see Table 2.

*Chrysotoxum orthostylum* Vujić sp. nov.  
(Figs 9a, 10a, 10b, 11b, 12a, b; 13b)

**Holotype.** male, FYR MACEDONIA, Golema poljana, 14.VI.1975, leg. S. Glumac, (S11248), (FSUNS).

**Paratypes.** KYRGYZSTAN: 1m, Tash-Arik, Talas, 4.VI.1992, leg. Jirousek, (S11448) (DD); FYR MACE-

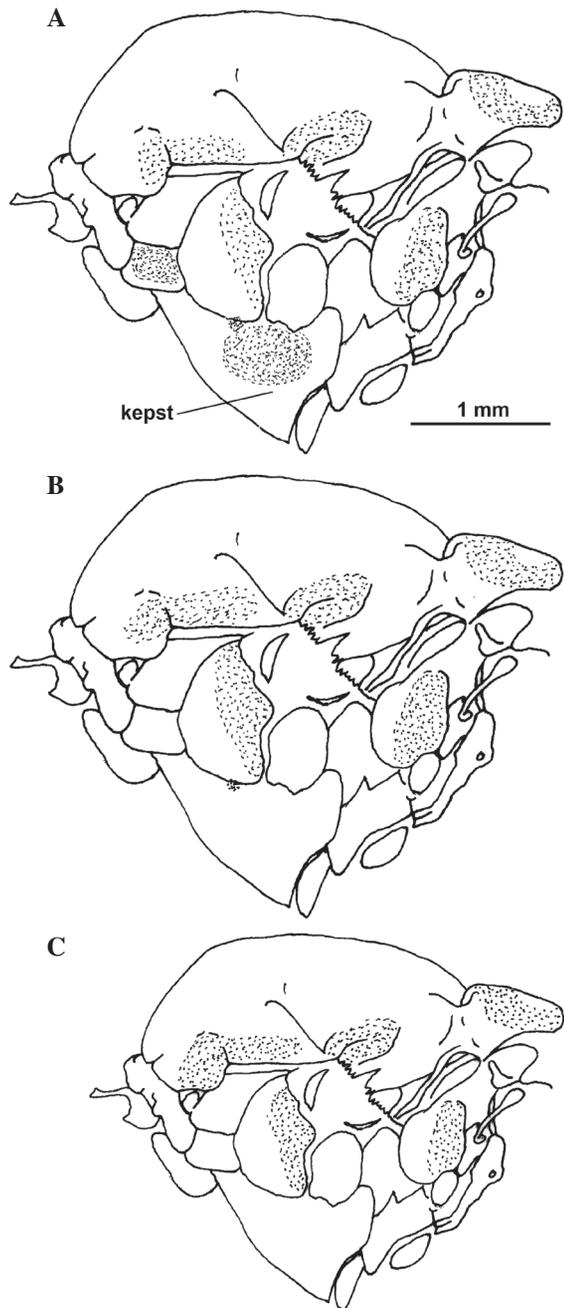


Fig. 9. Thoracic pleura, lateral view. A, *Chrysotoxum orthostylum* sp. nov.; B, *Chrysotoxum vernale*; C, *Chrysotoxum montanum* sp. nov. Dotted areas indicate yellow colour (kepst, katepisternum).

