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Identification of cryptic species belonging to the *Bombus lucorum* - complex: DNA barcoding and morphological approaches

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Identification of cryptic species belonging to the *Bombus lucorum* - complex: DNA barcoding and morphological approaches

by

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1. Summary

DNA barcoding has proven to be a suitable biological tool to obtain reliable results for identifying species. In this study, a group of cryptic bumblebees, namely the *Bombus lucorum* - complex, collected across the Austrian Alps, was analysed. This cryptic bumblebee group contains three species: *Bombus lucorum*, *Bombus cryptarum* and *Bombus magnus*, which are known to be common and widespread. A universal mitochondrial gene sub-region, the cytochrome oxidase I (COI) was used for the molecular analysis. The DNA barcoding method allowed for the identification of the collected individuals. The results showed that only two of the three species could be found in the sampling areas, namely *Bombus lucorum* and *Bombus cryptarum*. The obtained molecular data contributed to our understanding of the ecological distributions, as well as altitude preferences, of these species. According to the results, *Bombus lucorum* appeared to be more abundant and occurred in diverse altitude ranges, whereas *Bombus cryptarum* appeared to prefer high altitude levels and was distributed only in the western Alpine areas. Furthermore, a reinvestigation of certain diagnostic morphological characters and, finally, a comparison of molecular and morphological determinations were performed. These results revealed that most of the examined characters overlap between the species; likewise, differences in measurements taken from various body parts were not significant.

2. Introduction

Bumblebees (*Bombus,* Bombini, Apidae) are important pollinators (Goulson 2010) and occur mainly in the northern hemisphere, living in a wide range of habitats (Williams 1998). Some species even occur in South America and South-East Asia (Goulson 2010). Due to their ability to fly at low temperatures and due to their long tongues, many *Bombus* species assume remarkably important roles in agriculture, pollinating numerous crops and flowers (Molet et al. 2009, Goulson 2010).

Today, around 250 bumblebee species are reported globally (Williams et al. 2008, Goulson 2010) and are widespread throughout Europe, North America and Asia. Wide ranging species diversities occur in the alpine and arctic zones (Goulson 2010).

Molecular genetic data allow for discrimination between closely related species that are not easily identified by morphological characters (Goulson 2010, Carolan et al. 2012). Thus, it can be assumed that several described species may actually represent clusters of cryptic species. Cryptic species are defined as two or more single classified species resembling each other in their morphological appearance (Pfenninger et al. 2007). Even for experienced taxonomists, the identification of cryptic species on the basis of morphological characters may be impossible and the results are not always reliable (Williams et al. 2012).

The focus of the present study centres on a cryptic bumblebee species group, namely the *Bombus lucorum* - complex, which consists of three species: *Bombus lucorum* (Linnaeus, 1761), *Bombus cryptarum* (Fabricius, 1775) and *Bombus magnus* Vogt, 1911 (Scholl et al. 1983, Murray et al. 2008, Carolan et al. 2012). These species cannot be reliably differentiated in the field. To correctly identify them - which is important in biological and ecological studies - molecular biological methods have turned out to be useful instruments.

Recently, a molecular genetic method termed DNA barcoding has been developed to gain information for identification of species in taxonomic research. This method is performed using short standardised gene regions to identify species. A standard gene region was examined in this study, a fragment of the mitochondrial gene cytochrome c oxidase subunit 1 (COI) (Herbert et al. 2003).

The three species of the *Bombus lucorum* - complex are known to be among the most important wild plant and crop pollinators in northern Europe (Carolan et al. 2012). Of the three species, *Bombus lucorum* is the most common and is widespread in Europe. *Bombus cryptarum* is widespread in northern Europe and appears to be rather continental, occurring in mountain regions in high altitude ranges and has recently been found in the British Isles and Ireland (Murray et al. 2008). *Bombus magnus* is most abundant in the Atlantic region and less common in Eastern Europe (Rasmont 2012). In Austria, *Bombus lucorum* and *Bombus cryptarum* were found regularly, whereas the taxonomic status of *Bombus magnus* remains doubtful (Gokcezade et al. 2010).

Individuals belonging to the *Bombus lucorum* - complex can be recognised in the field by their colour pattern, which consists of a single band of lemon yellow hairs on the thorax (mesosoma), a second yellow band on the second tergite and a white "tail" on the fourth and fifth tergites of the abdomen (metasoma) (Gokcezade et al. 2010) (Figure 1). A reliable determination of these species by morphological characters is difficult and nearly impossible with respect to the females, i.e. workers and queens (Bertsch 2009, Carolan et al. 2012). The cryptic species of the *Bombus lucorum* - complex have been previously examined by means of molecular and morphological identifications. The results of the past studies indicate that these taxa represent genetically distinct lineages, although identification relying on morphological characters was not reliable (Murray et al. 2008; Bertsch 2009; Carolan et al. 2012).

One aim of this study was to identify specimens of the *Bombus lucorum* - complex by means of molecular genetics using the DNA barcoding method and to compare these results with data on the ecological requirements of the species in the Austrian Alpine areas. The data obtained should provide answers to questions regarding the distribution and altitude preferences, as well as plant preferences and habitat types.

The second part of the study was the determination of certain morphological characters of these bumblebees. Various characters, which have been shown to be relevant for diagnostic determination, were selected from previous studies (Bertsch 2009; Neumayer unpublished). Finally, a comparison of the obtained molecular and morphological data was performed. The comparison allows us to test the reliability of the morphological identifications.

3. Material and Methods

3.1. Field sampling

Bumblebees were collected from July 17th to August 28th in the summer of 2012, at following Alpine regions: Schneeberg, Gesäuse, Glocknergruppe (North and South), Karwendel, Kaunertal in the Ötztaler Alps, Silvretta and Hochobir (Table 1).

In total, eight sampling areas, known as habitats for species of the *B. lucorum* - complex, were sampled in different altitudes, ranging from 600 to 2300 m a.s.l. Most bumblebees were taken above 1600 m a.s.l. The collecting was performed under warm weather conditions. Specimens were collected irrespective of their sex. Sex determination was performed later in the laboratory.

Appendix 1 and 2 gives a detailed overview of the altitude ranges and the sampling concept.

Table 1. Sampling schedule of the seven sampling areas and numbers of the collected specimens of the *Bombus lucorum* - complex, as well as their sex.

Sampling location	**Transect range (m a.s.l.)**	**Sampling date**	**Collected by**	**Total number of specimens**	**Number of females**	**Number of males**
Schneeberg/NÖ	below 1000 to 1700	31.July 2012	Gereben-Krenn, Gokcezade, Bardakji	5	5	0
Gesäuse/Stmk.	below 1000	25.Aug.2012	Neumayer	2	1	1
Glocknergruppe North/Sbg.	1300 to 2300	17-18.July 2012	Neumayer	4	4	0
Glocknergruppe South/Ktn.	1000 to 2300	17.-18.July & 28.Aug.2012	Neumayer	29	23	6
Karwendel/T.	1300 to 2300	10.-11.Aug.2012	Neumayer	7	4	3
Kaunertal/T.	1300 to 2300	08.-09.Aug.2012	Gereben-Krenn, Krenn	17	16	1
Silvretta/Vbg.	1000 to 2000	04.-06.Aug.2012	Gereben-Krenn, Krenn	24	24	0
Hochobir/Ktn.	1000 to 2000	29.July 2012	Gereben-Krenn, Krenn	no bumblebees found	no bumblebees found	no bumblebees found

The individuals were identified as members of the *B. lucorum* - complex by visual inspection according to their colour patterns (Gokcezade et al. 2010) (Figure 1). The specimens' foraging behaviour was noted and assigned to certain categories (e.g. plant species, resting, pollen collection, nectar feeding, etc.) in the field.

Specimens were collected using an insect net and then transferred to tubes filled with 96% ethanol. At the same time, samples of the foraged plants were collected as a whole and labelled for subsequent determination. GPS coordinates were recorded for each individual sample.

All 88 bumblebees, as well as the foraged plants, were stored at -20°C.

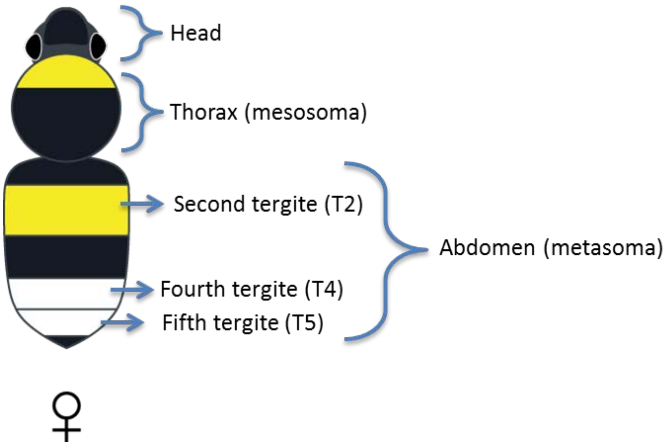


Figure 1. Schematic illustration showing the characteristic colour pattern of females from the *Bombus lucorum* - complex (modified from Gokcezade et al. 2010).

