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NOTES ON DISTRIBUTION OF *BOMBUS CRYPTARUM* (HYMENOPTERA, APOIDEA) IN MORAVIAN TERRITORY (CZECH REPUBLIC) AND ITS LABORATORY REARING

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Abstract

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B. cryptarum and *B. magnus* are among the so-called cryptic species whose identification is very difficult due to high interspecific variability of their morphological characteristics. This greatly limits possibilities for studying their biology, bionomics and ecology. The aim of this research was to contribute to knowledge about distribution of *B. cryptarum* in Moravia (Czech Republic) and to verify the possibility of its laboratory rearing. During 2006–2009, 26 collections were performed at Moravian localities. Of the 17 specimens that were assigned to *B. cryptarum* or *B.* aff. *cryptarum* based on morphology, the molecular analysis of mtDNA RFLP confirmed the identification of 10 speciemens. The molecular analysis even assigned to *B. cryptarum* one specimen determined morphologically as *B.* aff. *magnus*.

Of four queens captured in spring, and while applying a laboratory method used for breeding of *B. terrestris*, two complete nests were successfully reared, including queens of the 2nd generation that then set up their own brood. Species identification of these queens as *B. cryptarum* was confirmed by both molecular analysis and analysis of the marking pheromones of males among their offspring. A single *B. terrestris* worker was able to raise young queens of *B. cryptarum* of the brood.

Czech Republic, Moravian territory, faunistics, rearing, bumblebees, cryptic species, *Bombus cryptarum*, DNA analyses

The bumblebee *Bombus cryptarum* belongs to the subgenus *Bombus* s. str. in which there exist so-called cryptic species. Generic identification of these species is often very difficult due to the large interspecific variability of their morphological characteristics. In the Czech Republic, there are three other species of this subgenus: *B. terrestris* (Linnaeus, 1758), *B. lucorum* (Linnaeus, 1761) and *B. magnus* (Vogt, 1911). While existence of the species *B. terrestris* and *B. lucorum* has previously been generally accepted, *B. magnus* and *B. cryptarum* were considered to be merely morphological variants of the *B. lucorum* species (Williams, 1991, 1998). Not until recent studies based on molecular techniques and chemical analysis of labial glands for male pheromones (Bertsch *et al.*, 2004, 2005) has it been shown that these are indeed separate species. Generic classification of the bumblebees *B. cryptarum* and *B. magnus* on the basis of morphological characteristics, however, remains very complicated. This problem has been satisfactorily resolved only for the queens of these bumblebees (Bertsch *et al.*, 2004, 2005). Species identification of males and workers is very difficult and not always reliable, as to date there are no fully applicable identification keys (AMIET, 1996). This issue greatly limits the possibility to obtain information about the biology, ecology and distribution of these species, which is related in turn to limited possibilities for the species' protection.

From information collected in recent years, most of which is based solely on the readily identified queens, it appears that *B. cryptarum* is spread across Europe. This type is more common in the Benelux countries, northern and central Germany, Poland, and in some parts of Great Britain. Several records come also from Russia. It is less common in the southern parts of these countries. Information is still lacking, however, for many European countries (Bertsch *et al.*, 2004; Rasmont *et al.*, 1986; Rasmont, 1986, 2010).

Concerning knowledge of *B. cryptarum's* presence within the Czech Republic, the situation is similar to those in other European countries. The first faunistic information for *B. cryptarum* from the Czech Republic was published by Tkalců (1999). Since 2004, *B. cryptarum* and *B. magnus* began to be placed onto generic lists of Hymenoptera of the Czech Republic as two separate species (Přidal, 2004). Most existing findings of *B. cryptarum* come from wet overgrown meadows and pastures with bushes at altitudes ranging from 183 to 1000 m a. s. l. (Komzáková *et al.*, 2008).

Since 2006, we have been monitoring the occurrence of *B. cryptarum* and *B. magnus* in Moravia and analyzing the DNA of specimens captured while also examining the possibility of laboratory rearing for purposes of species protection as part of Ministry of Education Project No. 2B06007: Bumblebees as an active element of landscape biodiversity.

MATERIALS AND METHODS

Methods of capture. Bumblebee queens of the species *B. cryptarum*, as well as males and workers of the subgenus *Bombus* were collected individually by sweeping with a net at 26 selected localities in Moravia, which included submontane and lowland habitats (Tab I). The queens for laboratory rearing were collected in spring when seeking a nest. Collection of males and workers was conducted in summer. The captured specimens were identified by Antonín Přidal from Mendel University in Brno. Collected material has been stored in a dry state in entomological boxes and in this condition it was used for molecular analysis.

