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Pollen morphology and the flower visitors of *Chaerophyllum coloratum* L. (Apiaceae)

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Abstract – The pollen features of *Chaerophyllum coloratum* L., endemic to the Dinaric Alps, have been examined by both light microscopy and scanning electron microscopy in order to contribute to a better understanding of the taxonomic status of the species. Flower visitors have also been observed and analyzed with the aim of clarifying certain pollination aspects of the species including flower attractiveness especially to honeybees, and also in order to ascertain its contribution to the bee pasture. The pollen grains of *C. coloratum* are isopolar, radially symmetrical and medium sized. Polar axis (P) is 26.83±1.77 μm length, and equatorial diameter (E) is 9.17±0.57 μm length. P/E ratio amounts 2.90±0.10 indicating a perprolate shape. In an equatorial view, the grains are constricted in the equatorial region (bone-shaped), with obtuse polar caps. In polar view, they are triangular with obtuse angles and furrows in the sides of the triangle (interangular). The grains are tricolporate with three straight ectocolpi arranged regularly meridionally, of mean length 14.43±2.17 μm, each of which has one endopore. The characteristic internal thickenings around the protruding, clearly visible endopores (costae) in the constricted equatorial region are obvious in light microscopy. The ornamentation is psilate, irregularly rugulate ("cerebroid"), the exine surface is rather undulating. With regard to the observed flower visitors, the following pollination types occurred: melittophily, myophily, sapromyophily, cantharophily, and phalaenophily, and the most frequent pollinator was the honeybee.

Keywords: flower visitors, light microscopy, scanning electron microscopy, palynomorphology

Introduction

According to The Plant List (2010) the family Apiaceae belongs to the major group of Angiosperms, containing 347 genera and more than 3000 species and subspecies distributed worldwide (Tutin 1968). The genus *Chaerophyllum* L. (Apiaceae), the largest one in the subtribe *Scandicinae* belonging to the tribe *Scandiceae*, comprises about 45 species, native to Eurasia, North Africa and North America (Pimenov and Leonov 2004, Yilmaz and Tekin 2013). As opposed to other Apiaceae genera such as *Anethum, Angelica, Carum, Coriandrum, Daucus, Foeniculum, Levisticum, Peucedanum, Petroselinum, Pimpinella* etc., species of which are widely used in the Dinaric or Balkan countries as spice, medicinal or culinary plants (Tucakov 1996, Pelagić 2001), the use of *Chaerophyllum coloratum* has not been reported. But there are several species of this genus growing elsewhere that are recognized as either medicinal or food plants: in Turkey *C. bulbosum* L. (Polat et al. 2013) and *C. libanoticum* Boiss. et Kotschy (Demirci et al. 2007), in Mongolia *C. gracile* Freyn. Sint. (WHO 2013), in India – western Himalaya *C. villosum* Wall ex DC. (Singh 2012), etc.

The latest studies of *Chaerophyllum* species included their anatomy and morphology (Kowal et al. 1971, Kowal and Latowski 1973, Yilmaz and Tekin 2013, Reuther and Claßen-Bockhoff 2013), and the composition of essential oils and biological effects of physiologically active compounds (Gonnet 1985, Pedro et al. 1999, Baser et al. 2000, Dall'Acqua and Innocenti 2004, Nematollahi et al. 2005, Başer et al. 2006, Kürkçüoğlu et al. 2006, Kapetanos et al. 2008, Chizzola 2009, Lakušić et al. 2009, Razavi and Nejad-Ebrahimi 2010).

A fair number of palynological studies of the family Apiaceae date back to the mid-1950s. The first reports regarding the pollen morphology of some species were published by Erdtman (1952). Many Apiaceae species, originating from different regions or countries of almost all the continents, have already been palynomorphologically described. Thus, with a monograph about the pollen morpho-