3.2. Molecular analysis

The DNA of all 88 specimens collected was extracted. Afterwards, molecular analysis was performed to obtain DNA barcodes. Sequences of male individuals and those having low quality were excluded, so that in the end 57 barcodes were available for the remaining analyses.

3.2.1. DNA extraction

From each specimen stared in ethanol, all three legs from one side were removed using sterile forceps and scalpels.

The legs were allowed to dry at room temperature for 10 minutes on clean parafilm layers. The tissue preparation was performed under clean and sterile conditions to avoid DNA contamination.

The individuals were restored to their ethanol tubes at -20 C° for further morphological analysis.

Total genomic DNA was extracted using the “DNeasy Blood & Tissue extraction Kit” (Qiagen) following the manufacturer´s manual “Purification of Total DNA from Animal Tissues, spin column protocol”.

The leg tissues were fragmented in a 1.5 ml reaction tube with 180 µl ATL buffer (Qiagen) using polypropylene pestles. It was not easy to grind the legs completely; the legs could not be ground completely but they were fragmented as much as possible by cracking the cuticula. Then 20 µl Proteinase K (Qiagen) was added and the samples were digested by shaking at 56 C° overnight.

After lysis, the DNA extraction followed the manufacturer´s protocol (Purification of Total DNA from Animal Tissues, Spin-Column Protocol). The elution step was repeated once producing two DNA fractions (I and II) from each specimen. For the first eluate, 200 µl of elution buffer were added and 100 µl for the second eluate.

DNA samples were stored at -20°C.

3.2.2. Polymerase Chain Reaction (PCR) and Gel Electrophoresis

A fragment of the mitochondrial gene cytochrome oxidase subunit I (COI) was used in this study (Herbert et al. 2004). This DNA fragment was amplified using universal primers, which have proven to work successfully; the forward primer LCO and the reverse primer HCO (Folmer et al. 1994) (Table 2).

Table 2. Primers used in this study for the amplification of the COI region (Folmer et al. 1994).

Primer Name	**Sequence 5´-3´**
LCO-1490	GGTCAACAAATCATAAAGATATTGG
HCO-2198	TAAACTTCAGGGTGACCAAAAAATCA

PCR reactions were performed using the first DNA eluate (fraction I). A negative control without DNA was included in every PCR approach. Since the primers LCO-1490, HCO-2198 have been used in various former studies and proved to be successful, positive controls were not included in the PCR reactions.

The PCR reactions were carried out in a reaction volume of 25 µl using a 2 x "DreamTaq ® PCR Master Mix" (Fermentas), 10 µM of each primer and approximately 100 ng of DNA.

The cycling profile consisted of denaturation at 94°C for 180 s, followed by 30 cycles of 30 s at 95°C, 30 s at 48°C, 60 s at 72°C and a final extension period at 72°C for 300 s.

PCR products were analysed on 1.5 % agarose gels in 1 x TAE buffer. Each sample applied on the gel contained 3 µl of PCR product, 1.5 µl of 6 x Loading Dye (Fermentas®) and 1.5 µl SYBR Green (Sigma Aldrich®) (1:500 of the 10,000X stock in TAE). A 100 bp DNA ladder (Fermentas®) was used containing 3 µl ladder and 1 µl SYBR green (1:500).

The electrophoresis was performed at 13 volts per centimetre gel (Biorad®).

3.2.3. Purification and Gel Electrophoresis

Successfully amplified PCR products were purified for sequencing using the "Gene JET PCR Purification Kit # K0702" (Thermo Scientific). The purification followed a modified protocol. For the first purification step, 30 µl of binding buffer was added to 22 µl of the remaining PCR product, the solution was mixed well and incubated for 1 minute at room temperature. The remaining purification steps followed the manufacturer´s manual. For the last purification step, each sample was eluted in 35 µl of the supplied elution buffer.

The purified samples were applied on 1.5 % agarose gels in 1 x TAE buffer. The volumes used as well as the DNA ladder and voltage are the same as those used in the gel electrophoresis of PCR products.

3.2.4. Sequencing

Purified PCR products were sequenced at the "VBC- Biotech Service GmbH" company using the standard sequencing technique after Sanger on an ABI Prism 3100 and ABI Prism 3730XL capillary sequencer. (Sanger et al. 1975).

The primers used for sequencing were the same as used for the PCR reactions. Both primers forward LCO-1490 and reverse HCO-2198, were used to sequence the purified samples in both directions.

In total, 57 sequences were obtained in this study.

3.2.5. Sequence analysis, phylogenetic tree and molecular genetic distances

3.2.5.1. Sequence editing and alignment

The initial step of sequence alignment was to create a contiguous sequence by matching the forward and reverse reads of each sequence and checking them for ambiguous sites using SeqMan v.7. Then all sequences were uploaded into Bioedit v.7.1.7 and an alignment was performed using ClustalW (Higgins et al. 1994). The primer sequences were removed from all aligned sequences, leaving sequences regions consisting of 658 base pairs for further analysis.

For evaluation of species identities, a nucleotide blast search (Altschul et al. 1990) at the NCBI homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed for all contiguous sequences generated in this study. Search parameters included the whole arthropod database. The best matches according to E value and sequence identity were then considered for the species assignment.

3.2.5.2. Construction of the phylogenetic tree and haplotype network

For the construction of the phylogenetic tree, a final alignment including all sequences was imported into Mega v 5.1 (Tamura et al. 2011), and a Neighbour-Joining (NJ) bootstrap tree with 500 replicates was calculated using the following parameters: Kimura-2-parameter substitution model assuming a gamma distribution. The data set consisted of 57 sequences obtained in this study.

Geographical correlations between Austrian bumblebee haplotypes from this study and northern European haplotypes from the study of Carolan et al. (2012) were investigated. Sequence data of both studies were combined in a haplotype network. The data set consisted of 55 sequences of *B. lucorum* and *B. cryptarum* from this study and 67 sequences of *B. lucorum*, *B. cryptarum* and *B. magnus* from Genbank including sequences from Finland, Denmark, Ireland and Orkney Islands. All sequences were imported into Network version 4.6.1.1, and haplotype networks were constructed using default settings (Bandelt et al. 1999).

3.2.5.3. Calculation of genetic distances

The sequence alignment was imported into Mega v 5.1 and distances were calculated with the Kimura-2-parameter substitution model using default settings (Kimura 1980). Mega v 5.1 allows measurement of genetic distances between groups and within groups and construction of a DNA barcoding gap diagram.

Intraspecific and interspecific distances were calculated using Mega. The results of the pairwise comparisons were blotted into a DNA barcoding gap diagram.

3.3. Morphological analysis

Morphological examination was performed without prior knowledge of the DNA barcoding results of the genetic species determinations. The concept followed the investigation of certain morphological diagnostic characters and finally a comparison between the morphological and genetic determinations of the examined individuals.

Since only females (i.e. workers and queens) were used for the analysis, male specimens were sorted out after sexual determination. In addition, specimens which did not have available DNA barcodes were excluded from the morphological analysis as well, leaving a total of 55 specimens for the morphological analysis.

3.3.1. Preparation of bumblebee specimens for morphological analysis

For the morphological analysis, specimens were cleaned from pollen grains following the guide for processing bees kept in alcohol using the brochure of Droege (2010).

The bumblebees were taken out of the ethanol tubes and dried on clean tissues at room temperature. Then they were washed by stirring in warm tap water with a few drops of dishwashing soap, rinsed in pure water and finally with pure ethanol. Afterwards, the bumblebees were pinned with entomological pins which were inserted vertically through the right side of the thorax and provided with a label.

Finally, the specimens were dried and brushed to fluff the hair using a fine paint brush and hair dryer. The heat helped make the legs and wings relaxed so that they were able to be spread. The bumblebees were kept in an insect collection box at room temperature.

3.3.2. Morphological characters

Certain diagnostic morphological characters were selected from previous studies of the *B. lucorum* - complex (Bertsch 2009; Neumayer unpublished). A reinvestigation of the morphological character states was performed. Based on these diagnostic features; the sampled specimens were assigned to one of the species of the *B. lucorum* - complex.