Molecular determination of species using RFLP analysis. Genomic DNA was isolated from two legs of each specimen/individual using the commercially available DNeasy® Blood & Tissue Kit (Qiagen, Germany). A part of the mitochondrial CO1 gene (1064 bp) was amplified by PCR using the primer pair CO1_forward 5'-ATAATTTTTTTTATAGTTATA-3' and CO1_reverse 5'-GATATTAATCCTAAAAAA-TGTTGAGG-3' (Tanaka *et al.*, 2001). Individual PCR was carried out in 30 µl volumes containing 10x buffer (including 1.5 mM $MgCl_2$) (Finnzymes, Finland), 200 µM of dNTP mix (Finnzymes, Finland), 2 µM of each primer (Metabion, Germany), 0.2 U of DyNAzyme II DNA polymerase (Finnzymes, Finland), and 2 µl of template DNA. The PCR cycle was as follows: 93 °C for 1 min, followed by 30 cycles of 93 °C for 45 s, 45 °C for 1 min and 60 °C for 4 min, with a final 3 min extension period at 60 °C. PCR products were run out on a 1.5% agarose gel and visualized over UV light after staining in ethidium bromide. Amplified mtDNA fragments were digested using the restriction enzyme *Hinf* I. Each reaction contained 7.5 µl of PCR product, 1 U of *Hinf* I enzyme and 1 µl of 10x NEBuffer 2 (New England Biolabs Inc.). HPLC-grade water was added to obtain a total reaction volume of 10 µl. The samples were incubated at 37 °C for 4 h. Restriction fragments were electrophoretically separated on 2% agarose gel and visualized over UV light after staining in ethidium bromide. For comparison, DNA were used that were obtained from one *B. cryptarum* individual whose identity had been verified by the male marking pheromone, one *B. terrestris* individual from laboratory rearing at Masaryk University, and one *B. lucorum* individual captured in nature and identified based on morphological characters.

Determination using pheromones (Urbanova *et al.*, 2001; Terzo *et al.*, 2005; Rasmont *et al.*, 2005) was possible in males from laboratory rearing and was carried out by the Institute of Organic Chemistry and Biochemistry (IOCB) of the Academy of Sciences of the Czech Republic in Prague.

Rearing methodology was the same as for commercially bred species of *B. terrestris.* Captured queens were placed into a plastic rearing box with dimensions of 140 × 190 × 70 mm and kept in darkness at 27–28 °C and 60–80% relative humidity. They were fed 60% sugar solution (90% sucrose, 10% fructose) and fresh pollen, which was replaced daily. Only in the case of one wintering queen was dried and frozen pollen used. To stimulate laying, they were offered a cocoon of *B. terrestris*, or possibly *B. lucorum*. In 2007, after laying eggs one queen received as a helper a callow worker of *B. terrestris.* Once the first workers had hatched, the colonies were moved into wooden hives with dimensions 265 × 200 × 200 mm. Young queens emerging in the hives were moved along with unrelated males into a glass aquarium covered by a netting in daylight to mate. Queens whose mating was observed were removed from the terrarium and transferred to rearing boxes in darkness at room temperature. Here, they were provided with sugar solution in order to create energy reserves for the period of hibernation. Queens who had been observed to mate were left with males for approximately 10 days, after which they were moved to the same conditions as the queens who had mated.

Queens showed readiness to enter hibernation (i.e. filled abdomen and minimal physical activity) were after 7 days placed into a refrigerator at 4 °C. The queens, who remained remarkably active, were thereby stimulated to lay eggs as were the queens caught in the wild.

RESULTS AND DISCUSSION

During 2006–2009 as part of the project, we managed to capture a total of 17 bumblebee specimens (3 workers, 14 males). Based on morphological characteristics, they were assigned to *B. cryptarum* or species of *B.* aff. *cryptarum*. Four of the specimens (1 worker, 3 males) were identified as *B. magnus* or *B.* aff. *magnus*.

