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FIRST DESCRIPTION OF *Cypripedium* AND *Cypripedium calceolus* var. *citrina* B. HERGT IN ROMANIA: MORPHOLOGY, POLLINATION AND CONSERVATION STATUS

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Abstract

Cypripedium genus is represented by 59 species and nothospecies (species of hybrid origin), mainly distributed across North, Central and South America, Europe and Asia, but absent from Madagascar and Australia. The most important characteristic of this genus is their showy and large labellum, which transformed into the specific slipper-shaped, vividly coloured, inflated pouch. In Romania, the genus is represented by one species only, Cypripedium calceolus. The article contains the first description of a newly discovered population in Prahova County, as well as a complete illustration of a Cypripedium calceolus flower pollinated by a female bee of the Lasioglossum genus. The flowers are passive traps pollinated by bees of particular type and size. The article describes in detail different mimicry (deceit) pollination strategies employed by the orchid in order to exploit its insect pollinators (generalized food deception mimicry, shelter mimicry, nest/brood-site mimicry, rendezvous attraction). Using advanced techniques of ultra-macro photography, details of floral organs are illustrated in order to describe in great detail the intriguing deceptive pollination mechanisms and mimicry strategies employed by the Slipper Orchids to attract their highly specific and rather scarce pollinating insects. Along with the detailed description and illustration of the common single-flowered plants, we also describe rare Cypripedium calceolus f. *biflorum plants in Prahova County and present for the first time in Romania, a unique Cypripedium calceolus* var. *citrina individual, found in 2019, in Suceava County.*

Key words: *Cypripedium calceolus, Prahova, pollination, Lasioglossum, conservation.*

INTRODUCTION

Orchidaceae Juss. is the second largest family of flowering plants or Angiosperms/Angiospermae Lindl., 1830 (the major family is the Compositae or the Asteraceae Brecht. & J. Presl, 2017, with 36,701 accepted species). Plant databases describe *899 orchid genera*, comprising between *28,000* and *35,000 species of orchids* (Dressler, 1993). In Romania, the family covers three subfamilies, Cypripedioideae Kostel., 1831, Orchidoideae Lindl., 1826 and Epidendroideae Lindl., 1821. *Cypripedium calceolus* L. is the type species of the subfamily Cypripedioideae Kostel., 1831, synonym of Cypripedioideae Garay, 1960, tribe Cypripedieae Lindl., 1826, subtribe Cypripediinae (Lindl.) Meisn., 1842, genus *Cypripedium* L. 1753, section *Cypripedium*, subsection *Cypripedium*. Cypripedioideae is a *monophyletic* family (composed of organisms descended from a single ancestor) and consists of five genera: *Cypripedium*, *Mexipedium*, *Paphiopedilum*, *Phragmipedium* and *Selenipedium*, widely distributed throughout the temperate regions of Eurasia and North America, also in Central and South America (all the way to Southern Brazil and Bolivia), tropical Asia (from India to Taiwan), South-east Asia (from Indo-China to the Philippines, New Guinea and the Solomon Islands) (Cribb, in Pridgeon et al., 2009). The subfamily has no representatives in Madagascar and Australia. Cypripedioideae can be found from the sea level, up to the highest mountain peaks, as some species of *Cypripedium himalaicum* Rolfe and *Cypripedium tibeticum* King ex Rolfe) have been reported to grow up to 4900

m a.s.l., in the Himalayas (Maity et al., 2002). In Europe, Cypripedioideae subfamily is represented by *one genus* only, the *Cypripedium* L. genus. *Cypripedium* L. genus is represented by *59 species* and *nothospecies* (species of hybrid origin), mainly distributed across North and Central America (Guatemala and Honduras), Europe and temperate Asia (Russia, Siberia, China - the Himalayas, Mongolia, Korea, Myanmar and Japan), Sakhalin and Aleutian Islands (Cribb, in Pridgeon *et al.,* 2009). In Romania, the genus is represented by one species only, *Cypripedium calceolus* L.

The aims of the present study are: (1) to describe in detail the main morphological characteristics of a newly found population of *Cypripedium calceolus* in Prahova County, by using advanced techniques of ultra-macro photography; (2) to explore the intriguing deceptive pollination mechanisms and mimicry strategies employed by this orchid to attract its highly specific pollinating insects; (3) to summarize its conservation status and (4) identify the main threats it faces in Romania, (5) suggesting conservation strategy measures to ensure the future protection of this species.

MATERIALS AND METHODS

1. Study species: *Cypripedium calceolus* found in Prahova County is the object of this study.

2. Morphometric/biometric analyses: In order to describe this newly found population as comprehensive as possible, a number of morphological characters were taken in consideration and morphometrically analysed. Prahova County population was compared to a control population found in Harghita County.

3. Study sites and populations counts: Studies were conducted in three sub-alpine sites on wet to dry, calcareous substrates: (a) Valea Rea - Sinaia, Baiu Mountains, Prahova County; altitude 700-1000 m a.s.l.; deciduous woodland; 40-45 individuals (De Angelli & Anghelescu, 2020); (b) Depresiunea și Munții Ciucului, Natura 2000 protected area, code: ROSPA0034, Harghita and Bacău Counties; altitude 600-1100 m a.s.l.; pine-beech forests; 150-200 individuals (Anghelescu et al., 2021b); (c) Rodnei Mountains National Park (national park and protected area, category II IUCN), code: RO02 (EUNIS), Bistrița Năsăud and Maramureș Counties; altitude 630-990 m a.s.l.; mixed (coniferous, deciduous) woodland, meadows; several hundred individuals.