The morphological analysis included the following two main parts:

1. Selection of the characters, i.e. checking for the presence or absence of the character state in relation to the corresponding species (Figure 2). Table 3 gives a detailed overview of differences in appearance of the characters. The following three characters were chosen:
a) The “yellow collar”: Length of collar extension below the wing base, the width between the tegulae of the mesosoma (Figure 2). The yellow hairs do not extend below the tegulae in *B. lucorum*, but they extend below the tegulae in *B. cryptarum*, although they are not widely spread (Table 3).
b) The “surface structure of T2”: Surface structure of the cuticula of the second tergite of the metasoma in the middle of the hind margin (Figure 2). The surface structure is obliquely grooved in *B. lucorum* and vertically grooved in *B. cryptarum* (Table 3).
c) The “shape of lamella”: The shape of the lamella of the labrum between the lateral projections (Figure 3). This trait is narrowly U- shaped in *B. lucorum* and V- shaped in *B. cryptarum* (Table 3).

2. Measurements of certain body parts according Figures 2 and 3. (Bertsch 2009; Neumayer unpublished). The following three body parts were measured in all individuals:
a. Width of thorax between the tegulae (Figure 2).
b. Diameter of the median ocellus (Figure 3).
c. Length of the malar space, i.e. the space from the base of the complex eye to the base of the mandible (Figure 3).
The measurements of body parts were performed using a stereomicroscope (Nikon SMZ-U, zoom 1:100) equipped with a measuring-ocular. To ensure accurate measurements, the recommendations of Bertsch (2009) were followed:
The measurements were done at the same magnification for each part and calculated in millimetres (mm) using the units given in the measuring ocular. Subsequently, the data was analysed using IBM® SPSS® Statistics 19. A Mann Whitney-U-test was performed to test for significant differences in the measured body parts ($P < 0.05$).

The individuals were photographed to show the differences in their morphological characters. Due to difficulties in obtaining clear pictures for some body parts, photo series with differently sharpen layers were taken for each specimen afterwards; the photos were then stitched using “Helicon Focus version 5.2 pro” to obtain one completely focused micrograph.

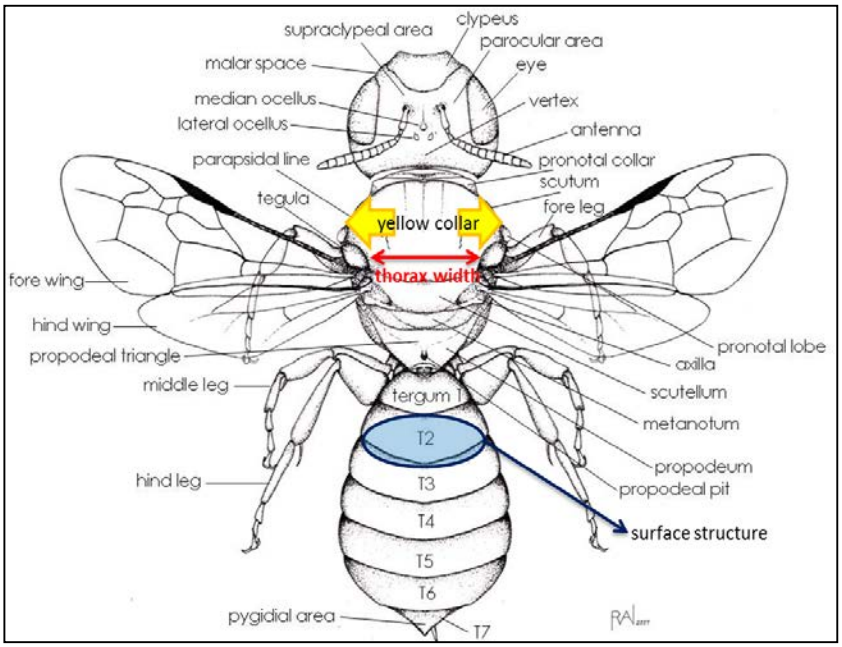


Figure 2. Overview of the selected characters for reinvestigation and measurement; yellow collar of the thorax (yellow label), the surface structure of the 2nd tergite T2 (blue label) and the thorax width (red label) (modified from Droege 2010).

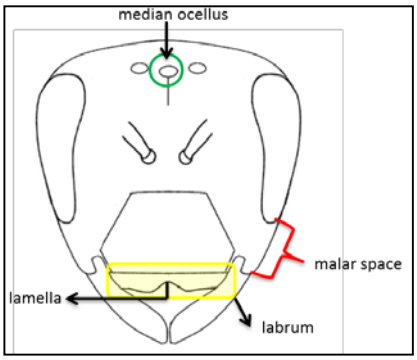


Figure 3. Front view of the head of female *Bombus sp.* showing selected characters for the analysis; diameter of the median ocellus (green label), shape of the lamella of the labrum (yellow label) and the length of the malar space (red label) (modified from Gokcezade et al. 2010).

Table 3. Differences between the diagnostic characters of *B. lucorum* - complex according to Bertsch (2009) and Neumayer (unpublished). See corresponding Figures 4, 5, and 6.

Species	Yellow collar	Surface structure of T2	Shape of lamella
B. lucorum	does not extend below the tegulae	grooved obliquely	narrowly U- shaped
B. cryptarum	extends below the tegulae but is not widely spread. A black narrow s-shaped hairline appears within the collar but was not perceptible in the Alpine specimens.	intensely grooved vertically	V- shaped
B. magnus	extends further below the tegulae and is widely spread	intensely grooved vertically, in addition coarse and wrinkled	broadly U- shaped

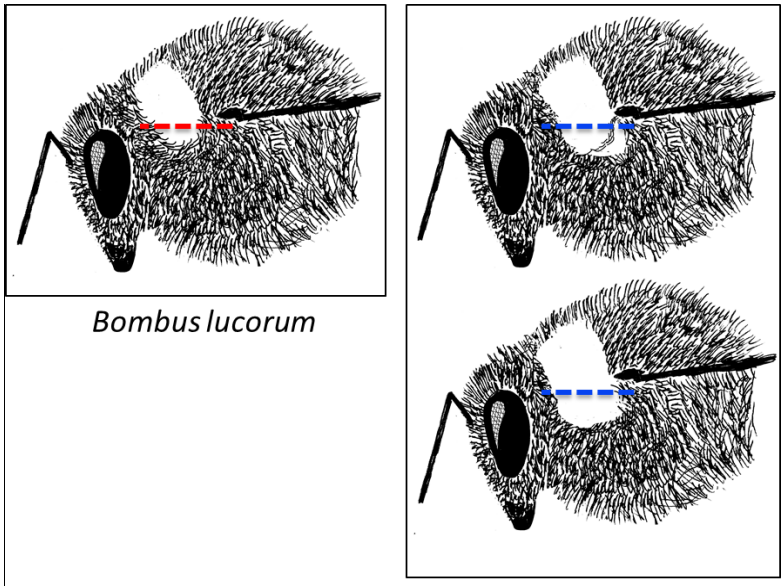


Figure 4. Patterns of lateral hair growth of the thorax (mesosoma): the yellow collar does not extend below the tegulae in *B. lucorum* (red label) but does so in *B. cryptarum* (blue label) (drawings modified from Neumayer unpublished). The upper drawing of *B. cryptarum* shows the s-shaped hairline through the yellow hair growth of the collar (which was not detected in the Alpine specimens).

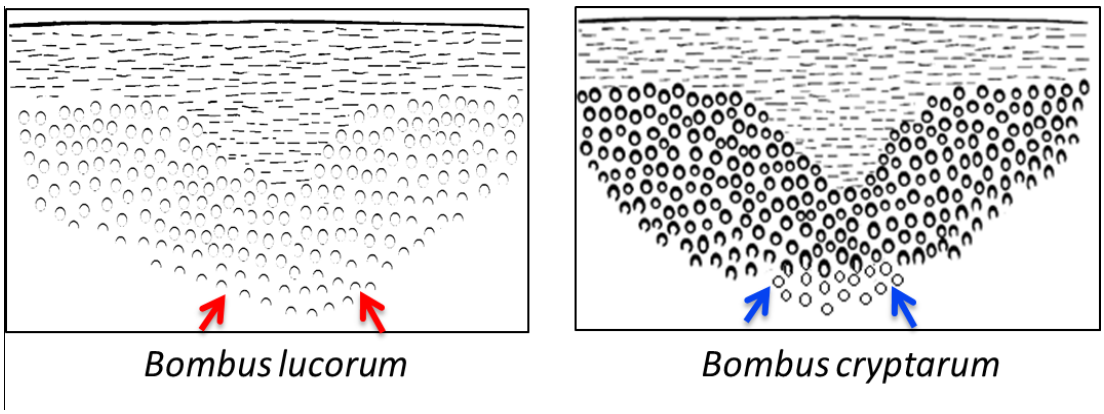


Figure 5. Surface structure on the middle of the edge of the 2nd tergite of the metasoma. The grooves are oblique in *B. lucorum* (red label), while they are deep and clearly rounded in *B. cryptarum* (blue label) (drawings modified from Neumayer unpublished).