In 11 cases, restriction digestion of a part of the mtDNA CO1 gene with the enzyme *Hinf* I resulted in two restriction fragments which differed in length, one being 500 bp and the other 550 bp. The presence of these two bands confirmed the identity of *B. cryptarum*. Two individuals were assigned to *B. terrestris*. In 3 individuals identified on the basis of morphology as *B. magnus* and 5 specimens identified as *B. cryptarum* or *B.* aff. *cryptarum* digestion did not occur, and the result was the undigested PCR product 1064 bp in length. This suggests that these most likely are not individuals of *B. cryptarum*, because in the given sequence restriction sites characteristic of these species were not found. But whether or not these are *B. magnus* remains to be verified.

A reliable method for determining the species' generic identity is by chemical analysis of the labial glands for male pheromones, but this requires sampling the head tissue immediately after death, which in this case was possible only for specimens obtained from laboratory rearing.

The presence of *B. cryptarum* was found across Moravia at altitudes between 200 and 1 000 m. a. s. l., but they are rather rare (see Table I.).

As stated in the methodology section, four queens were available to verify the laboratory rearing of *B. cryptarum*. Two queens were captured in the spring of 2007 (one at the site Zálesí, between Moravský Beroun and Krahulčí, and one at the site Obecní díly, nad Šternberkem směr Nové Dvorce (Municipal lands outside of Šternberk in the direction of Dvorce). At the second of these sites, two queens were found in spring 2008. Queens found in 2007 were determined only morphologically and are not listed in the table.

In 2007, one of the queens died after 8 days in the laboratory, probably due to ill health. The second queen established brood and after 7 days she was given a callow worker of *B. terrestris* to help her with care of the brood. Adoption of the *B. terrestris* worker by the *B. cryptarum* queen and subsequent cooperation in the care of the brood were apparently successful. After establishing the egg cell, however, the queen died and the worker raised five young queens of the orphaned brood. Since at that time *B. cryptarum* males were not available, queens were offered *B. lucorum* and *B. terrestris* males to mate. Although de Jongh (1982) and Rasmont & de Jongh (1985) had reported queens mating with *B. terrestris* and *B. lucorum* males, we recorded no interspecific mating. Two of these five young queens of the 2nd

I: *Localities, date of collection and comparison of morphological identification and mtDNA analysis*

Locality	Date of collection	Morphological identification	PCR-RFLP
NP Podyjí, pod Šobesem	17. 8. 2006	*B. cryptarum*	*B. terrestris*
NP Podyjí, Zadní Hamry, pod Led.slujemi	17. 8. 2006	*B. cryptarum*	*B. cryptarum*
CHKO Žďárské vrchy, Světnovské údolí	2. 9. 2006	*B. cryptarum*	*B. cryptarum*
CHKO Žďárské vrchy, Světnovské údolí	2. 9. 2006	*B. magnus*	-
CHKO Žďárské vrchy, Světnovské údolí	2. 9. 2006	*B. magnus*	-
CHKO Žďárské vrchy, Světnovské údolí	2. 9. 2006	*B. magnus*	-
Slavice, okr. Třebíč	11. 6. 2007	*B. cryptarum*	-
CHKO Žďárské vrchy, Světnov louka	13. 6. 2007	*B. cryptarum*	*B. cryptarum*
CHKO Bílé Karpaty, Strání	4. 8. 2007	*B. cryptarum*	*B. terrestris*
Obecní díly, nad Šternberkem směr Nové Dvorce	28. 4. 2008	*B. cryptarum*	*B. cryptarum*
Obecní díly, nad Šternberkem směr Nové Dvorce	28. 4. 2008	*B. cryptarum*	*B. cryptarum*
CHKO Žďárské vrchy, Radostín	5. 7. 2008	*B.* aff. *cryptarum*	*B. cryptarum*
NP Podyjí, Mašovice	29. 7. 2008	*B.* aff. *cryptarum*	*B. cryptarum*
Orlické hory, Šerlich- Orlické záhoří	16. 8. 2008	*B.* aff. *cryptarum*	-
CHKO Žďárské vrchy, Radostín	31. 8. 2008	*B.* aff. *cryptarum*	*B. cryptarum*
Obecní díly, nad Šternberkem směr Nové Dvorce	12. 8. 2009	*B.* aff. *cryptarum*	-
Obecní díly, nad Šternberkem směr Nové Dvorce	12. 8. 2009	*B.* aff. *cryptarum*	-
CHKO Jeseníky, Karlov	12. 8. 2009	*B.* aff. *cryptarum*	-
CHKO Žďárské vrchy, Stržanov	14. 8. 2009	*B.* aff. *magnus*	*B. cryptarum*
CHKO Žďárské vrchy, Světnovské údolí	14. 8. 2009	*B.* aff. *cryptarum*	*B. cryptarum*
CHKO Žďárské vrchy, Světnovské údolí	14. 8. 2009	*B.* aff. *cryptarum*	*B. cryptarum*

NP = national park, CHKO = protected landscape area

generation in the autumn showed a behavior that usually leads to eggs laying but they laid no eggs and eventually died.