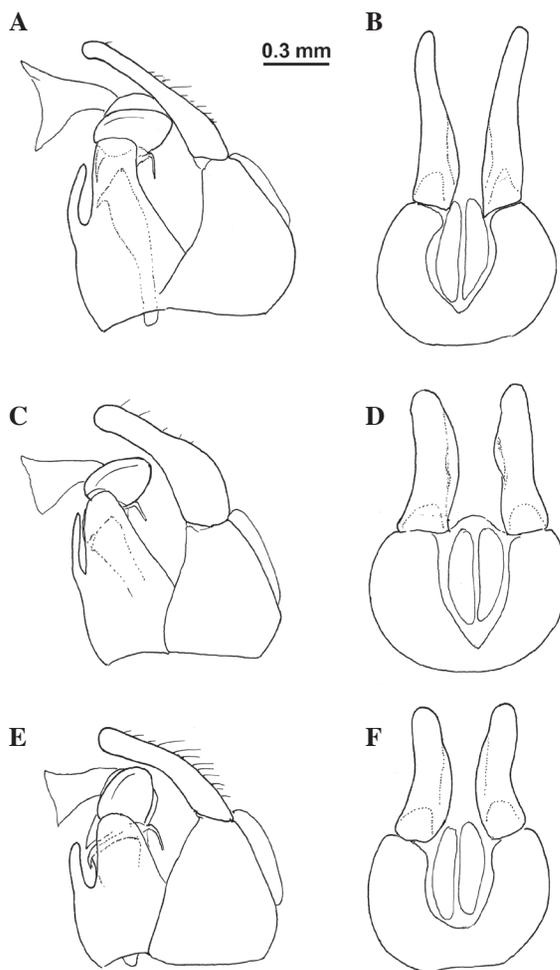


Fig. 10. Male genitalia. A, *Chrysotoxum orthostylum* sp. nov., lateral view; B, *Chrysotoxum orthostylum* sp. nov., dorsal view; C, *Chrysotoxum festivum*, lateral view; D, *Chrysotoxum festivum*, dorsal view; E, *Chrysotoxum vernale*, lateral view; F, *Chrysotoxum vernale*, dorsal view.

DONIA: 3m, Kočani, vlažna livada, na lišću *Lapsana* - *Chrysanthemum*, 3.VI.1960, leg. Glumac (S11246, S11251, S11448); 1m, NP Pelister, 24.VII.1956. (S11247); 1f, Carevec, Bistra planina, 1.VII.1972, leg. Ćingoc (S11249); 1m, Kožuf, Golema poljana, 14. VI.1975 (S11248); 1m, Goten planina, 27.V.1988, leg. Krpač (S11252); 1m, 2f, Mavrovo, Carevec, 17.VI.1994, leg. Mihajlova (S11250, S11253, S11254); MONTENEGRO: 1m, Durmitor, Kanjon Sušice, 6.VII.1994, leg. Radenković (S11447); SERBIA: 1m, Kopaonik, Samokovska reka, 22.VI.1991, (S06316); TURKEY: 1m, Gurun, Mezikiran Gecidi, 10.VII.1997, leg. Mazikiran Geçidi (S11245).

**Diagnosis.** Large to medium size species (14-17 mm, n = 9); r = 1:0.9:1; scutum with short black pile which are either intermixed with long yellow pile (male) or not (female); katapisternum with a yellow macula extending on most of its dorsal margin (Fig. 9A); posterior corners of terga 3 and 4 developed in thorn-like processes (Fig. 12A); male surstylus elongate, 3.7× longer than wide (Fig. 13B); surstylus as long as hypandrium (excluding superior lobes and aedeagus) (Fig. 10A); epandrium plus surstylus 1.5-1.6 times longer than hypandrium (excluding superior lobes and aedeagus) (Fig. 10A); in female frons black, with two rectangular pollinose maculae.

Highly significant differences in wing shape were shown between *C. orthostylum* sp. nov. and *C. vernale* and *C. montanum* sp. nov. mtDNA COI haplotype of

*C. orthostylum* sp. nov. is genetically the most divergent to *C. vernale* and *C. montanum* sp. nov.

**Description.** Male (holotype). L = 14.5mm.

**Head.** Eye pile straight and yellow, denser and longer on the dorsal half of the eye surface; vertical triangle black, with black pollinosity on the anterior corner and silvery-white pollinosity posteriorly, black-haired except for some yellow pile anteriorly; eye contiguity about 17-facets long; frontal triangle black, with long, black and yellow pile intermixed; antenna black, with black pile on scape and pedicel; arista dark brown and bare; r = 1:0.9:1; face tuberculated, yellow and yellow-haired; face with a central black vitta extending from antennal bases to mouth edge (vitta as wide as a fourth of the face width); genae yellow with yellow pile; occiput white pollinose, with long yellow pile.

**Thorax.** Scutum black with two central, silvery-pollinose vittae extending for two thirds of the scutum length from its anterior margin; scutum with long yellow pile, intermixed with short black pile on the posterior part; katapisternum with a yellow spot on the dorsal margin (Fig. 9A); scutellum yellow with a black central disc, covered with long yellow and short black pile; legs yellow except the black basal third of pro- and mesofemora; leg pile yellow; wing wholly microtrichose; halter completely yellow; cells R<sub>1</sub> and R<sub>2+3</sub> brown pigmented.