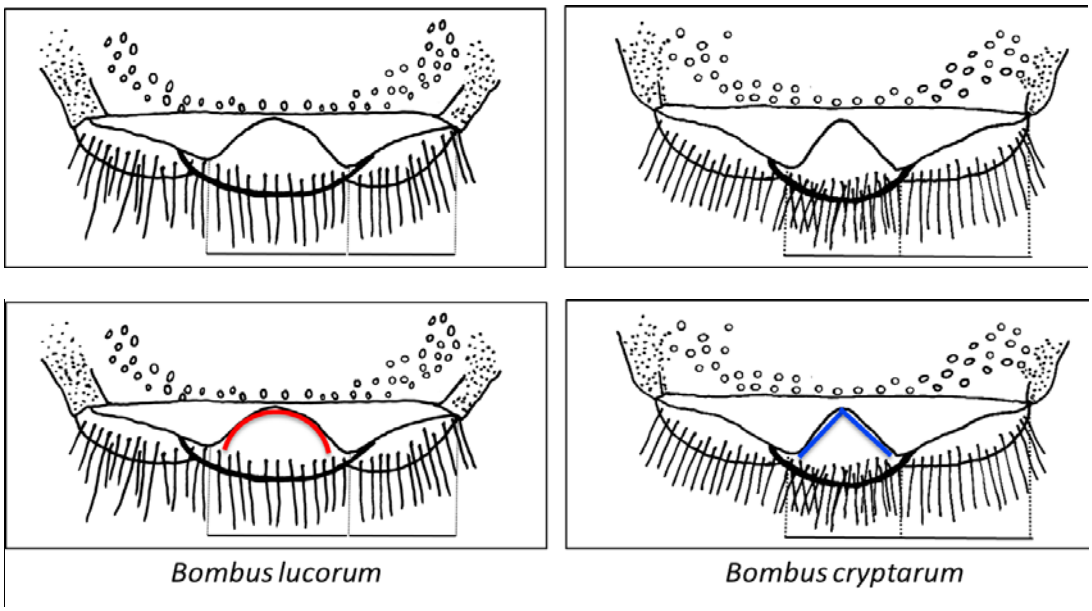


Figure 6. Shape of the lamella of the labrum between the lateral two projections that is u- shaped in *B. lucorum* (red label) and v- shaped in *B.cryptarum* (blue label) (drawings modified from Neumayer unpublished).

4. Results

4.1. Sampled bumblebees

A total of 88 individuals were collected from the sampling areas in Austria, namely: Schneeberg (5 individuals), Gesäuse (2 individuals), Glocknergruppe North (4 individuals), Glocknergruppe South (29 individuals), Karwendel (7 individuals), Kaunertal (17 individuals) and Silvretta (24 individuals). 68 % of bumblebees were taken from altitudes above 1600 meters a.s.l. and 32 % from below 1400 meters a.s.l. (Appendices 1 and 2).

For the molecular and morphological analyses, not all individuals were suited since there were male bumblebees and unsequenced specimens, so that 55 individuals from the *Bombus lucorum* - complex and two individuals from outgroups (*Bombus terrestris*) were included in the final analyses (Appendix 3).

4.2. DNA Barcoding

Contiguous sequences of 658 bp in length were obtained from 57 individuals, comprising 55 sequences of individuals the *B. lucorum* - complex and two sequences of *B. terrestris* (which were used as outgroups). They were used for the phylogenetic tree construction and calculation of the genetic distances.

In the Blast search, the best five matches for each contiguous sequence scored an E-value of 0.0 and sequence identity was always above 99 %. Based on these results, all specimens were associated to those given from the Genbank. Blast search results revealed that the sequences of the 55 bumblebees consisted of 38 individuals of *B. lucorum* and 17 individuals of *B. cryptarum*.

Therefore, according to the genetic data, only two of the three species of the *B. lucorum* - complex, namely *B. lucorum* and *B. cryptarum*, were sampled from the examination areas in the Austrian Alps. No fresh specimens of *B. magnus* could be obtained.

The total sample consisted of 69.1 % of *B. lucorum* and 30.9 % of *B. cryptarum* according to NCBI blast search results.

The obtained genetic data of the DNA barcoded specimens of *B. lucorum* and *B. cryptarum* could be utilized for further biological investigations concerning altitude preferences and distribution in the Alpine regions.

While *B. lucorum* is widely spread in all altitude ranges and more abundant than *B. cryptarum*, the latter shows a preference for habitats located above 2000 meters a.s.l. (Figure 7). Furthermore, despite the low yield of collected specimens, it is clear that *B. cryptarum* is distributed in the whole western sampling area, especially on the southern slopes of the Glockner group (Figure 8).

4.3. Habitat types and forage plants

An examination of the habitats shows that most of the bumblebees were collected from krummholz zones (37.5 % of collected specimens), followed by various meadow habitats (31.8 % of collected specimens), Alpine grass and different waysides (Appendix 2).

Investigation of the foraging plants revealed that various plant species were visited by the bumblebees. The most frequently visited plant was *Calluna vulgaris*, on which 34 % of *B. lucorum* and 38 % *B. cryptarum* were found. Further, 28 % *B. lucorum* and 19 % *B. cryptarum* were visiting different *Trifollium* species. The predominate activity of the bumblebees was nectar feeding, followed by pollen collection (Appendix 2).

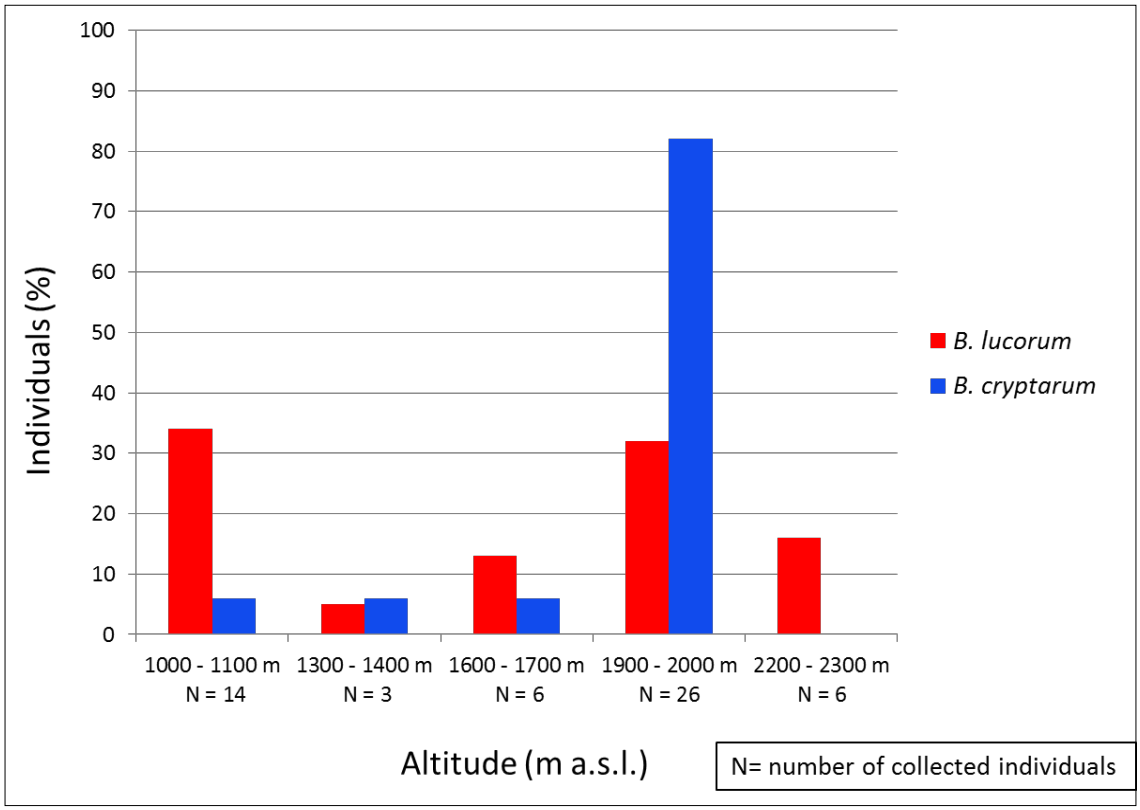


Figure 7. Percentages of the total collected bumblebees of the *B. lucorum* - complex from the different altitudes of the sampling areas in 2012. The graph gives the five selected altitude ranges (m a.s.l.). *B. cryptarum* (blue) occurs in high habitats above 2000 meters a.s.l., while *B. lucorum* (red) occurs in all altitude ranges.