4. Pollination monitoring: Monitoring was made for a total of 8.5 hours in 2017 and 2018, in Prahova County, 2.3 hours in 2019, in Suceava County and 4.5 hours in 2021, in Harghita County. The observer (NA) was initially located approximately 2-3 m above the subjects (groups or individual plants). Once the insects were observed to have entered the labellum, the observer (NA) approached the flowers to a distance of about 1-10 cm. The behaviour of visitors was recorded on digital photographs, from the moment they entered the labella, until they left the flowers. No insects or plants were collected or harmed in any way.

5. Digital photographic equipment: Individual plants were photographed with body cameras: Canon 5D Mark III, Nikon D3 and Nikon D850. lenses: Nikon Micro NIKKOR 60 mm, Venus Optics Laowa 100 mm 2X Ultra Macro and Canon MP-E 65 mm 1-5x Macro Lens. Additional equipment: Manfrotto Tripod, Litra Torches 2.0s. Adapted Helion FB tube was used for Automated Focus Bracketing. Images were analysed using Adobe Photoshop CC 2021, Helicon Focus and Zerene Stacker Software.

RESULTS

1. Type: *Cypripedium calceolus* is a long-lived herbaceous, terrestrial, perennial, geophyte. It is considered one of the most attractive terrestrial species of orchids in Eurasia.

2. Geographic range: *Cypripedium calceolus* can be found in northern England and Scandinavia, eastern France, Northeast Spain, Germany, Northern Italy and Eastern Europe eastwards to European Russia, all the way to Primorye Region and Sakhalin Island, and from Crimeea to Siberia, Mongolia, Northeast China, Korea and Japan's Rebun Island (Averyanov, 2000). A small population of about forty *Cypripedium calceolus* individuals has been recently found to occur in an isolated area in Algeria (Nemer et al., 2019). This species is totally absent in the evergreen, warm and humid Mediterranean zone (Terschuren, 1999).

3. Binomial name: The official, accepted name of this species was published in 1753, by the Swedish botanist Carl Linnaeus/Carl von Linné (1707-1778), author abbreviated as 'L.'.



Figure 1. *Cypripedium calceolus* L. f. *biflorum* Rouy 1912 occurring within Prahova County population; note the fine trichomes and the bifid/bidentate *synsepalum* © NA

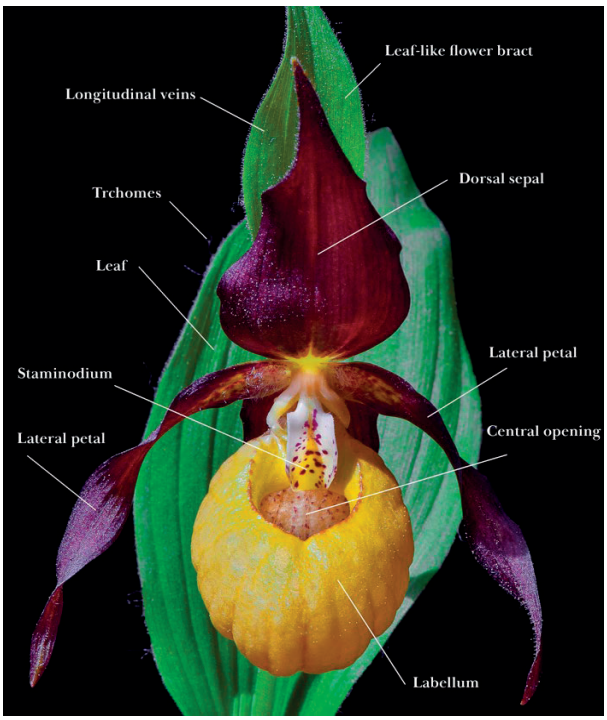


Figure 2. *Cypripedium calceolus* - Schematic explanation of the perianth morphology; the leaves and bract are covered in glandular hairs - *trichomes*, which secrete *cipripedin*, a quinone that causes allergic reactions (dermatitis) © NA



Figure 3. The unique individual, *Cypripedium calceolus* L. var. *citrina* B. Hergt, 1899 found Suceava county © NA



Figure 4. Flower detail - the flowers appear entirely yellow, totally missing the *anthocyanins* (red-purple pigments). The hover flies of the family Muscidae Latreille, 1802 *hoover* the oily exudates on the shiny surface of the labellum, attracted by the fruity smell of the flower © NA

4. Etymology: The *generic name*, *Cypripedium*, has its origins in Greek mythology in the words *Kýpros* (Cyprus), which refers to the other name of Aphrodite, the *Cyprian* (she was born in Cyprus), and *pédilon* (*pedium* in Latin), which means *slipper* or *open shoe*, a reference to her *small slipper*, hence the vernacular names of this genus, the Slipper Orchids or the Lady's Slipper Orchids. The *specific epithet*, *calceolus*, also has its origins in Ancient Greek, meaning a *small pebble* or *calcar*, thus referring to the *calcareous soils* where this species usually occurs.