**Abdomen.** Elongate, parallel-sided (Fig. 11B); shiny black with short black pile, except for the yellow-

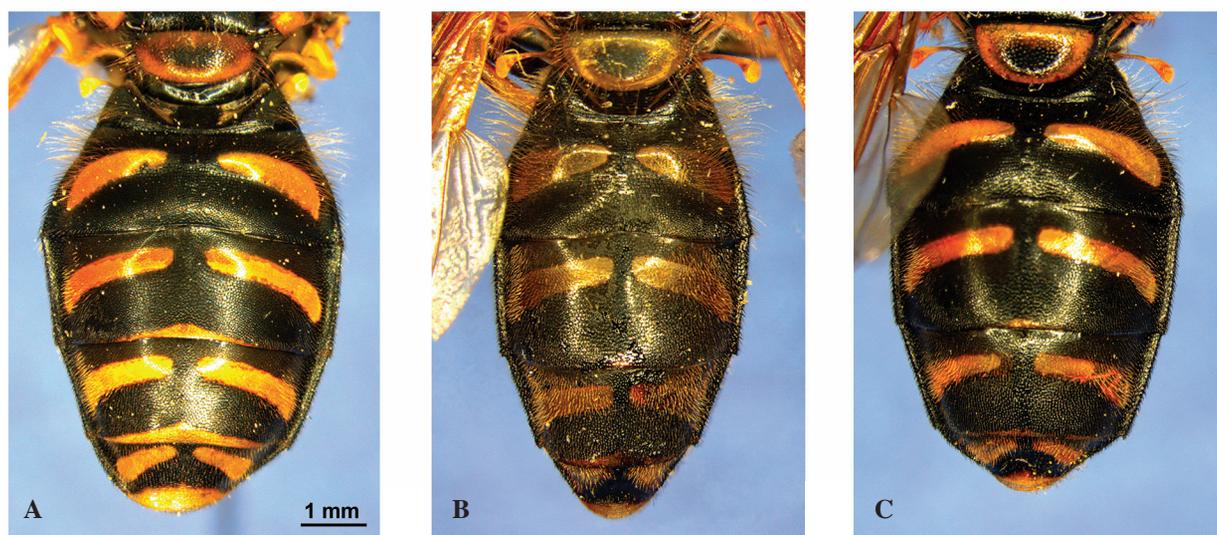


Fig. 11. Abdomen, dorsal view. A, *Chrysotoxum montanum* sp. nov.; B, *Chrysotoxum orthostylum* sp. nov.; C, *Chrysotoxum vernale*.



Fig. 12. Terga 3-5, dorsal view, *Chrysotoxum orthostylum* sp. nov. A, male; B, female (t, thorn-like process).

haired tergum 1 and anterior part of tergum 2; all terga with two yellow and yellow-haired fasciae (Fig. 11B); posterior corners of terga 3 and 4 developing in thorn-like processes (Fig. 12A); all sterna shiny black and yellow haired; anterior margin of sternum 3 with two interconnected yellow fasciae not reaching the lateral margins; anterior margin of sternum 4 with two yellow maculae not reaching the lateral margins.

**Genitalia.** (Fig. 10A, B): Elongate surstylus, 3.7× longer than wide (Fig. 13B); triangular-shaped hypandrium, 1.2 times longer than its basal width (Fig. 10A).

**Female.** Similar to the male except for the following characters: frons black with two rectangular pollinose maculae; frons with short black pile; scutum only with short black pile.

**Geographic distribution.** FYR Macedonia, Kyrgyzstan, Montenegro, Serbia, Turkey (Fig. 14B).

**Etymology.** The noun in apposition *orthostylum* means ‘straight stylus’ and refers to the fact that the male surstylus is straight and more elongated than that in similar species.

**Flight period.** Mid May to mid August.

**Habitat and flowers visited.** *Chrysotoxum orthostylum* sp. nov. has been found flying with other *Chrysotoxum* species in the grassy edges of conifer forests at high altitudes in Serbia and Montenegro. In FYR Macedonia, *C. orthostylum* sp. nov. has been found resting on *Lapsana* and *Chrysanthemum* leaves in wet meadows.