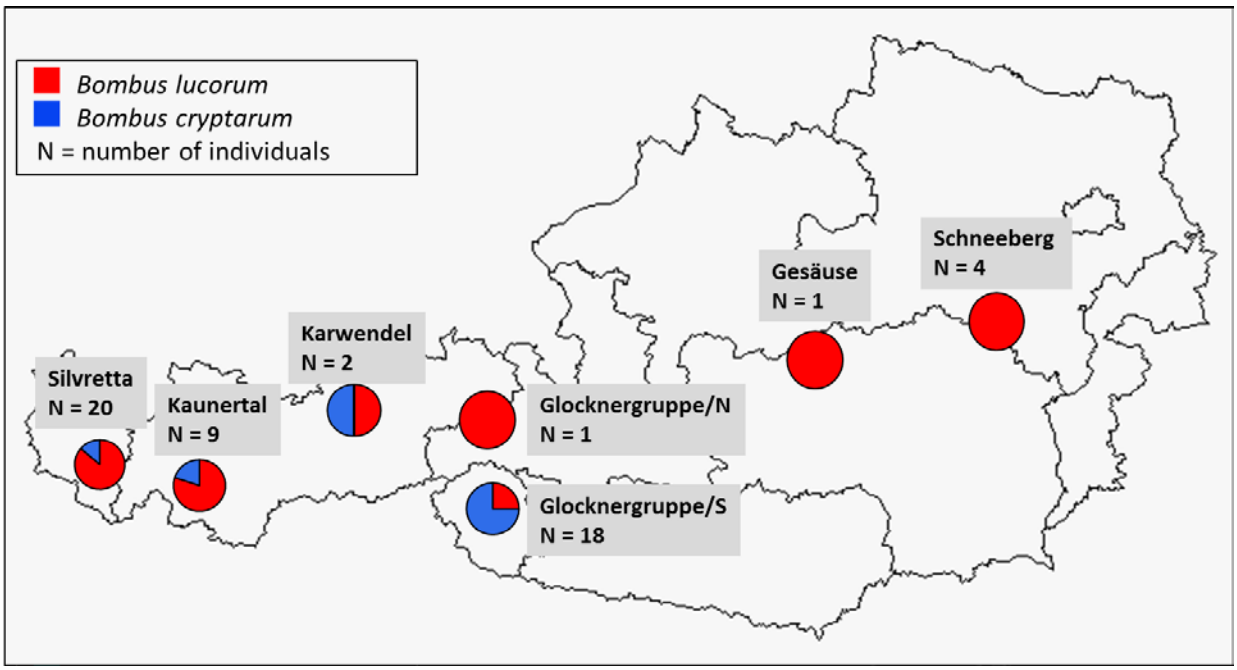


Figure 8. Occurrence of *B. lucorum* (red label) and *B. cryptarum* (blue label) in the studied areas in the Austrian Alps according to the sampling in 2012. Total number of examined individuals N=55.

The NJ tree consisting of 55 sequences shows two distinct clades, one for *B. lucorum* including 38 sequences and the other for *B. cryptarum* including 17 sequences, which of both were fully supported in the bootstrap analysis. In addition, two sequences of the outgroup *Bombus terrestris* are included (Figure 9).

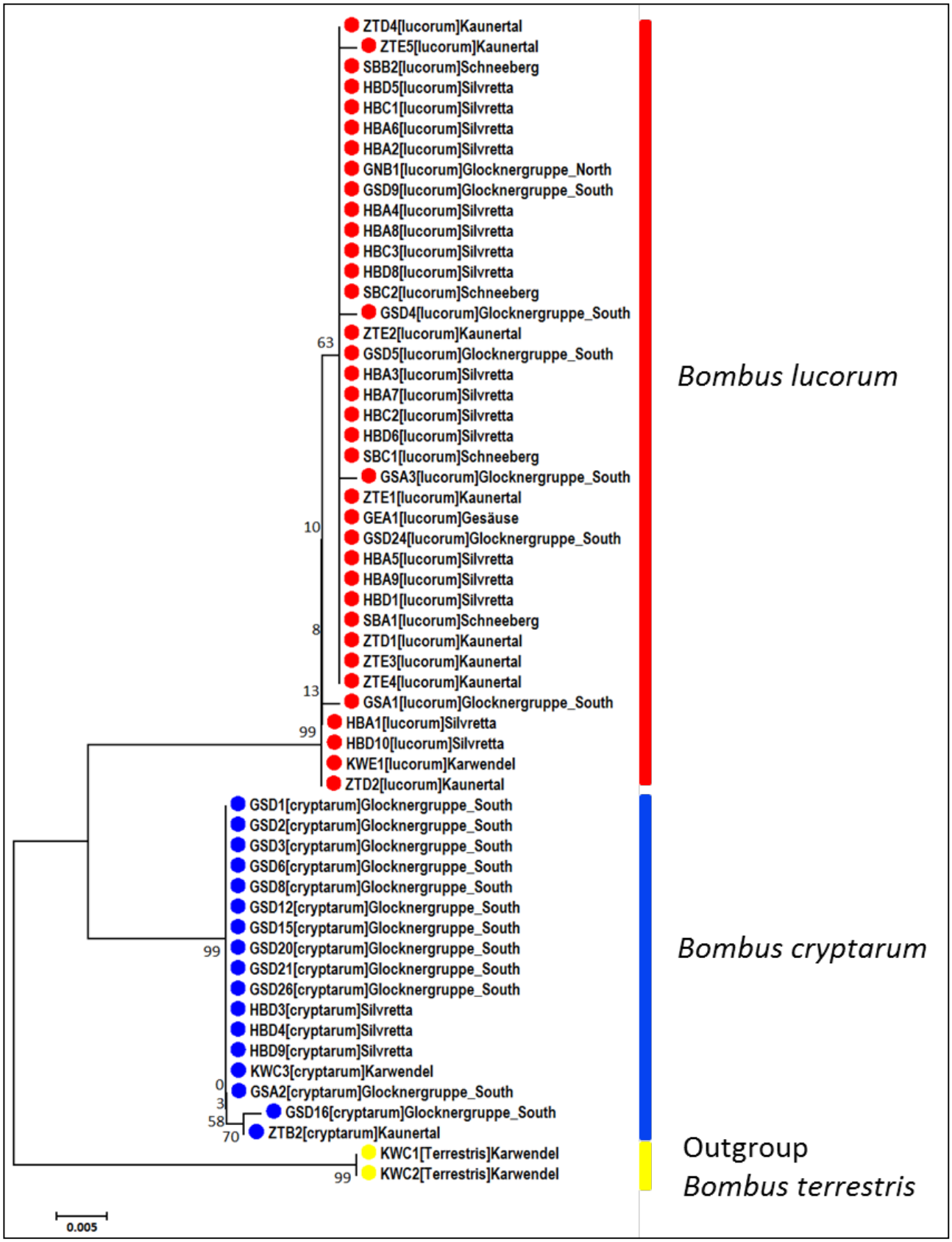


Figure 9. NJ tree including 55 sequences generated in the present study. Bootstrap values are shown at the branch nodes and highly support two distinct main clades: *B. lucorum* (red labels, 38 sequences) and *B. cryptarum* (blue labels, 17 sequences). *B. terrestris* was used as outgroup (yellow labels, 2 sequences).

Genetic distances between *B. lucorum* and *B. cryptarum* ranged from 3.7 to 4.4 %, whereas intraspecific distances ranged between 0 and 0.5 %. The corresponding diagram does not show an overlap between curves of divergences among and within species and, therefore, a clear so called DNA barcoding gap exists, demonstrating the clear genetic separation of *B. lucorum* from *B. cryptarum* (Figure 10).

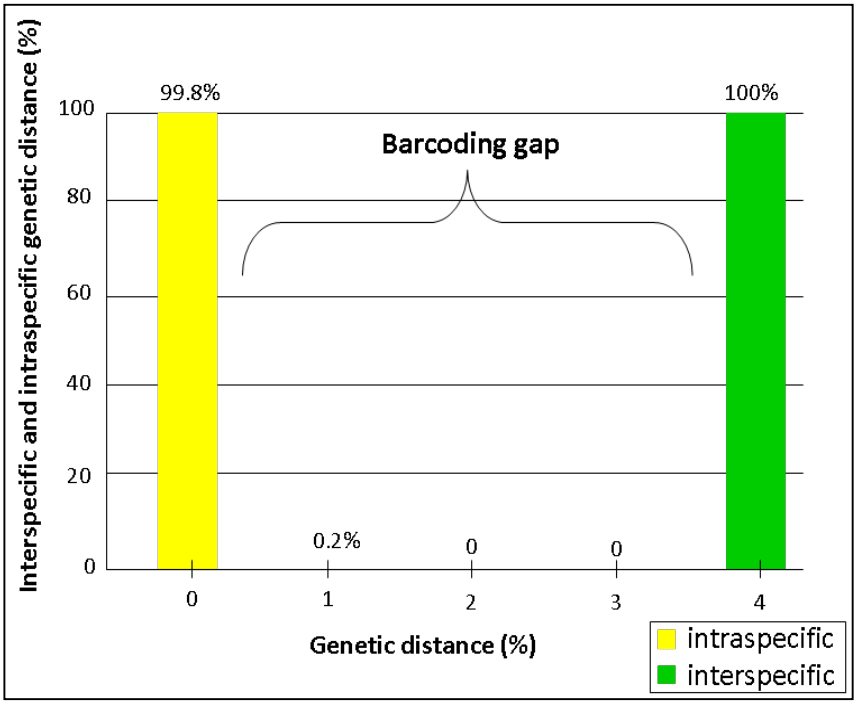


Figure 10. Genetic distances within groups (intraspecific, yellow bar) and between groups (interspecific, green bar). The graph shows very well separated distances resulting in a clear gap in the category range from 0.5 to 3.7 %. This suggests the genetic separation of *B. lucorum* from *B. cryptarum*.