5. Morphology. **Stem**: In orchids, *no primary root* is formed. The *underground organs* as well as the *aerial organs* are all produced by the different parts of the *stem*. **Hypogeal stem**: The *underground part of the stem* forms a long, *branchy* and *thick*, *horizontal* ***rhizome***. **Adventitious roots**: The ***rhizome*** produces *adventitious roots*, some covered in *root hairs* or *rhizoids*. They originate either from the axillary buds of the scale leaves, or directly from the main stem (axis) of the rhizome, developing just below a node. From the *terminal buds* of the branchy rhizome, the *aerial organs*, the *leaves* and the *aerial (epigeal) part of the stem* emerge. **Epigeal stem**: The *flowering stem* is slender and tall, glandular-pubescent. **Basal leaves**: 3-5 yellowish-brown scaly sheaths emerge basally. **Cauline leaves**: 3-5 vivid-green, alternate or spirally oriented, widely elliptical, more or less densely coarsely hairy and glandular, acuminate leaves are formed. They grow along the length of the stem, sheathing it. **Flower bracts**: Large, erect and leaf-like, lanceolate to ovate, glandular, acuminate, usually longer than the ovaries and shorter than the flowers. **Trichomes**: Dense glandular hairs, which cover the stem, leaves, bracts and perianth parts (Figure 1). **Inflorescence**: It is usually a large, single-flowered *raceme*. Occasionally, some plants may produce 2-3 flowers. **Individual flower**: It is the largest flower in all European genera, very brightly coloured and extremely attractive (Figure 2). **The Perianth**: It consists of two whorls of 3 sepals (2 lateral & 1 median) and 3 petals (2 lateral & 1 median - the labellum). **Sepals**: They are crimson-red, elongated, oval-lanceolate and *sinuate* (slightly twisted). The lateral sepals are fused and form a *synsepalum* with a forked-tip (bidentate), rarely entire (Averyanov, 2000), which hangs downward, below the labellum. The median (dorsal) sepal is broad, lanceolate, either erect or bent slightly forward over the central opening of the labellum. The margins of the sepals are pubescent, with longer hairs at the base and shorter, denser hairs (trichomes) toward the tips. **Petals**: The *two lateral petals* are usually similarly coloured, crimson-red or dark brown. They are ribbon-like, occasionally slightly spirally twisted toward apex, linear-lanceolate and held horizontally, more or less densely hairy/downy basally (trichomes). **Labellum**: The median, modified petal is bright-yellow, ovoid and large, very attractive. It has the characteristic shape of a *pouch* or *shoe*, filled with air. The high contrast between the brownish perianth and the golden labellum enhances the attractiveness of the flowers. On the top of the pouch, there is an oval exit-like, termed as the *central opening*, with its margins/rim turned inward and covered in a slippery substance. At the base of the opening, there are *two lateral (auriculate) openings*, which are formed on each side of the gynostemium (Figure 5). The inner walls of the labellum are lined with long, translucent *trichomes*, pointing toward the two lateral openings. Their basal, epidermal cells contain *anthocyanins* and are red or purple-pigmented, mimicking drops of reddish nectar scattered on the bottom of the pouch. The *trichomes* secrete little drops of oil, but are not usually chewed or eaten by the insects (Figure 6). However, Stoutamire (1967) reported chewed hairs. Laterally, the labellum walls present translucent patches termed *windows*, which play an important role in insect pollination (Figure 7). They are composed of colourless, transparent epidermal cells, that lack both pigmentation and intracellular spaces (Claessens & Kleynen, 2011). These are designed to allow sufficient light into the pouch to keep the trapped, diurnal insect active long enough to emerge from the pouch (Figure 8). Without the light from the windows, the trapped insect would likely go to sleep and perhaps die without escaping. **Spur**: No spur is present. **Nectar**: No nectar is secreted. **Scent**:

The flowers emit a soft, rose-like or fruity, orange-like perfume, depending on the soil composition. It has been shown that only the sepals and lateral petals are the main sources of scent production.

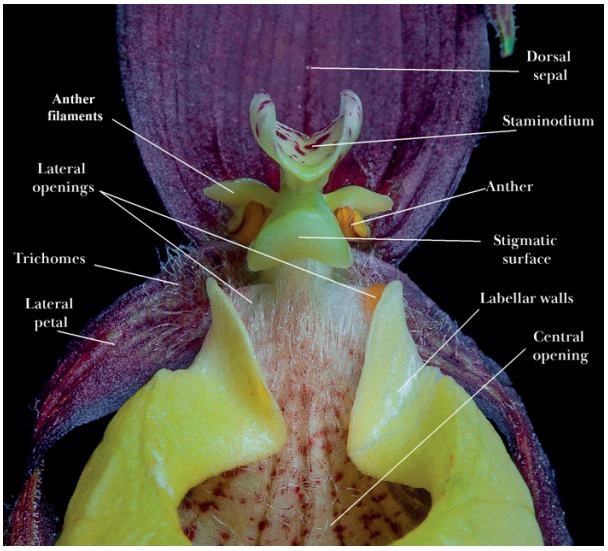


Figure 5. Schematic explanation of the reproductive organs, situated below the staminodium, flanking the lateral openings if the labellum © NA



Figure 6. Detail of the translucent, oil secreting trichomes that cover the inner surface of the labellum. Note the *purple-red pigmented basal epidermal cells* that mimic *drops of nectar* and the individual pollen grains © NA

The labellum emits a very weak chemical stimulation. Analyses of the floral fragrance showed that the volatile compounds contain large quantities of *acetate* (*dodecyl acetate*), chemical compounds that are also found in pheromones used by many bees and bumblebees (Tengö & Bergström, 1977).

The Gynostemium: The gynostemium is yellowish-green, thick, three-lobed with a short base. It is formed by the partial fusion of the androecium (male) and gynoecium (female).



Figure 7. Detail of the translucent, lateral windows that allow the light to penetrate the inside of the labellum. Note the *prominent venation* of the thin, labellar walls © NA

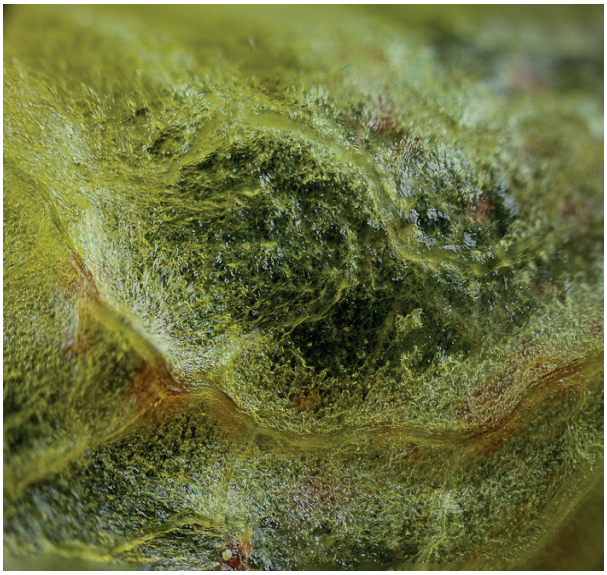


Figure 8. Detail of a window composed of a thin layer of unpigmented epidermal cells. The transparent patches help orientate the insects towards the lateral openings, a phenomenon known as *phototaxis*. © NA