**Taxonomic notes.** *Chrysotoxum orthostylum* sp. nov. can be distinguished from *C. montanum* sp. nov. and *C. vernale* by the abdomen shape, which is slender and nearly parallel-sided in *C. orthostylum* sp. nov. (Fig. 11B) but more oval in other species (Fig. 11A, C). In addition, the posterior corners of tergites 3 and 4 in *C. orthostylum* sp. nov. are developed into thorn-like processes (Fig. 12A, B), while in similar species these

corners are blunter. The katapisternum of *C. orthostylum* sp. nov. has a yellow macula (Fig. 9A), which is more reduced or even absent in *C. vernale* and *C. montanum* sp. nov. (Fig. 9B, C). Males of *C. orthostylum* sp. nov. can be separated from other species by the long surstylus, which is 3.3-3.7× (n = 5) longer than wide in *C. orthostylum* sp. nov. (Fig. 13B) but 2.8× or less in the other species (Fig. 13A, C). In addition, the shape of the hypandrium apex is rectangular in *C. orthostylum* sp. nov. (Fig. 10A) and more triangular in *C. festivum* (Fig. 10C) and *C. vernale* (Fig. 10E).

**Variability.** In *C. orthostylum* sp. nov. the colour of legs ranges from dark yellow to black in basal third of pro- and mesofemora and in basal two thirds of metafemora. In addition, southern specimens (FYR Macedonia) have wider and lighter yellow fasciae on terga than specimens from more localities further north.

*Chrysotoxum vernale* Loew, 1841  
(= morphotype A *sensu* Nedeljković, 2011)  
(Figs 8a, 9b, 10e, 10f, 11c, 13c)  
syn. *collinum* Rondani, 1857: 202  
syn. *flavipenne* Palma, 1864: 40  
syn. *fuscum* Giglio-Tos, 1890: 160  
syn. *vernaloides* Giglio-Tos, 1890: 161

*Chrysotoxum vernale* was described from an unspecified but large (“Unter vielen Exemplaren...”) number of specimens, both males and females. Some males studied by Hermann Loew were collected in the place where he was working at the time of the description (“...hier gefangen...”): Poznań (“Posen”), Poland. Apart from this, no other data about the type locality of *C. vernale* are given in the original description. In the ZMHB there are 27 specimens of *C. vernale* belonging to Loew’s collection (J. Ziegler, in lit.); of these

specimens only 20 are labelled as ‘Coll. H. Loew’ and further information is not provided in the available data bases at the ZMHB (Sven Marotzke, in lit.). These specimens were examined here to confirm the colour of scutum pile, which was yellow. Two named specimens (male and female) of *C. vernale* from the ‘old collection’ (“Alte Sammlung”) at the NHMW were also examined; the female is labelled as ‘*Chrysotoxum vernale* f Loew\*’ (handwritten). No information about the locality where the NHMW specimens were collected is available, but Loew’s type material is usually marked with an asterisk (\*) (A. Vujić, pers. obs.). We

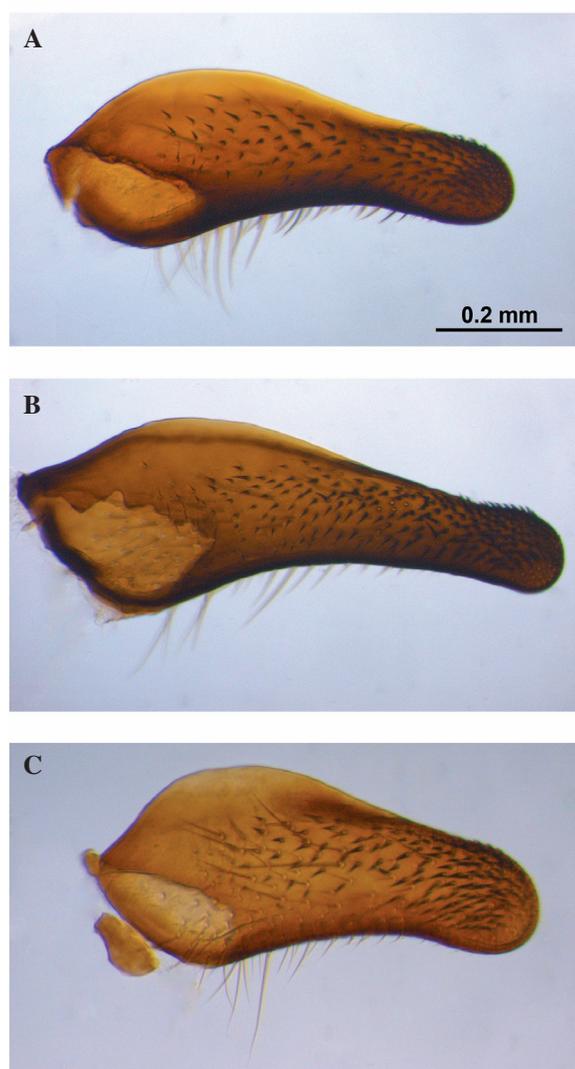


Fig. 13. Right surstylus of the male genitalia, lateral view. A, *Chrysotoxum montanum* sp. nov.; B, *Chrysotoxum orthostylum* sp. nov.; C, *Chrysotoxum vernale*.

consider that the specimens examined both in ZMHB and NHMW belong to the type series of *C. vernale* and the NHMW male is designated as lectotype in order to stabilize this species concept.