4.4. Comparisons between morphological identification and DNA barcoding determination

The morphological analyses of the individuals revealed that not all character states are reliable for species determination in the sampled bumblebees, since mismatches occurred when comparing the morphological determinations with those based on DNA barcodes. The yellow collar, surface structure of T2 and the shape of the lamella were examined in all DNA barcoded individuals of *B. lucorum* and *B. cryptarum*. In addition, measurements of the selected body parts, the thorax width, the diameter of the median ocellus and the malar space showed overlaps between the two species and no significant differences.

Results of the morphological data of the examined 55 individuals are shown in appendix 3.

Table 5 shows results of matches and mismatches of the comparisons between comparisons of DNA barcodes data and morphological data of the examined specimens.

Table 5. Results showing total numbers of matches and mismatches in the comparison of genetic with morphological determinations of the examined specimens consisting of *B. lucorum* and *B. cryptarum*. Total number of analysed individuals = 55. For full details, see Appendix 3.

Morphological character	**Number of matches (N)**	**Number of mismatches (N)**	**Matches (%)**	**Mismatches (%)**
Yellow collar	47	8	85.5	14.5
Surface structure of T2	45	10	81.8	18.2
Shape of lamella	55	0	100	0

4.4.1. Yellow collar

Reinvestigation of the lateral yellow collar character matched for 85.5 % of total examined bumblebees (N=55 consisting of: *B. lucorum* N=38, *B. cryptarum* N=17) according to comparisons of the obtained morphological and genetic determination data (Figure 11).

The determination did not match for 14.5 % of the individuals representing an overlap in this character. Therefore, 3.6 % were incorrectly identified as *B. cryptarum* (N=2) whereas their genetic identities show that they belong to *B. lucorum* (Figure 12). Additionally, 10.9 % of the specimens were incorrectly identified as *B. lucorum* (N=6) whereas they belong to the *B. cryptarum* species according to their DNA barcodes (Figure 13).

Following figures give examples of differences in the yellow collar character:

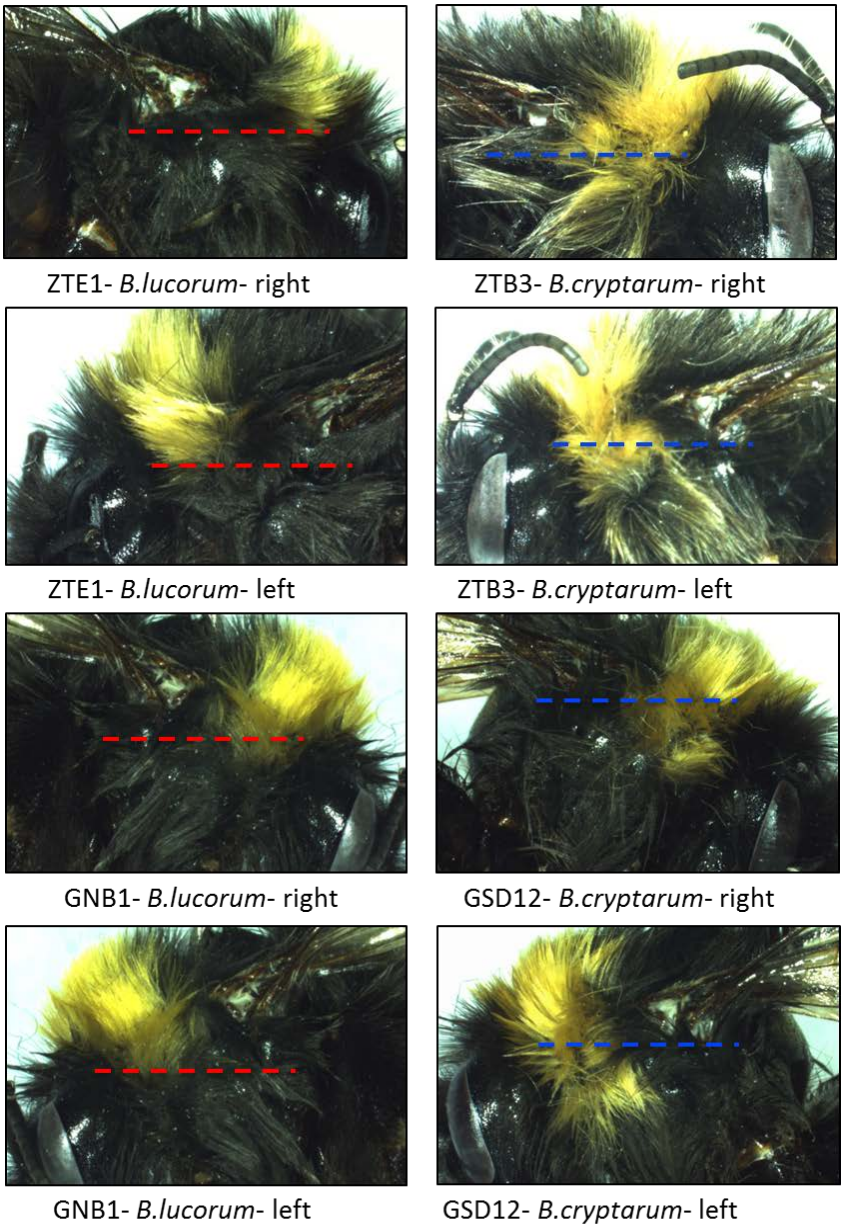


Figure 11. Extent of the yellow collar on the thorax. Pictures were taken from both left and right sides of the examined individuals. The figures show the diagnostic difference of the length of the yellow hair growth, which does not extend below the tegulae in *B. lucorum* (red labels) and extends below the tegulae in *B. cryptarum* (blue labels). Individual specimen code precedes species name.

Figures 12 and 13 show mismatches in individuals of *B. lucorum* and *B. cryptarum* between the species determination based on the “yellow collar” trait compared to the determination using DNA barcoding in both studied bumblebee species.

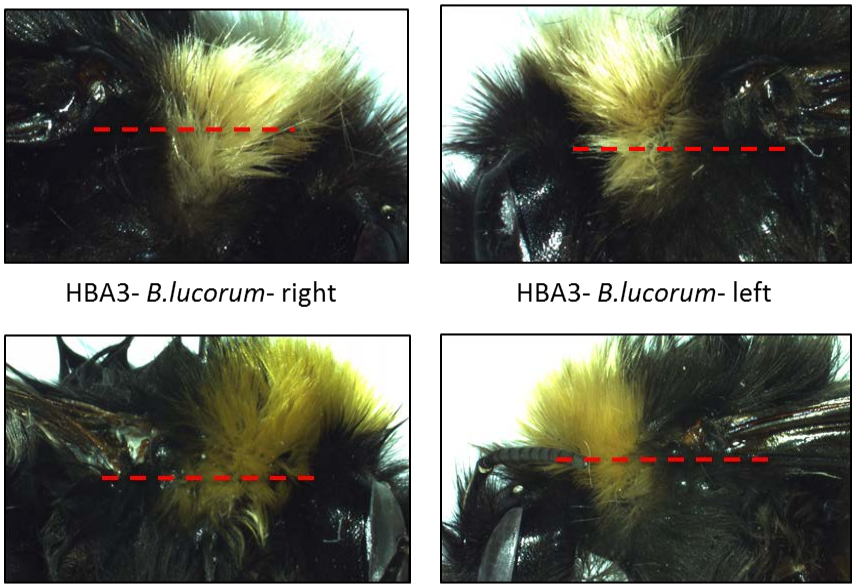


Figure 12. Mismatch of morphological determination based on the character of the "yellow collar" in *B. lucorum* individuals and their determination using DNA barcodes. The yellow hair growth extends below the tegulae (red labels). This character has previously been used to identify *B. cryptarum*. Individuals shown in this figure did not match with their respective morphological data and DNA barcodes. Individual specimen code precedes species name.