In contrast, both the queens obtained in 2008 began unhesitatingly to establish egg cells on the *B. terrestris* cocoons. They raised their own workers and after transfer to the hive were developed well. After producing several generations of workers, the families began to produce variably colored males and later young queens as well. The colonies' time courses of development, numbers of queens, and numbers of individuals in the nests are shown in Table II. At the time of producing young queens, there were young males in both colonies, so it was possible to attempt their mating under laboratory conditions. Mating was observed in only 6 of the total number of 32 queens. One queen died soon after mating. In addition, queens after mating did not show typical behavior for queens of *B. terrestris,* which generally are ready to enter hibernation. Seven queens were still wintering in the refrigerator and the others were placed in the breeding room, where seven started to lay eggs. Three queens which had produced brood nevertheless died after a few days, two raised only the first generation of workers, and the remaining two raised only males.

Of the seven queens that were wintering in the laboratory in early September, just one was still living at the beginning of the following February. She did not establish the egg cells, however, and died a few days after coming out of hibernation.

Chemical analysis performed at IOCB of the male labial glands' pheromones from both colonies (Table II) and from males reared by queens of the second generation confirmed that they were *B. cryptarum.*

II: *Time behavior and number of individuals in colonies originating from B. cryptarum queens collected in 2008*

Colony	Date of collection	1st egg cell	1st worker	1st queen	Number of queens produced in colony	Total number of individuals on date 26. 8. 08
A	28.4.08	7.5.	28.5.	14.7.	12	290
B	28.4.08	6.5.	28.5.	27.6.	10	160

SUMMARY

The species *B. cryptarum* and *B. magnus* are among the so-called cryptic species whose identification is very difficult due to their high interspecific morphological variability. During 2006–2009, the occurrence in Moravia of the cryptic species *B. magnus* and *B. cryptarum* was observed. Bumblebees were collected at 26 habitats of submontane and lowland types. Collection was conducted in spring, when the queens could be obtained for laboratory rearing, and in summer, when male and worker were obtained for determination. Males and workers were determined based on morphological characteristics. Consequently, their generic affiliation was verified by mtDNA RFLP analysis at the Research Institute for Fodder Crops, Ltd. and Agricultural Research, Ltd. in Troubsko, Czech Republic. The young queens collected were used for rearing at the Faculty of Science, Masaryk University, in Brno.

By means of morphological determinations, 17 specimens were assigned to the species *B. cryptarum* or *of B.* aff. *cryptarum* and 4 specimens were assigned to the species of *B.* aff. *magnus.* Molecular analysis of a part of the mtDNA CO1 gene using RFLP confirmed the identity of *B. cryptarum* in 11 individuals while 2 individuals were identified as *B. terrestris.* One individual morphologically designated as *B.* aff. *magnus* was identified as *B. cryptarum*. In 8 individuals, digestion did not occur in a part of the mtDNA CO1 gene, thus indicating a high probability that this was not of the *B. cryptarum* species. Whether or not it is *B. magnus* remains to be verified.

Four *B. cryptarum* queens were captured during 2007 and 2008 (two in each year). These were taken to the laboratory for experiments using methods practiced for *B. terrestris.* In 2007, one queen died. A *B. terrestris* worker was added to the second one, which already had its own brood. Subsequently, that queen also died and the worker raised five young queens from the brood. In 2008, both captured queens succeeded in rearing two colonies, which produced sexual specimens. Attempts at mating of the young queens were only partially successful. Moreover, the mated queens did not show typical pre-hibernation behavior common for *B. terrestris.* Some of the queens subsequently began to lay eggs and raised a number of workers and males. Of the seven queens wintering like *B. terrestris* only one survived, and after a few days that one died in the laboratory.

The correct identification of these two bumblebee colonies as *B. cryptarum* was confirmed by analyzing the marking pheromone from males of the second generation.

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