*Chrysotoxum flavipenne* was described from a single male. The description provided by Palma (1863) is detailed enough and indicates that the scutum pile are yellow (“Torace...rivestito nel dorso da gialla peluria...”) and the thoracic pleuron has a yellow macula (“...ampia macchia triangolare dello stesso colore su ciascun fianco”). The repository collection of the *C. flavipenne* holotype is unknown to the authors of the present paper; it might be deposited in the entomological collection at the Museo Zoologico, Centro Musei delle Scienze Naturali, Naples, Italy but this collection is now being inventoried (Roberta Improta, in lit.). Giglio-Tos (1890) diagnosed *Chrysotoxum fuscum* and compared it with *C. vernale* of Loew. Giglio-Tos (1890) also noted the existence of *Chrysotoxum vernaloides* as a variety of *C. fuscum*. According to the original description of *C. fuscum* the colour of the scutum pile is yellow (“Thorace fulvo piloso”); the variety *vernaloides* differed from other *C. fuscum* specimens in characters other than the thoracic pile (Giglio-Tos, 1890). Sommaggio (2001) proposed *C. fuscum* and *C. vernaloides* as junior synonyms of *C. vernale* upon examination of the type material and designated lectotypes for these two names. These two lectotypes are deposited at the entomological collection of the Museo Regionale di Scienze Naturali, Torino, Italy. However, this museum suffered a fire in 2013 and the collections are still inaccessible to users; Giglio-Tos’ collection is undamaged (Marinella Garzena, in lit.). Based on the original descriptions of *C. flavipenne*, *C. fuscum* and *C. vernaloides*, as well as on the nomenclatural and taxonomic advances made by Sommaggio (2001), we confirm these three names as synonyms of *C. vernale*. In addition, the holotype of *C. collinum* was examined by the authors of the present study on the basis of four images made by Dr Luca Bartolozzi (LSF): dorsal, lateral and ventral overall views and anterior view of the face. This specimen was also confirmed to be the typical *C. vernale*.

#### Material examined.

*Lectotype* (designated here): 1m labelled as ‘vernale, Schlerin’/ ‘Alte Sammlung’ / S11470 (NHMW).

*Paralectotypes* (designated here). If labelled as 16/547/ *vernale* f Loew Alte Sammlung/ Austria Alte Sammlung/ *Chrysotoxum vernale* f Loew\* (NHMW). 20 specimens labelled as ‘Coll H. Loew’ and seven