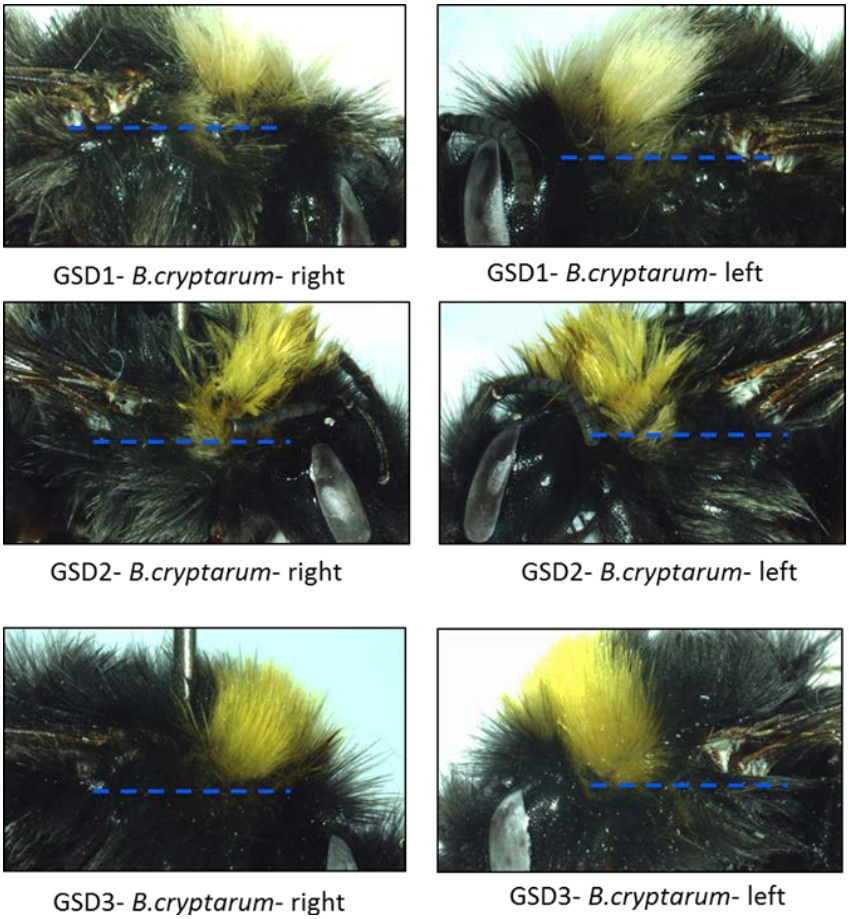


Figure 13. Legend see below

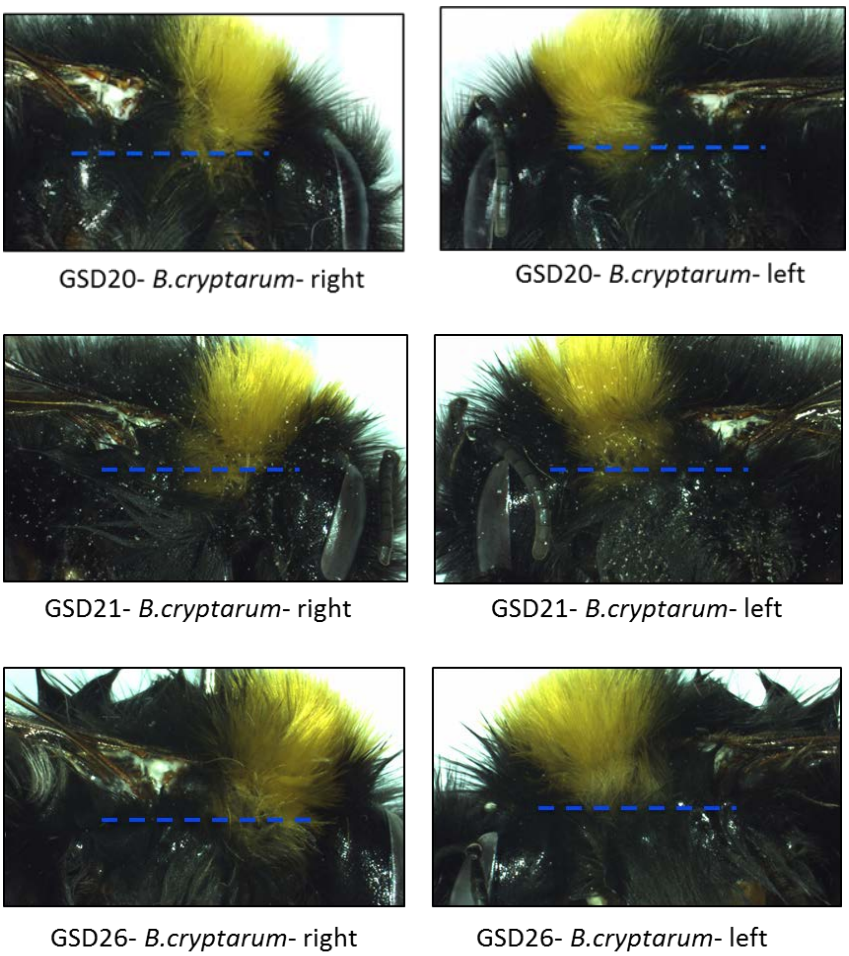
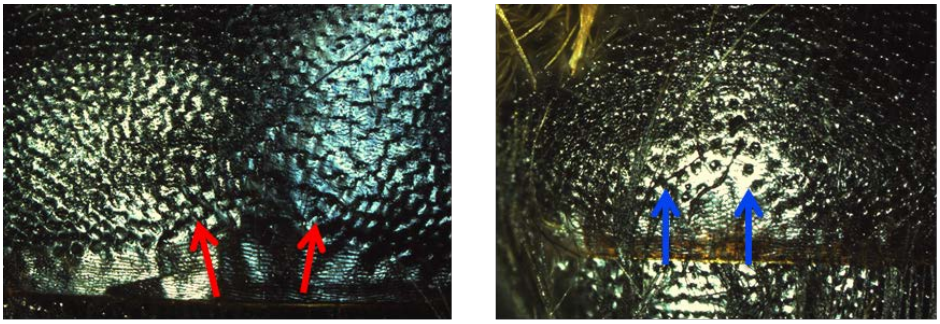


Figure 13. Mismatch of morphological determination based on the character of the "yellow collar" in *B. cryptarum* individuals and their determination using DNA barcodes. The yellow hair growth does not extend below the tegulae (blue labels). This character was previously believed to identify *B. lucorum*. Individuals shown in this figure did not match with their respective morphological data and DNA barcodes. Individual specimen code precedes species name.

4.4.2. Surface structure of the second tergite (T2)

Reinvestigation of the surface structure on the 2nd tergite (T2) of the metasoma turned out to be successful for 81.8 % of all examined individuals according to comparisons between the obtained morphologic and genetic data (Figure 14).

The determination of 18.2 % of the individuals was not successful according to the comparison with the DNA barcodes. Therefore, 12.7 % of the specimens were incorrectly determined as *B. cryptarum* (N=7) and 5.45 % were incorrectly determined as *B. lucorum* (N=3). Thus, the surface structure of T2 represents an overlap between species characters, as well.

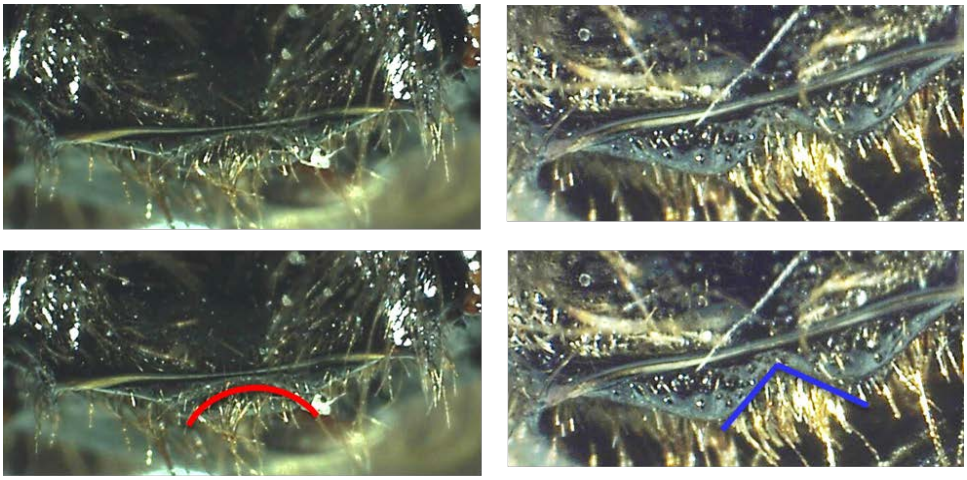


Bombus lucorum *Bombus cryptarum*

Figure 14. Surface structure of the cuticula showing the structure on the 2nd tergite (T2) in the area of the hind margin. Diagnostic differences between examined individuals of *B. lucorum* (red label) and *B. cryptarum* (blue label) are shown. While the surface is obliquely grooved in *B. lucorum*, it is deep and vertically grooved in *B. cryptarum*.