Androecium: Is represented by *two fertile stamens*, making *Cypripedium calceolus* the only *diandrous* orchid of the ***Dinadrae***, the other name given to the Cypripedioideae family. The stamens are placed laterally to the gynostemium, on two side lobes. **Anthers**: Each stamen carries a fertile anther. The

stamen filaments extend beyond the anther, forming a thorn-like acute termination. The anthers block the small, side openings found at the base of the labellum, laterally to the stigma.
Pollen grains: The anthers produce the *sticky* or *agglutinated*, *individual (free) pollen grains*, called *monads*, held together within the anther by a sticky fluid called *elastoviscin* (Pacini & Hesse, 2002).



Figure 9. Detail of the unique staminodium. It is tongue-shaped and points towards the inside of the labellum. It is marked with red patched of cells © NA

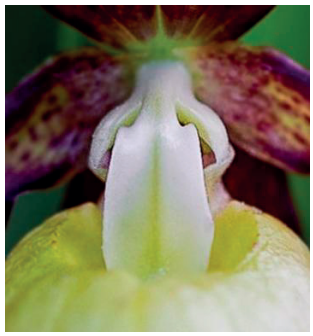


Figure 10. Detail of a rare, unpigmented, totally white staminodium. As a rule, staminodia vary significantly in the amount of pigmentation, which makes them more attractive to pollinators © NA



Figure 11. Close-up of the red pigmented epidermal patches that present stomata, in the staminodium groove © NA

Staminode: It resulted from the transformation of the third, degenerated, non-fertile anther, being placed on top of the gynostemium. It is yellowish-white, large, elongated, tongue-shaped (petaloid), oblong-ovate, presenting a central ridge, rounded at apex, with a wide, often thick base, borne on a short stalk (Figures 9-10). It has a bright, whitish-yellowish colour and is adorned with patched of pigmented epidermal cells that form a pattern of crimson-red spots and stripes that function as false nectar-guides (Figure 11).
On the adaxial (upper) side it presents many stomata inside the groove (Szlachetko *et al*., 2020). It is very slippery, tongue-like and points downwards toward the central opening of the labellum.
Gynoecium: The structure of the gynoecium is unlike any other European orchid. **Stigma**: Is located below the anthers and formed on a short pedicel (termed pedicellate) slightly curving downwards, tongue-shaped (Figure 12).

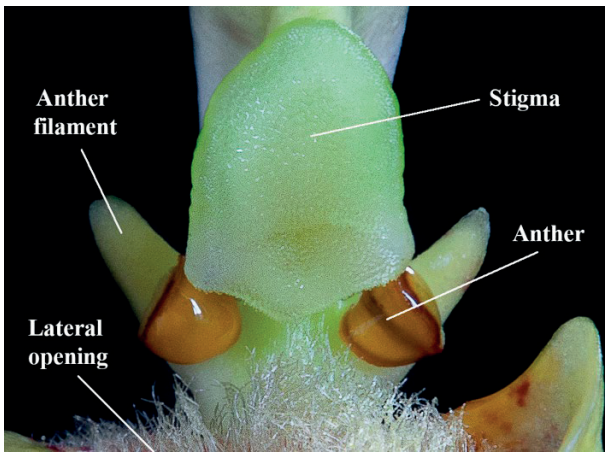


Figure 12. Detail of the two fertile anthers and the stigmatic, concave, heavily papillate surface © NA

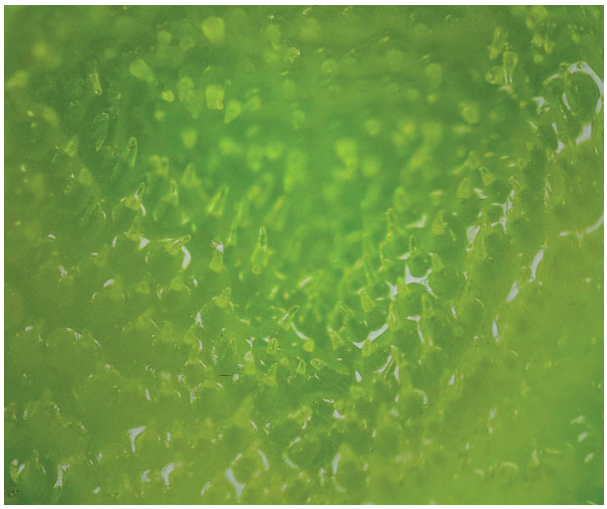


Figure 13. Clos-up of the conical, glandular papillae with their bases emerged in papillary, viscous exudate © NA

It is large, triangular, three-lobed, with a significantly larger middle lobe with a rounded apex, concave in the centre. In contrast to all other European orchids, *Cypripedium calceolus* has a dry stigma, which is not soaking in stigmatic fluid. On its surface, however, there

are minuscule, oblique, conical *papillae* that point downwards and backwards, surrounded by small amounts of viscous glandular/papillate exudate, which brush the pollen grains from the insects' backs (Figure 13). **Ovary**: Is green, slender, downy (covered in very fine hairs) and has a straight, shorth and thick pedicel. It is unilocular with parietal placentation (Szlachetko et al., 2020), slightly curved, but not twisted (Figure 18).

6. Morphomeric/biomeric analyses: All morphological measurements were undertaken in the field, between 2017-2021. In total, 18 morphological characters were measured directly. Morphological characters used for analysis included most of the characters used previously in Shirokov (2020). The quantitative measurements encompass all organs except the hypogeal parts of the stem (rhizome) (Table 1).