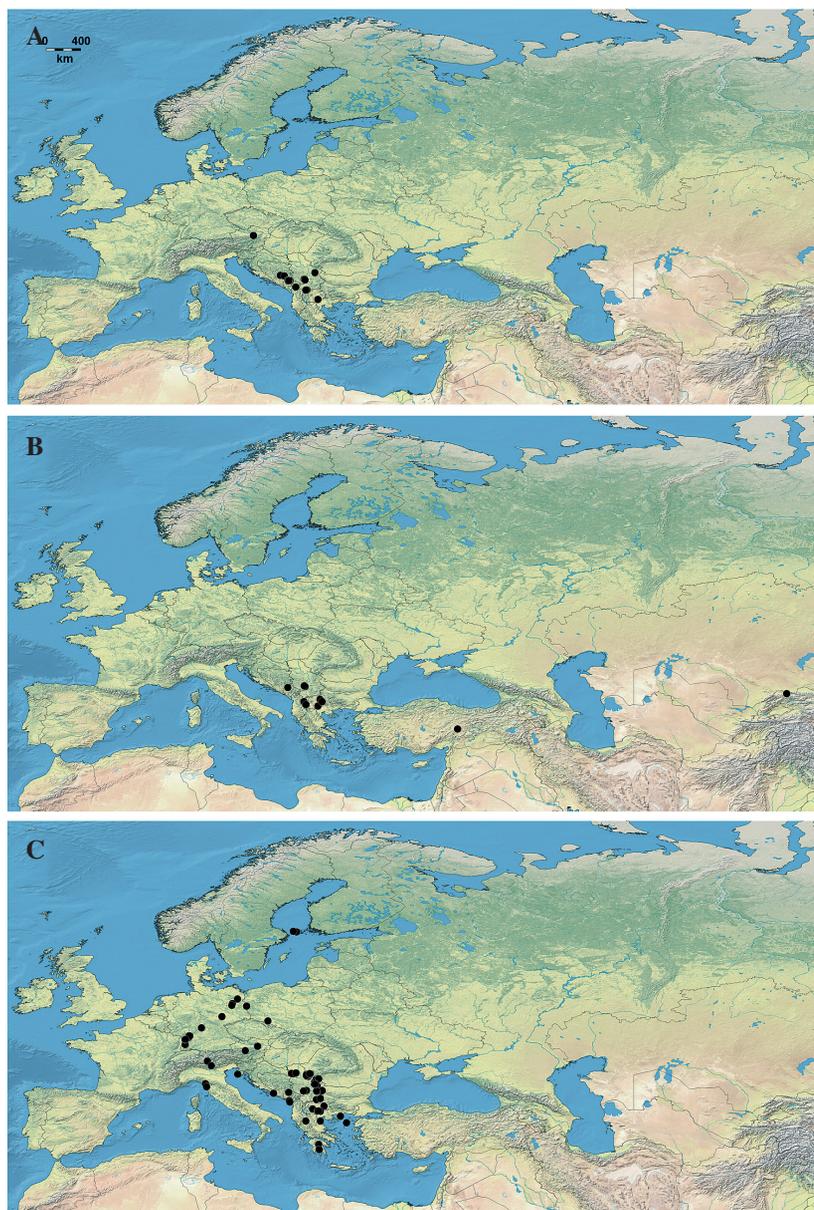


Fig. 14. Distribution maps of the material examined. A, *Chrysotoxum montanum* sp. nov.; B, *Chrysotoxum orthostylum* sp. nov.; C, *Chrysotoxum vernale*.

specimens not labelled but supposed to be part of Loew's collection and have been collected in Poznań (J. Ziegler, in lit.); 10 of the 20 'Coll H. Loew' specimens are labelled as 'Wien Schiner' (4 specimens), 'Florenz Mann' (3 specimens), 'Syrakus Zeller' (1 specimen), 'Spanien' (1 specimen), 'Paris' (1 specimen) (ZHMB).

*Additional material.* Holotype of *Chrysotoxum colinum*: m, Museo "La Specola" coll. Rondani HOLO-TYPUS (red label)/ 357/ *Chrysotoxum vernale* (= *col-*

*linum* Rnd.)/ rev. (overwritten by hand on 'det.') M. Daccordi 1985 [LSF]. For further non-type additional material see Table S1.

*Diagnosis.* Large to medium size species (11-15 mm,  $n = 10$ );  $r = 1:0.6:1.5$ ; vertical and frontal triangle with yellow pile; scutum with long yellow pile (male) (Fig. 8A); katapisternum with a yellow macula (Fig. 9B); scutellum mainly with long yellow pile; basal third of pro- and mesofemora black; surstylus 1.2-1.3 times

longer than hypandrium (excluding superior lobes and aedeagus) (Fig. 13C); epandrium plus surstylus 1.1-1.2 times longer than hypandrium (excluding superior lobes and aedeagus) (Fig. 10E); in female, frons black, with two rectangular pollinose maculae. *Chrysotoxum vernale* has larger wings than *C. montanum*. Additionally, it has wide temperature, precipitation and altitude range.

*Geographic distribution.* (only localities confirmed in the present study): Austria, Croatia, Finland, Germany, Greece, Italy, FYR Macedonia, Montenegro, Poland, Serbia (Fig. 14C).

*Flight period.* Mid April to mid-August.

#### *Adjustments to the Palaearctic Chrysotoxum key*

Refinements to couplets in the key to the Palaearctic *Chrysotoxum* in Violovitsh (1974) are provided below, based on the results presented here, as well as those in Nedeljković *et al.* (2013). *Chrysotoxum montanum* sp. nov., *C. vernale* and specimens of *C. orthostylum* sp. nov. with femora black basally would key out to *C. vernale* (*sensu lato*) in Violovitsh (1974); specimens of *C. orthostylum* sp. nov. with femora wholly yellow would key out to *Chrysotoxum festivum* (Linnaeus, 1758) (*s. l.*). *Chrysotoxum festivum* and *Chrysotoxum tomentosum* Giglio-Tos, 1890, which also have yellow fasciae on terga 2-4 not reaching the lateral margins of tergum, would key out to *C. festivum* (*s. l.*).