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logy of Angiosperms, Cerceau-Larrival (1959) expanded the knowledge of the pollen features of Apiaceae of France and North Africa. From the same point of view, Ting (1961) described some American species, Cerceau-Larrival (1962a, b, 1963, 1965, 1981) Mediterranean species, Nilsson et al. (1977) North European species, Van Zeist et al. (1977) Asian species, Punt (1984) Northwest European species, Chester and Raine (2001) South European and North African species. In most cases, the researchers investigated species indigenous to a local area, region or country. For instance, Perveen and Qaiser (2006) studied species from Pakistan, Yousefzadi et al. (2006) and Amjad and Akkafi (2012) species from Iran, Pehlivan et al. (2009), Mungan et al. (2011), Dogan Guner et al. (2011) and Yilmas and Tekin (2013) those from Turkey and De Leonardis et al. (2008, 2009) species from Italy. However, only a few palynological studies concerning the genus *Chaerophyllum,* including the following 7 species have been carried out: *C. astrantiae* Boiss. & Balansa*, C. aureum* L.*, C. bulbosum* L.*, C hirsutum* L., *C. reflexum* Lindl., *C. villosum* Wall. ex DC. and *C. temulentum* L. (Punt 1984, Chester and Raine 2001, Perveen and Qaiser 2006, Yilmaz and Tekin 2013).

According to the available literature data, there is only one published paper about *C. coloratum*, concerning the composition of essential oils of ripe fruits and umbels (Vajs et al. 1995). However, the pollen features of this species have previously been quite unknown. Pollination biology of the Apiaceae related to flower preferences of insects has also received little attention, and has been studied mostly in economic plants and only a few wild species: *Heracleum sphondylium, Seseli farrenyi*, *Thaspium* sp, *Zizia* sp. and *Daucus carota* (Bell and Lindsay 1978, Lindsey 1984, Lindsey and Bell 1985, Lamborn and Ollerton 2000, Langenberger and Davis 2002a, b, Rovira et al. 2004, Zych 2006).

The detailed description of the pollen characteristics of this taxon in the present study contributes to taxonomic and melissopalynological research as well as to the pollen atlas of the region. By providing evidence about the flower visitors, the current study aims to examine certain pollination aspects of the species including flower attractiveness, especially to honeybees, and also to establish its contribution as a melliferous plant to bee pastures.

Material and methods

Distribution of the study species and flower morphology related to pollinator attraction

Chaerophyllum coloratum L. is an endemic plant, geographically restricted to the Dinaric Alps (Croatia, Bosnia and Herzegovina, Montenegro, Kosovo, Albania), and growing in sunny and dry habitats, amongst shrubs, on rocky grassland and similar Mediterranean-climate terrain (Šilić 1990). The plant is protected by the National Law of Montenegro (Official Gazette of RM, No. 76/06), and is classified as a species of international importance (Stevanović et al. 1995). Based on the available literature data (Rohlena 1942, Šilić 1990, Bulić 1994, Karaman 1997, Tomović 2007, Lubarda 2013), relevant herbarium collections (Herbarium of the University of Montenegro – TGU, Herbarium of Tirana University – TIR, Herbarium of the Faculty of Forestry, University in Banja Luka)**,** the Flora Croatica database (Nikolić 2014), and personal field observations, a distribution map of the species has been prepared and is presented in Fig. 1. As a basic layer, the UTM grid map of the Balkans was used.

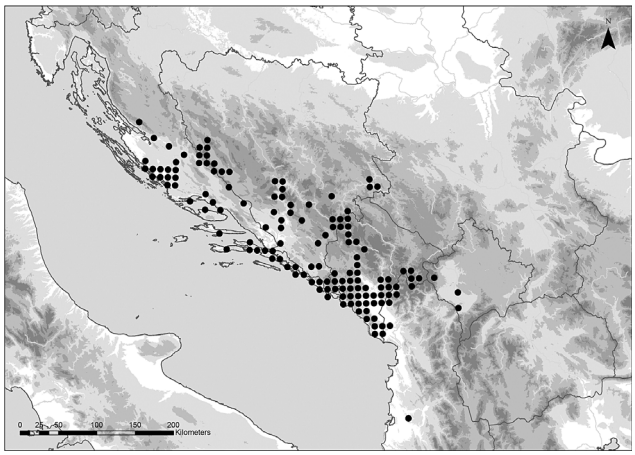

Fig. 1. Distribution map of *Chaerophyllum coloratum,* marked with dots.