Table 1. Average values of the main morphometric characters of *Cypripedium calceolus* individuals in Prahova and Harghita Counties; the unique individual *Cypripedium calceolus* var. *citrina* was analysed in Suceava County. Note: all measurements were done in centimetres (cm) unless otherwise stated

Morphological character	*C. calceolus* Prahova	*C. calceolus* Harghita	*C. citrina* Suceava
Shoot height	21.8-55.5 (72.2)	22.3-58.7 (74.5)	22.2
Leaf length	12.25-16.5 (18.4)	12.5-17.2 (18.6)	7.8-11.3
Leaf width	6.9-9.8 (12)	6.8-10.1 (13.1)	4.2-5.7
Bract length	2.3-5.2 (6.2)	2.4-5.4 (6.7)	5.1
Bract width	1.7-2.7 (3.1)	1.7-2.9 (3.7)	2.8
Flower height	3.8-6.6 (8.6)	3.8-6.8 (8.9)	5.3
Flower width	3.8-5.5 (6.6)	3.9-6.2 (6.7)	3.2
Dorsal sepal length	3.5-4.8 (5.7)	3.5-5.1 (6.4)	3.9
Dorsal sepal width	1.4-1.5 (1.8)	1.4-1.6 (1.9)	2.3
Synsepalum length	3.4-4.7 (5.3)	3.4-4.9 (5.4)	2.4
Synsepalum width	1.4-1.6 (1.8)	1.4-1.8 (1.9)	1.9
Lateral petal length	4.1-5.8 (7.1)	4.0-5.8 (7.8)	3.7
Lateral petal width	1.3-2.6 (2.9)	1.4-2.7 (3.0)	1.1
Labellum length	2.4-4.2 (5.1)	2.3-4.7 (5.8)	2.8
Labellum width	1.5-2.4 (3.2)	1.5-2.8 (3.4)	1.9
Staminode length	0.4-1.2 (1.8)	0.3-1.4 (1.8)	0.5
Staminode width	0.3-0.9 (1.1)	0.3-0.9 (1.3)	0.3
Diameter of lip orifice	1.2-2.1 (2.6)	1.2-2.3 (2.8)	0.7

7. Chromosome numbers: 2n = 20, 22.

8. Flowering time: May-July, depending on the altitude. The flowers' longevity is estimated between 11-17 days, a considerable long time for a temperate orchid (Savina, 1964).

9. Pollination: **(a) Compatibility**: *Cypripedium calceolus* is a *self-compatible* species. **(b) Type of pollination - Allogamy**: In the wild, *Cypripedium calceolus* is an exclusively *allogamous*, *entomophilous* species, which depends entirely on insect pollinators for *seminal reproduction* (fruit set and seed production). **(c) Insect behaviour**: Five anthophilous insect species were recorded as flower visitors but most of them only alighted or rested on the flower. It has been shown that the bees belonging to Halictidae and Andrenidae families are the sole pollinators of some *Cypripedium* orchids. In Romania, only bees belonging to the genus *Lasioglossum* Curtis, 1833 (Family Halictidae) were found entering the labellum and subsequently escaping from the lateral openings, covered in a smear of sticky pollen grains. The average time spent inside the labellum was 1-3 min. **(d) Deceptive pollination mechanisms and mimicry strategies**: *Cypripedium calceolus* does not produce any rewards for the pollinating insects (edible nectar or pollen), hence its pollination mechanisms are based exclusively on ***mimicry*** and ***deceit*** strategies. Nilsson (1979) noticed that *Lasioglossum* bees became visually attracted (visual stimulation) from afar by the bright yellow labellum and by the patterns of the contrasting crimson-red spots that function as false nectar guides, mimicking the presence of food (nectar) - this type of pollination strategy is termed ***generalised food deception mimicry*** (Figure 14). At short distance, the approaching bees start to show an undulating flight, caused by the olfactory attractant (olfactory stimulation), the fruity scent of the flower, which contains acetates. Both *Lasioglossum* males and females secrete acetates in order to attract each other and mate (Przybyłowicz, 2012). It is supposed that *Cypripedium calceolus* flowers partially mimic the male pheromones and deceive the females to land on the labellum, by the promise of finding suitable mating partners - this type of pollination strategy is termed ***rendezvous attraction***, a precursor of ***sexual deceptive mimicry*** found in genus *Ophrys* (Anghelescu et al., 2021a).



Figure 14. A Holarctic species of hoverfly *Cheilosia scutellata* (Fallen, 1817) (Family Syrphidae Latreille, 1802), just landed on the slippery labellum, trying hard to get a grip on its slippery surface, before plunging inside the pouch © NA

Once landed, the insects loose grip of the extremely slippery, greasy, oily surface of the labellum and plunge straight into the golden pouch, which functions as a passive trap - this type of pollination strategy is termed a ***passive trap mimicry*** (Figure 15).



Figure 15. A female bee belonging to the genus *Lasioglossum* Curtis, 1833 (Family Halictidae Thomson, 1869), which just fell inside the labellum. After a few moments of confusion, the bee will eventually find its way out of the pouch © NA

After failing to find any nectar, they try to climb the walls of the labellum (impossible due to the inward turned rims/margins) or attempt to open their wings (impossible due to the lack of space).