*Chrysotoxum festivum*, *C. orthostylum* sp. nov. (femora wholly yellow), and *C. tomentosum*

Males – change step 46

46 (47). Femora of all legs yellow.

- a (b). Posterior corners of terga 3 and 4 developed into thorn-like processes (Fig. 12A); male surstylus elongate, 3.7× longer than wide (Fig. 13B) ..... *C. orthostylum* sp. nov.
- b (a). Posterior corners of terga 3 and 4 without or with very inconspicuous thorn-like processes; male surstylus 1.5-2.8× longer than wide.
- c (d). Scutum with long yellow pile only (figure 5A: Nedeljković *et al.*, 2013); mouth edge yellow ..... *C. festivum*
- d (c). Scutum with long yellow pile and short black pile intermixed (figure 5B: Nedeljković *et al.*, 2013); mouth edge black ..... *C. tomentosum*

*Chrysotoxum montanum* sp. nov., *C. orthostylum* sp. nov. (femora black basally), and *C. vernale*

Males – change step 47

47 (46). Pro- and mesofemora dark brown to black at least basally.

- a (b). Abdomen slender, 1.8× longer than wide; posterior corners of terga 3 and 4 developed into thorn-like processes (Fig. 12A); male surstylus elongate, 3.7× longer than wide (Fig. 13B) ..... *C. orthostylum* sp. nov.
- b (a). Abdomen more oval, 1.4-1.6× longer than wide (Figs. 11A, C); posterior corners of terga 3 and 4 without or with very inconspicuous thorn-like processes; male surstylus 2.5-3× longer than wide (Figs. 13A, C).
- c (d). Scutum with yellow pile only (Fig. 8A); katapisternum with a yellow macula (Fig. 9B) ..... *C. vernale*
- d (c). Scutum with black pile only (Fig. 8B); katapisternum completely black (Fig. 9C) ..... *C. montanum* sp. nov.

*Habitats and flowers visited.* In Europe, *C. vernale* is found in the following habitats: forests; open woodlands and scrubs on well-drained sites, from *Betula* invaded heathland to *Fagus* woodlands to *Genista florida*/*Quercus pyrenaica* maquis; also in well-drained, unimproved grasslands, including montane and alpine grasslands (up to 2500 m). It is known to visit white flowers of Umbelliferae, as well as those of *Caltha*, *Crataegus*, *Euphorbia*, *Helianthemum*, *Sorbus*, *Valeriana* (Speight, 2013).

*Taxonomic notes.* To separate this species from the similar *C. montanum* sp. nov. see Table 2.

*Chrysotoxum festivum*, *C. orthostylum* sp. nov. (femora wholly yellow), and *C. tomentosum*

Females – change step 36

36 (37). Femora of all legs yellow.

- a (b). Abdomen slender, 2× longer than wide; posterior corners of terga 3 and 4 developed into thorn-like processes (Fig. 12B) ..... *C. orthostylum* sp. nov.
- b (a). Abdomen more oval, 1.6-1.7× longer than wide; posterior corners of terga 3 and 4 not developed into thorn-like processes, or very inconspicuously developed.
- c (d). Pollinose maculae on frons triangular ..... *C. tomentosum*
- d (c). Pollinose maculae on frons rectangular ..... *C. festivum*

*Chrysotoxum montanum* sp. nov., *C. orthostylum* sp. nov. (femora black basally), and *C. vernale*

Females – change step 37

37(36). Pro- and mesofemora dark brown to black at least basally.

- a (b). Abdomen slender, 2× longer than wide; posterior corners of terga 3 and 4 developed into thorn-like processes (Fig. 12B) ..... *C. orthostylum* sp. nov.
- b (a). Abdomen more oval, 1.6-1.7× longer than wide; posterior corners of terga 3 and 4 not developed into thorn-like processes, or very inconspicuously developed.
- c (d). Pollinose maculae on frons triangular ..... *C. montanum* sp. nov.
- d (c). Pollinose maculae on frons rectangular ..... *C. vernale*