C. coloratum L. is an annual to biennial aromatic herbaceous plant, up to 1 m tall (Fig. 2). The species has a hollow, furrowed, sparsely hairy, ribbed (branched in the upper part) stem, distinctive sheathing leaves pinnately divided 3 times into linear leaflets, and intensively yellow flowers ar-

Fig. 2. *Chaerophyllum coloratum*, the general *habitus* of the study species.

ranged in compound umbels. Tiny flowers recognizable by typically inflexed petal apex, are produced in mid-spring, followed by linear, aromatic non-fleshy fruits. Involucral leaves are absent, while leaves of involucelum are permanent and linear. Flowers in the outer and central part of the umbellet are perfect (hermaphrodite) and open first, whereas the inner florets are functionally male and open next. The male stage is defined as the pollen-presenting phase and the female stage as the phase with receptive stigmas. In the post-floral stage, the ovaries of all hermaphrodite flowers elongate to a certain degree irrespective of their fertilization status. Thus it is easy to distinguish even unfertilized hermaphrodite flowers from functionally male flowers, which may have rudimentary styles and ovaries, but never elongate after anthesis. The proportion of male flowers varies between branch orders. In some cases fruit set in the terminal umbel is nearly 100% and it decreases towards the lower branches (Reuther and Claßen-Bockhoff 2013). Peripheral flowers are usually slightly zygomorphic, while inner ones are actinomorphic. The stamens alternate to the petals, and arising from an epigynous nectary disk. Nectar is produced in both flower phases.

Sampling and analysis of pollen

The plant material was collected in Montenegro (territory of Podgorica Municipality, 42°22'46.08"N, 19°13'20.8"E) in May 2013. The flowers (anthers) were collected at full flowering stage from 10 plants of wild populations. The voucher specimen (No 504520) was deposited in the herbarium collection of the Faculty of Natural Sciences and Mathematics, University of Montenegro TGU.

The pollen morphology was examined by both light microscopy (LM) and scanning electron microscopy (SEM). For light microscopy, the pollen grains, prepared according to the standard acetolysis method (Erdtman 1952), were mounted in glycerine jelly and observed with a Leica DMSL microscope equipped with a digital camera (Leica DC 300) and Leica IM1000 software. For SEM study, the pollen grains were covered with gold (in BAL-TEC SCD 005 Sputter Coater, 100 seconds in 30 mA) and observed using JEOL JSM- 6390 LV electron microscope at an acceleration voltage of 20 kV. Pollen grains were photographed in polar and equatorial views, and observations and measurements were done on a sample of 50 or more grains for each morphological character.

The following features describing pollen grains were examined: size, shape, ornamentation, apertures, polarity, symmetry, length of polar (P) and equatorial axis (E) (in SEM) and exine thickness (in LM). The terminology used to describe the pollen grains is based on Erdtman (1971) and Punt et al. (2007).

Flower visitors

With the intention of detecting the flower visitors of *C. coloratum*, plants were observed at five sites at the territory of Podgorica Municipality (Zagorič 42°27'35"N, 19°15'40"E, Gorica 42°27'00"N, 19°16'49"E, Srpska gora 42°22'46"N, 19°13'19"E, Tuzi 42°20'47"N, 19°20'43"E, Bioče 42°32'14"N, 19°29'03"E), 3 times during anthesis, from 3rd to 20th May, during the daytime. Flower visitors were shot with digital camera Nikon Colorpix P500 and caught with an entomological net and entomological exhauster for further identification.

Results

Pollen morphology

The pollen grains of *C. coloratum* are isopolar, radially symmetrical and medium sized (Figs. 3, 4). The mean length of the polar axis (P) is 26.83±1.77 μm, and the mean of the equatorial diameter (E) is 9.17±0.57 μm. The ratio of the length of the polar axis to the equatorial diameter (P/E) averaged 2.90±0.10 making the pollen grain shape perprolate. When observed in polar view, the grains are triangular with obtuse angles (Figs. 3B, 4B), and furrows in the sides of the triangle (interangular), in equatorial section fossaperturate. In an equatorial view, the grains are bone-shaped, having equatorial constriction and obtuse polar caps (Figs. 3A and 4A).

The grains are tricolporate, with three straight colpi, each of which has one endopore positioned in the indentations between the mesocolpial lobes. Ectocolpi of mean length 14.43±2.17 μm are meridionally arranged, indistinctly tapering towards to the poles. They are narrow, slit-like, sunken and rather short ca 1/2 polar distance. In the side view, the inner and the outer contours of both mesocolpial and colpial side are slightly concave, as can be seen in

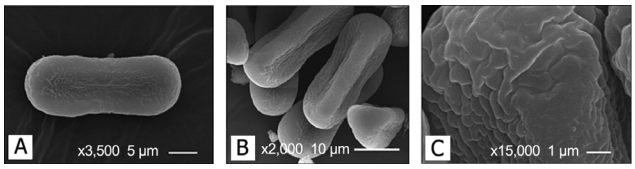

Fig. 3. Scanning electron micrographs of perprolate pollen grains of *Chaerophyllum coloratum*. In equatorial view, the pollen grains are constricted in the equatorial region (bone-shaped), with short straight furrows (colpial side – A; mesocolpial side – B). In a polar view (B), the grains are triangular, with the furrows in the sides of the triangle (interangular). A detail of undulating exine surface showing psilate, irregularly rugulate ("cerebroid") ornamentation (C).