The inner surface is oily and marked with red blotches and deep veins, which mimic a brood-site cavity (marked with nest tunnels or veins), thus inducing some females to search for a suitable brood-site. This type of pollination strategy is termed ***brood-site mimicry*** (Nilsson 1979; Li et al., 2006). The same phenomenon was observed in China's Sichuan Province where queen bumblebees searching for nest sites enter the labellum of *Cypripedium tibeticum* that mimic the dark entrance of a mouse hole, a common nest site for bees (Li et al., 2006). The oily hairs give the insects a good grip and serve as guides, facilitating their forward movement towards the two auricular, lateral openings. The *translucent windows* play an important part in guiding the female bees that instinctively follow the brighter patches of light that get larger near the anthers, a phenomenon called *phototaxis*. Insects of the right size (medium size) manage to climb the ladder of hairs and force their way through the narrow posterior openings, thereby touching the orchid's anthers. Pollen grains embedded in *elastoviscin* are deposited on the insect's body (thorax) (Figure 16). During a successive visit, after falling again inside another *one-way passage trap*, the sticky pollen grains will be scraped off by the downward pointing papillae of the dry stigma, successfully accomplishing the orchid's pollination. In some cases, various species of bees transform this *passive trap* into a warm, night-time *shelter*. During periods of rainy weather or cold nights, the ambient temperature inside the pouch may exceed the outside temperature by up to 3^0C during the early morning hours, so bees may use them as a sleeping or resting place - this type of pollination strategy is termed ***shelter mimicry***.



Figure 16. The pollination sequence of a *Cypripedium calceolus* flower by a solitary female bee belonging to the genus *Lasioglossum.* Climbing the ladder of oily trichomes and following the light guidance of the transparent labellar windows, the bee reaches one of the two lateral openings and squeezes its medium-sized body through. In the process its thorax gets smeared with a *layer of sticky pollen grains* (*monads*). In Romania, this is the first complete description and illustration of a *Cypripedium calceolus* flower being pollinated by a female bee of the genus *Lasioglossum.* © NA

10. Orchid mimics - Active camouflage

Despite the wide variety of mechanisms of deception displayed by the Slipper Orchids to attract and exploit their pollinators, it comes a time, when, orchids, in turn, may be exploited by other, very clever masters of mimicry: the goldenrod crab spiders, *Misumena vatia* (Clerck, 1757), (Family Thomisidae Sundevall, 1833). The young females, which temporary reside the orchid's flowers, are able to change and adapt their colour at will, a phenomenon termed as active camouflage.

These spiders change colour based on visual cues and spiders with impaired vision lose this ability (Insausti, 2012).

The standard colour of this spider is white. However, when they find a suitable yellow residence, they start secreting a liquid, yellow pigment into the body's outer cell layer, a process that requires 10-25 days to complete.

Thus, during the flowering period, when *Cypripedium* flowers are in full bloom and the bright golden pouches shine in the sun, the witty females become completely yellow (Figure 17).

Perfectly *camouflaged* from visual detection and with no need of a web (*Misumena* are webless spiders), they prey on any *naïve* insect that lands on the deceiving, golden trap (Chittka, 2001).



Figure 17. The perfect golden mimic, *Misumena vatia* uses the yellow labellum to hunt small insects. They are also known as *crab spiders* because of their unique ability to walk sideways as well as forwards and backwards. They use the *venom* from their *fangs* to immobilize their prey, although they are harmless to humans © NA

11. Mycorrhizal associates and nutrition: During its adult stages, *Cypripedium calceolus* develops numerous green leaves and uses photosynthesis to produce most of its necessary

carbohydrates, becoming *partially* to *fully autotrophic*. In the same time, similar to other green-leaved species, this orchid is likely to obtain some of its carbohydrates from an external source, *heteroterotrophically*, using a dual strategy that combines *photo-assimilation* and *myco-heterotrophy*, simultaneously or over time. This combined form of nutrition has been termed *mixotrophy*. Its *adventitious roots* and *rhizoids* are strongly colonised by *mycorrhizal hyphae* of the Tulasnellaceae family, for which this orchid presents a rather high specificity (Rasmussen, 1995). Recent studies also showed that *Cypripedium calceolus* is occasionally associated with other four species of saprotrophic fungi belonging to *Rhizoctonia* genus (Gebauer et al., 2016). These fungi species are mainly wood-rotting *saprotrophs*, which feed on decomposing dead organic matter, but some are *ectomycorrhizae* that simultaneously form *ectomycorrhizal associations* with the neighbouring trees, mainly oak (*Quercus robur* L.) and beech (*Fagus* L.) and other photosynthetic plants, from which they derive their carbohydrates and ultimately pass them to the orchid. In exchange, the *ectomycorrhizal fungi* pass water and minerals to the trees. The capacity of switching between fungal partners, i.e., from *Tulasnella* (fungi which form *ectomycorrhizal associations* with the neighbouring trees) to *Rhizoctonia* (*saprotrophs*) seems to be necessary for orchids growing under low light conditions that need to secure most efficiently their carbohydrate supplies.

12. Fruit: It is a single, elongated, ovoid, erect, *pod*. Fruit set varies from year to year, greatly depending on the presence of the pollinators and on the exposure to sunlight. In sunnier locations, the fruit set may be four times higher than in the less exposed ones. In average is between 15.2% (Kull, 1998) (Figure 18).

13. Seed: The seeds usually mature by July-August. They are numerous, minute, dust-like, wind-dispersed (Figure 19). The average seed size is 0.94 ± 0.29 millimetres in length and 0.2 ± 0.03 millimetres in width. The average number of naturally pollinated seeds per *capsule* is 1435 (Arditti & Ghani, 2000).

14. Growth cycle: *Cypripedium calceolus* is thought to be a particularly slow-growing plant, taking between 6-10 years (Rasmussen, 1995), 12 years (Kull, 1999) or even 16 years (Terschuren, 1999) of growth before it actually produces the first flower.