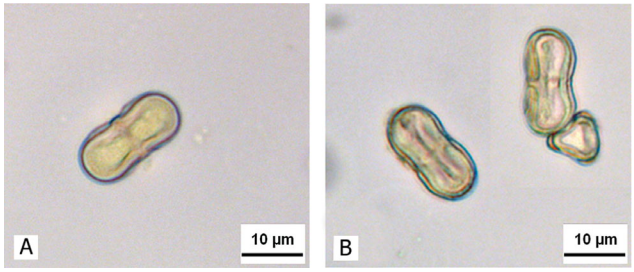

Fig. 4. Light microscopy micrographs of *Chaerophyllum coloratum* pollen grains having protruding clearly visible pores in the constricted equatorial region: equatorial view of colpial side (A) and mesocolpial side (B). In polar view (B), the grains have triangular outline.

light microscopy (Fig. 4A). The characteristic internal thickenings surrounding the protruding clearly visible endopores (costae) in the constricted equatorial region are obvious in light microscopy (Fig. 4B).

The sculpturing pattern, clearly visible in SEM, is psilate, irregularly rugulate ("cerebroid") (Fig. 3C). The exine surface is rather undulating and exine thickness averaged 0.95±0.15 μm.

Flower visitors

Flower visitors observed on this plant species could be classified as primary and secondary pollinators or accidental visitors (Figs. 5–7) belonging to all four insect orders: Hymenoptera (bees, wasps, and ants), Diptera (true flies), Lepidoptera (moths) and Coleoptera (beetles). Regarding the observed flower visitors, the following pollination types occurred: melittophily including pollination by honeybees (Fig. 5A), myophily (Figs. 5B, C, D) and sapromyophily including specialized and non-specialized fly pollinators (Fig. 5E), phalaenophily (moth pollination) (Fig. 5F) and cantharophily (beetle pollination) (Figs. 6A, B, C, D). Snails (Fig. 7A), ants (Figs. 7B, C) and spiders (Fig. 7D) were also observed but they are not associated with the pollination process.

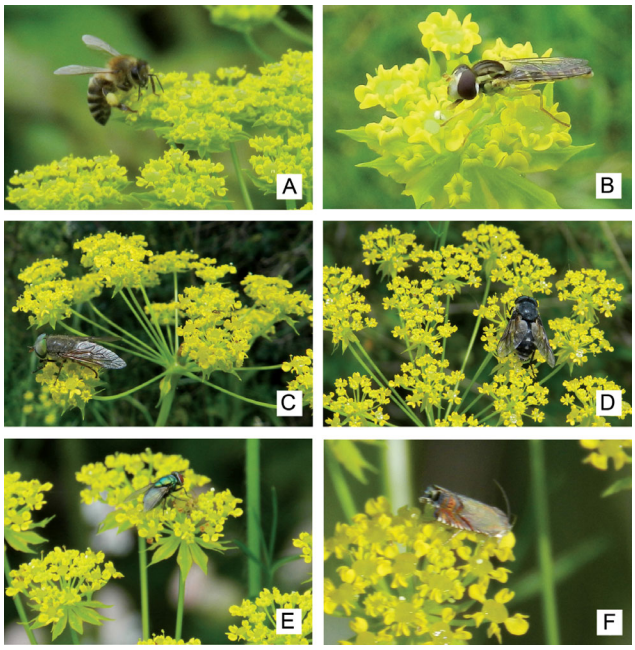

Fig. 5. The flower visitors of *Chaerophyllum coloratum:* A – Apidae (*Apis mellifera*); B – Syrphidae (*Sphaerophoria* sp.); C, D – Tabanidae (C – *Tabanus* sp., D – *Dasyrhamphis* sp.); E – Calliphoridae (*Lucilia* sp.); F – Lepidioptera (Tortricidae).

The flower visitors of *C. coloratum* were hymenopterans (*Apis mellifera*, Fig. 5A; and ants: *Camponotus vagus*, *C. aethiops*, Fig. 7B, and *Crematogaster sordidula*, Fig. 7C), dipterans (mostly true flies: Calliphoridae – *Lucilia* sp., Fig. 5E; Syrphidae – *Sphaerophoria* sp. and Tabanidae – *Tabanus* sp., *Dasyrhamphis* sp., Fig. 5B) coleopterans (mostly Mordellidae – *Mordella brachyura,* Fig. 6D; Cantharidae – *Cantharis* sp., Fig. 6B; and Cetoniidae – *Cetonia aurat*a, Fig. 6C, and *Oxytrea funesta* Fig.6A), and the least frequent were butterflies – moths (Fig. 5F) and neuropterans.

The majority of the visitors preferred the staminate phase of umbels, ants preferred the female phase, while coleopterans and bees visited flowers during both phases. Usually, inflorescences were routinely visited by certain insects individually, but in some cases, such is *Mordella brachyura,* many individuals were observed searching for nectar on a single inflorescence at the same time.

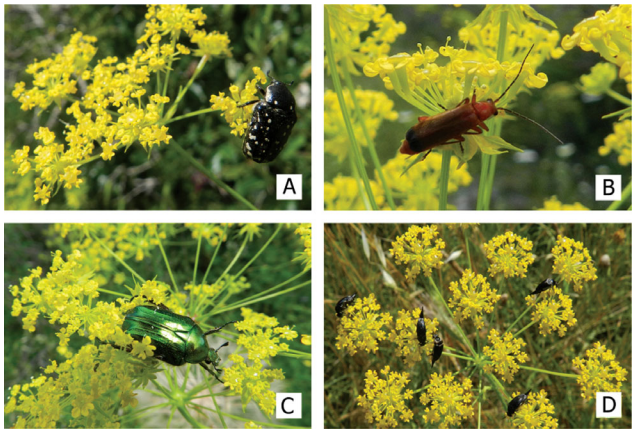

Fig. 6. Coleopteran pollinators of *Chaerophyllum coloratum*: Cetoniidae (A – *Oxythyrea funesta*, C – *Cetonia aurata*); B – Cantharidae (*Cantharis* sp.), D – Mordellidae (*Mordella brachyura*).

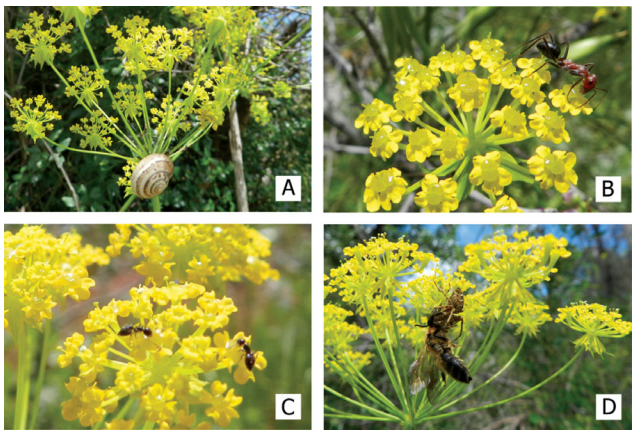

Fig. 7. Accidental visitors of *Chaerophyllum coloratum*: Gastropoda (A); Formicidae: *Camponotus vagus* (B) and *Crematogaster sordidula* (C); *Aranea* (D).

Discussion

Pollen grains of Apiaceae species are mostly stenopalynous, radially symmetrical, isopolar and prolate to perprolate in shape (Yousefzadi et al. 2006, Erdtman 1952). With respect to aperturation, they are generally tricolporate possessing three slit-like ectocolpi, each of which has one pore, and very distinctive and broad band-like costae (Punt 1984, Perveen and Qaiser 2006). As pointed out elsewhere, the tectum is commonly striate-rugulate or simple striate (Punt

1984) or, as stated by Perveen and Qaiser (2006), psilate to sparsely or densely granulate.

There are different criteria for classification of the pollen grains of Apiaceae, such as P/E ratio, exine sculpturing pattern, etc. For instance, Cerceau-Larrival (1962) proposed 5 pollen types occurring within the family based on shape index: subrhomboidal (type 1, P/E: 1–1.5), subcircular (type 2, P/E: 1–1.5), oval (type 3, P/E: 1.5–2), subrectangular (type 4, P/E: 2), and equatorially constricted (type 5, P/E: over 2). According to this classification, pollen grains of *C. coloratum* fit into type 5.