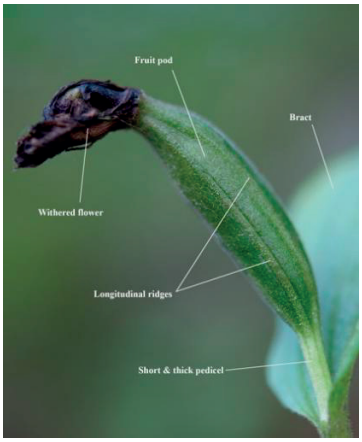


Figure 18. Detail of the *fruit pod*. It is elongated, green, untwisted, presenting three longitudinal ridges, the remains of the three carpels that fused and formed the characteristic syncarpous ovary © NA



Figure 19. Detail of the *seed capsule*. It is dry and partially opened along the three lines of dehiscence, through which the seeds will be released and windborne © NA

Nevertheless, the life expectancy of some individuals, after the first appearance above ground, can reach an age of about 30-100 years with some clumps reaching an average life span of 330-350 years (Kull, 1988; Nicolè, 2005), more than enough for successional generations to occur in the habitat.

DISCUSSIONS

This is the first description of a medium-sized population of *Cypripedium calceolus* that occurs in Prahova County. The population was discovered by Dan Anghelescu (De Angelli & Anghelescu, 2020) approximately 50 years ago, during a mountain trip.

However, the location was kept secret and no official descriptions were made at the time, since it occurs/grows in a vulnerable site, which needs to be highly protected, in order to preserve the plants. Although, exact and detailed records were not periodically kept, during the 50 years of monitoring, gradual increasing in size and spatial distribution of the population were observed. The population kept increasing from its initial size of 15-18 individuals to approximately 40-45 individuals

in 2020. Initially, it developed on an area of about 10m^2, which increased to approximately 20-30 m^2. The area, found at an altitude of 700-1000 m a.s.l., is characterized by varied light conditions and surrounded by *Fagus sylvatica* and *Acer pseudoplatanus*. In the same area various other alpine species may be found, such as *Allium ursinum, Galanthus nivalis, Anemone nemorosa, Helleborus purpurascens, Dryopteris filix-mas, Isopyrum thalictroides, Ranunculus serpens* subsp. *nemorosus, Arnica montana*. Due to the fact that the location remains completely unknown to the public, we hope that the Lady Slippers will continue to survive undisturbed and increase their numbers (close monitoring will be performed). The only threat posed is excessive shading, caused by vegetation succession (massive development of tree canopy, which, in time, may change the light condition and affect orchid's population survival). To date, this is the only *Cypripedium calceolus* population known to occur in Prahova County. The population described in Harghita County differs in terms of its floral community, occurring in a shady area, dominated by *Pinus sylvestris* and *Picea abies*. Other representatives are: *Viburnum lantana, Euonymus verrucosus, Corylus avellana, Vaccinium myrtillus, Aquilegia vulgaris, Fragaria vesca, Platantera bifolia*. However, it should be stressed that the height structure of vegetative shoots in Prahova County is almost similar to those in Harghita County, although those in Harghita proved to be more robust, taller and produce a higher share of flowering shoots. Lastly, our studies included the rare *Cypripedium calceolus* var. *citrina*, found in Suceava County (Figure 3). The Swiss priest and botanist Alphonse Rion (1809-1856) named it *forma flava*, in his *Guide du Botaniste en Valais* (1872). In 1899, the German botanist Bernhard Hergt named it variety *citrina* (var. *citrina*) from the Latin *citrus* (lemon tree), a reference to its uniform, bright yellow colour (Figure 4). In *citrina* plants, the *sepals* and *lateral petals* totally lack the *anthocyanin pigments* (pH dependent, red-purple pigments) and consequently, appear completely yellow, similar to the labellum (De Angelli & Anghelescu, 2020). In Romania, this is *the only specimen known* to have ever flowered in the wild. In the spring of 2020, this *unique specimen was uprooted and stolen* from its natural habitat. *The thief was never caught by the local authorities*. This unfortunate story brings me to the last part of this article, the conservation status and protection measures of *Cypripedium*.

Conservation status: All orchids are included under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora **(CITES)**. In Europe, *Cypripedium calceolus* is also listed on Annexex II and IV of the Habitats Directive 92/43/EEC/1992 and under Appendix II of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). It is fully protected under Schedule 8 of the Wildlife and Countryside Act 1984 and is listed on Schedule 4 of the Conservation Regulations/The Conservation of Habitats and Species Regulations 2017 (Bilz et al., 2011; Jakubska-Busse et al., 2021). Despite having a large distribution area covering most of Eurasia, in some parts of Europe many populations suffered a significant decline in the number of individuals (García et al., 2010), due to numerous natural and anthropic threats. Nevertheless, most of the populations are stable or even increasing in other parts due to conservation and protection measures that have been implemented. Therefore, this orchid has been assessed as a species of **Least Concern (LC)** by the International Union for Conservation of Nature (IUCN) *Red List of Threatened Species* 2014. Regardless of the apparent reassuring threat status (LC), in Europe, *Cypripedium calceolus* is protected at national level in most countries and collection of the species is forbidden. Many populations are included in Natura 2000 sites and other forms of protected areas (Rankou, 2021). *Cypripedium calceolus* has never been recorded and thus considered as absent in seven countries (Albania, Cyprus, Iceland, Ireland, Malta, North Macedonia, Portugal) and it was declared extinct in five countries (Belgium, Greece, Liechtenstein, Luxemburg, The Netherlands). It is classified as Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) in 22 out of the 35 countries of Europe. It is considered as Near Threatened (NT) in five countries (Austria, Estonia, Finland, Slovak Republic, Norway) or as of

Least Concern (LC) in only three European countries [Italy, Russia (European part), Sweden] (Jakubska-Busse et al., 2021).