Perveen and Qaiser (2006) investigated the pollen of 50 species representing 27 genera of Pakistani Apiaceae and on the basis of tectum features distinguished three distinct pollen types: *Bupleurum gilessii*-type, *Pleurospermum hookeri*-type and *Trachyspermum ammi*-type. The authors analyzed two *Chaerophyllum* species – *C. reflexum* Lindl and *C. villosum* Wall. ex DC and classified them into *Bupleurum gilesii* type characterized by a striate-rugulate tectum. Based on their results, pollen characters such as exine thickness, colpal membrane and the outline of a pollen grain seen in polar view (amb) are of little taxonomic value. The mentioned authors reported fossaperturate and perprolate pollen grains in *C. reflexum*, but planaperturate and prolate grains in *C. villosum*. In view of the above, pollen grains of *C. coloratum,* described as psilate, irregularly rugulate ("cerebroid") and fossaperturate, are more like those of *C. reflexum*.

Punt (1984), who thoroughly studied the palynomorphology of the North European Apiaceae, recognized 50 pollen types. The author pointed out that ornamentation features are of little value in pollen identification among the family members. By analyzing exine structure, this author classified *Chaerophyllum* pollen into 3 types: *C. hirsutum* type (including *C. hirsutum* and *C. temulentum*)*, C. bulbosum* type (*C. bulbosum*) and *C. aureum* type (*C. aureum*), all three having a psilate, irregularly rugulate tectum. The first type includes grains with distinct colpi 1/2 to 2/3 of polar distance, columellae shorter at the equator than at the poles, and the tectum smooth or very slightly undulating in the equatorial area, "cerebroid" in SEM. The second type, involving small grains of mean polar axis length of 28 μm, is characterized by indistinct and very small columellae in the mesocolpial region distincly increasing in size towards the poles, and a thin tectum distinctly undulating in the equatorial region, also "cerebroid" in SEM. In the third type, the endoaperture is a short colpus, columellae short but distinct at poles increasing in length and decreasing in width towards shoulders and equator, and tectum slightly undulating in equatorial area. The results of the present study concerning the characteristics of the exine surface of *C. coloratum,* correspond to those of Punt (1984) who found a similar sculpturing pattern in the *C. bulbosum* and *C. hirsutum* types, but to some extent a different pattern in *C. aureum,* whose tectum has also been described as psilate, irregularly rugulate, but not designated as "cerebroid*"*.

The characteristics of the pollen grains of *C. coloratum* with respect to type, number and position of apertures, as well as colpi length, correspond to those of *Chaerophyllum* species as described by Punt (1984). The main palynomorphological features that distinguish *Chaerophyllum* species from each other are *size* and the shape index **(**P/E). Pollen grains of *C. coloratum* are more similar in size to those of *C. temulentum* (average 28.8 μm), *C. aureum* (average 26.5 μm) and *C. bulbosum* (average 28 μm), than to those of *C. hirsutum,* which are larger, with an average *size* of 35.5 μm. In a polar view, *C. coloratum* grains are triangular, as in the three above-mentioned pollen types, whereas in an equatorial view, inner and outer contour of both, colpial and mesocolpial sides, is concave without any sexine extension above the endoapertures, as has been observed in *C. hirsutum* pollen type. Notwithstanding the generally recognizable shape at the family level, the P/E index may be an important interspecific distinguishing parameter. Hence, in *C. temulentum*, *C. bulbosum*, *C. aureum* and *C. hirsutum* this ratio ranges from 2.16 to 2.46, while in *C. coloratum* it is higher (2.90), since the pollen is more constricted at the equator. This pronounced equatorial constriction contributing to the higher P/E ratio, causes a distinctive bone-shaped outline that makes *C. coloratum* pollen recognizable. This shape is pronounced to a lesser extent in the majority of other species within the genus, as opposed to *C. hirsutum* whose grains are sometimes more elliptical.

Within the large diversity of flower visitors, due to the open flowers with exposed nectar-secreting gland and sexual parts, the family Apiaceae has usually been regarded as non-specialized in terms of pollination biology (Zych 2006). According to Niemirski and Zych (2011), Umbelliferae are mostly visited by flies, but may also be pollinated by beetles, bees or other hymenopterans. Flowers are visited by a wide range of insects, but not all of them are equally effective at transferring and depositing pollen. Bell and Lindsay (1978) reported that only four species of bumblebees (*Bombus* spp.), and some other hymenopterans, were seen pollinating *Angelica* species. Niemirski and Zych (2011) observed 72 insects from 7 taxonomic orders visiting flowers of the same species, of which only 30% had special pollen-carrying structures. Grace and Nelson (1981) indicated that out of 80 insect taxa that visited *Heracleum spondylium* and *H. montegazzianum* flowers, only 31 carried a significant pollen load. Zych (2007) reported that the most efficient pollinators of *H. spondylium* were dipterans (blow flies *Lucilia* sp.*;* hoverflies *Eriozona syrphoides, Eristalis* sp.*,* and *Meliscaeva cinctella,* and true flies *Phaonia angelicae* and *Thricops nigrifrons*), bumblebee (*Bombus terrestris*), and beetles *Stenurella* and *Dasytes.*

The current study suggests that *C. coloratum* floral characteristics, such as colour, scent, the availability of pollen and nectar, flower arrangement in flat-topped compound umbel, etc., serve as attractants to diverse insect pollinators belonging to the orders of Hymenoptera, Diptera, Lepidoptera and Coleoptera. These taxonomic groups differ inpollination efficiency, the coleopterans being known as the least specialized pollinators (Mačukanović-Jocić 2010) despite the number and abundance of beetle species observed in the present study and some previous investigation carried out on Apiaceae (Zych 2006). Ants, snails and spiders were also observed on *Chaerophyllum* flowers but, since they are not associated with the pollination process primarily due to

their inability to fly and ineptness at transferring pollen, they can be considered accidental visitors. In a similar study concerning the pollination of *Heracleum sphondylium*, Zych (2006) identified Syrphidae and Calliphoridae as the most important pollinators. From the pollination point of view, *C. coloratum,* being an aromatic plant, is most appealing to Apidae, followed by Syrphidae, and to a lesser extent to carrion flies. According to the present study, the most frequent visitor to *Chaerophyllum* flowers was the honeybee, which was also registered as an important pollinator of some other Apiaceae, such as *Trachymene incise* (Davila and Wardle 2002) and *Carum carvi* (Langenberger and Davis 2002b), but not in the case of *Heracleum sphondylium* (Zych 2006). Unlike Zych (2006) who noticed a few butterflies from the families Nymphalidae, Papilionidae and Pierideae visiting *Heracleum* flowers, the present study mentions only moths with short mouthparts feeding on *Chaerophyllum* flowers. The low visitation rate of butterflies can be explained by their long proboscises, which are more suitable for nectar-sucking from deep floral tubes (Krenn et al. 2005).

Due to the fact that the present study was not based on long-term monitoring of pollinators' activity and pollen load analysis, questions addressing the diversity of flower visitors need to be answered. Another peculiarity of umbellifers is their dichogamy that leads to the presence of temporally unisexual flowers. Such "temporal dioecism" may influence the behaviour of flower visitors (Zych 2007). In the case of *Heracleum spondilum* (Zych 2007), flowers in the staminate phase were visited significantly more often than those in the pistillate phase. This preference is especially noticeable in Dipterans. However, a study of fly pollination of *Angelica sylvestris* indicated that these insects did not show any preference regarding plant sexual phases (Niemirski and Zych 2011). Results of the current study on *C. coloratum* showed that a majority of the visitors preferred the staminate phase of umbels, ants preferred the female phase, while coleopterans and bees made visits during both phases.

The current paper is a part of an extensive research project into apiflora and its impact on the bee pasture of the selected region. The provided palynomorphological information contributes towards a better understanding of the taxonomic status of *C. coloratum*. Additionally, the data obtained will be helpful in the forthcoming melissopalynological analysis that will facilitate the identification of the floral sources of different kinds of honey, and will enable the creation of the pollen atlas of the main taxa found in honey types produced in the Balkans. Qualitative analysis of pollen in honey originating from the research area would allow the determination of its botanical and geographical origin (assuming the entire pollen spectrum being consistent with the flora of a particular region). By determining the spectrum of pollen types in honey and calculating the relative frequency of *C. coloratum* as the respective percentage with respect to the total number of pollen grains, it would be possible to confirm or deny the attractiveness of this species to honeybees.

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