In Romania, *Cypripedium calceolus* has been protected since 1938 by the High Royal Decree part I, no. 42 of February 20, 1938, given by King Carol II. Today, this species is included in Annex No. III, Law no. 462/2001, on the regime of protected natural areas, conservation of natural habitats, wild flora and fauna. In 1972, in Flora României were mentioned in total of 109 localities of *Cypripedium calceolus* (Săvulescu et al., 1972). However, as expected, many habitats have changed over the past 50 years, either naturally (vegetation succession) or due to human intervention. As a consequence, in some of the previously mentioned locations, the populations were absent or found counting much smaller numbers of individuals (Pop, 2006; Balázs et al., 2016). The European University Information Systems (EUNIS) data for Romania shows 21 Natura 2000 sites with *Cypripedium calceolus* occurring in only four sites located in the lower lands and the rest located in the upland regions (Jakubska-Busse et al., 2021). However, in the *Synthetic report concerning the state of conservation of species and habits of community interest from Romania*, the general assessment of the state of conservation for *Cypripedium calceolus* is considered favourable with unknown tendency (Mihăilescu et al., 2015).

Nevertheless, despite the accidental destruction of some locations, all is not lost. In recent years, an increasing number of new sites were discovered, most of which were never registered in the in the EUNIS database or officially published in local magazines. Just to give a few examples, various new, healthy, medium-sized to large populations were newly discovered in Prahova, Brașov, Mureș, Argeș, Buzău, Covasna, Harghita and Dâmbovița counties, either in the wild or on private properties. For example, as of 2021, Harghita County is counting more than 20 populations of Slipper Orchids, some newly discovered. Another example may be the newly discovered population of Slipper Orchids in Piatra Craiului range, at 1650-1700 m a.s.l. (20 July 2021, Bordea Ionuț, *personal communications*). The populations are usually local and isolated, small to large in size, counting from 3 to over 100 individuals in some cases, covering from 4-6 m^2 to 40-100 m^2. In most cases, the local people are very protective of the orchids and, at times, quite reluctant to advertise or register them, in order to preserve the plants. Other examples of recently registered populations of *Cypripedium calceolus* may be: two populations near Sovata, in Mureș county, within Ursu Lake Reservation and Sărături Arboretum (Pop, 2006); a small population of *Cypripedium calceolus* in The Cheile Șugăului-Munticelu Natural Reserve in Neamț County (Oprea et al., 2007); a new population in Piatra Craiului Mountains, in Rucăr village area (Marinescu et al., 2012); a re-discovered population after more than 80 years since the last record, in a Natura 2000 site named Făgetul Clujului-Valea Morii in Cluj County (Balázs et al., 2016) and four medium-sized populations found within the Natura 2000 site ROSCI0135 Pădurea Bârnova-Repedea (Stoica et al., 2017). Since the newly discovered sites of *Cypripedium calceolus* orchids keep adding up each year, a complete list of locations will be published in due time, in the future.

Threats: In Romania, as well as in most European countries, *Cypripedium calceolus* falls both under natural and anthropogenic threats. The most damaging or even lethal *natural threats* are prolonged *spring frosts*, *droughts* and *vegetation succession processes*. In recent years, it has been common ground, due to climate change, to have extended winters and experience alternating *frost/snow periods* in late spring or even in early summer, mostly in the subalpine or alpine regions where this orchid grows. The young shoots, leaves and floral stems are usually irreversibly damaged by the prolonged periods of frost and, in time, the destruction of several individuals may be very harmful to the entire populations. *Drought* caused by the lack of rains during extended periods of time, during hot and dry summers, is very harmful as well, since this species prefers constant moderate moisture or even wet substrates, for the normal development of the young seedlings and adult individuals, as well as for the seed germination and mycorrhizal associations (Corkhill, 1996). In Romania, the most damaging natural factor is *vegetation succession*, by which the

competing neighbouring trees, shrubs and other herbaceous species overgrow and invade the sites, asphyxiating and ultimately killing the orchids. It is important to keep in mind that, despite its preferences for shady places, it has been shown that *Cypripedium* plants *need a certain amount of light* in order to flower and ultimately to survive. In cases where the shade increased substantially due to the growth of neighbouring trees or vegetation, the plants first became *etiolated* (grew pale due to the partial loss of chlorophyll, the photosynthetic pigment) and then gradually produced fewer flowers and smaller stems. After few more years, all the plants disappeared permanently from above ground (Rasmussen, 1995). The *anthropogenic threats* include *habitat destruction*, *agriculture*, *inappropriate forest management* such as *uncontrolled deforestation* and *clear cutting*, *overgrazing* (which affects the individuals), the abandonment of traditional grazing activities (which leads to natural vegetation succession processes and therefore increased competition for this orchid), *real estate development*, *road and trail construction* and most of all, *plant collection* or *illegal uprooting* for gardens by the so-called *orchid enthusiasts* for its very attractive, large flowers that are likely to be used for both commercial and personal purposes. *Pollution* may also do long term damage to entire populations, since, the soil pH usually changes and the increased acidity of the substrates affects not only the pre-existing individuals (which prefer alkaline, calcareous substrates), but also destroys the specific mycorrhizal associations, thus limiting seed germination.

Conservation measures: In order to save the existing populations and also help the propagation and formation of new ones, appropriate measures should be taken, such as: habitat protection (prevention of habitat loss, alteration or disturbance), protection of sites, constant monitoring and surveillance of the existing populations and sites, constantly educating and raising public awareness of the existence and protection of orchids, fencing the vulnerable sites to protect the orchid species from collection and/or herbivores, proper sites management (mowing meadows, controlled grazing, forest proper management to prevent vegetation succession). Due to high ornamental qualities of *Cypripedium calceolus* orchids, the development of *successful micropropagation* and *cultivation techniques* of mature or immature seed germination techniques (Anghelescu et al., 2020) may lead to the protection of the wild specimens (Obdržálek, 2009; Korczyński & Krasicka-Korczyńska, 2014). In this way, both domestic cultivation and also reintroduction of plants to their primary habitats will be facilitated/possible (Ramsey & Stewart, 1998; Rasmussen & Pedersen, 2011). However, one should keep in mind that if optimal cultivation conditions are given, it may take 3-5 years or more, from seed germination to producing the first flowering stem (Perner, in Pridgeon et al., 2009).

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