4.4.3. Shape of the lamella of the labrum

Comparison between morphological data regarding this character and the corresponding genetic data showed a match for all examined specimens in both species. All species could be accurately determined in relation to this character and matched the respective genetic determination. Therefore, a match of 100 % was obtained. The following figure shows the difference of this character between *B. lucorum* and *B. cryptarum* (Figure 15).



Bombus lucorum *Bombus cryptarum*

Figure 15. "Shape of the lamella" in examined individuals showing the difference of the U-shaped lamella in *B. lucorum* (red label) and V- shaped lamella in *B. cryptarum* (blue label) between the lateral projections of the labrum.

4.4.4. Measurements of certain body parts

The body size, i.e. thorax width and proportions of the selected body parts; diameter of median ocellus and malar space were studied in order to search for differences within the *B. lucorum* - complex. The results of the measured values varied among the examined species, and neither *B. lucorum* nor *B. cryptarum* showed significant differences ($p < 0.05$) according to the Mann Whitney- U-test of the statistical analysis (Table 4).

The results show similar mean values in all cases, also *p*-values given from the statistical analysis do not show significant differences ($p < 0.05$). Thereby, no significant differences in any of the measured body parts could be demonstrated. The following graphs illustrate the data obtained from measuring the three body parts (Figures 16, 17 and 18).

Table 4. Measurement results of thorax width, diameter of the median ocellus and malar space for each of *B. lucorum* and *B. cryptarum* respectively (mean value ± standard deviation, minimal and maximal values). Results of the *p*-values given from Mann Whitney-U-test are also shown.

	Thorax width (mm)			**Diameter of median ocellus (mm)**			**Malar space (mm)**		
	Mean value ± SD	**Min**	**Max**	**Mean value ± SD**	**Min**	**Max**	**Mean value ± SD**	**Min**	**Max**
Bombus lucorum **N= 38**	3.92 ± 0.5	3.0	5.3	0.27 ± 0.03	0.2	0.36	0.52 ± 0.12	0.38	1.0
Bombus cryptarum **N= 17**	3.86 ± 0.44	2.6	4.6	0.26 ± 0.02	0.22	0.3	0.52 ± 0.05	0.44	0.7
***p*- value**	0.914			0.514			0.607		

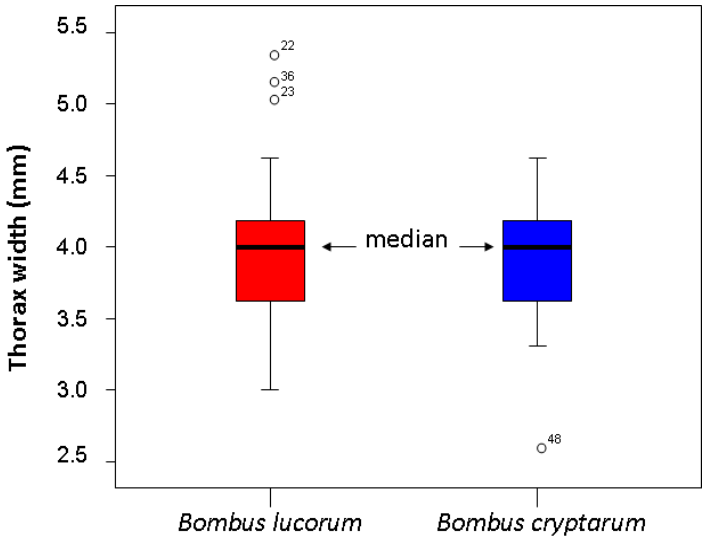


Figure 16. Comparison of thorax width in the examined species *B. lucorum* (red label) and *B. cryptarum* (blue label) according to the u-test (p = 0.914). Thorax width measurements failed to show significant differences between the two species.

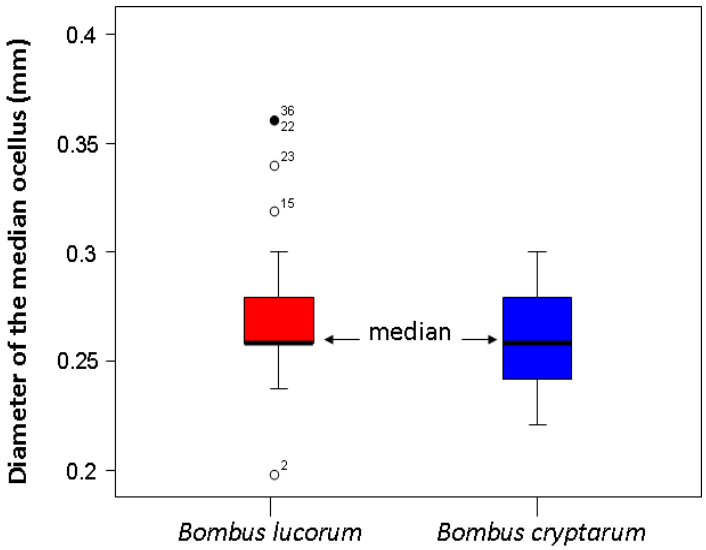


Figure 17. Comparison of diameter of the median ocellus in the examined species *B. lucorum* (red label) and *B. cryptarum* (blue label) according to the u-test (p = 0.514). The measurements do not show significant differences between the species.

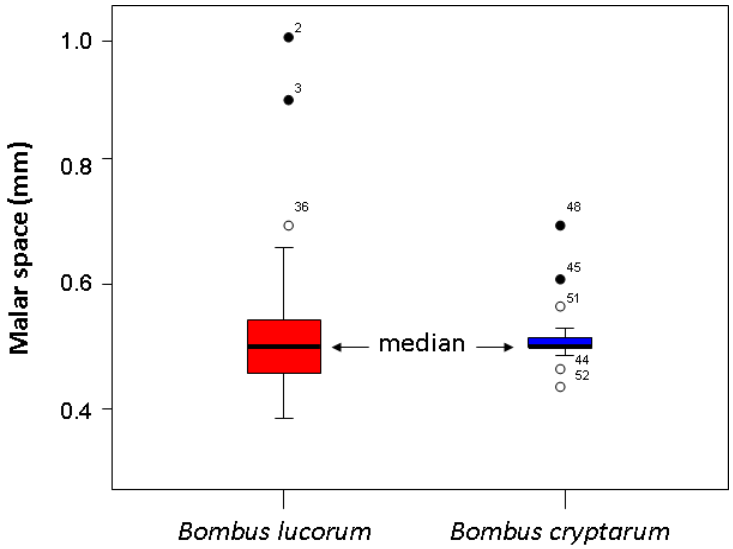


Figure 18. Comparison of malar space in the examined species *B. lucorum* (red label) and *B. cryptarum* (blue label) according to the u-test (p = 0.607). The character does not show significant differences between the species.

5. Discussion

5.1. Molecular genetic determination of the cryptic species of the *Bombus lucorum* - complex by DNA barcoding

5.1.1. *Bombus magnus*

Results of the DNA barcoding clearly demonstrated the absence of one of the three species of the *B. lucorum* - complex, namely *B. magnus*, from the sampling areas in the Austrian Alps in the year 2012. Despite the rich availability of *Calluna vulgaris* in the sampling areas, which is known to be preferred by *B. magnus* (Waters et al. 2010), no individuals of this species could be found. The absence of this species is not surprising, since it is particularly rare in Austria (Gokcezade et al. 2010).

The taxonomic status of *B. magnus* seems to be in dispute. Prior studies considered this species to prefer lowlands (Hagen et al. 2003), as well as heathlands and moors (Goulson et al. 2004). This might explain the lack of *B. magnus* in this study, since the sampling areas did not include lowland regions and moors. It would be worthwhile for further investigations to extend the sampling region to include moorland habitats, as well as to conduct species monitoring.

5.1.2. *Bombus lucorum* and *Bombus cryptarum*

It was clearly shown that molecular methods can obtain reliable data to identify the two cryptic species *B. lucorum* and *B. cryptarum*. The phylogenetic NJ tree shows two clearly separate clades of the *B. lucorum* - complex, namely of *B. lucorum* and *B. cryptarum*, which demonstrates that these species are distinguishable monophyletic species. These results are supported by previous studies of these cryptic species, which also verified species discrimination based on DNA barcodes (Murray et al. 2008; Bertsch 2009; Waters et al. 2010; Carolan et al. 2012).

In addition, the DNA barcoding gap was used as a tool to support the latter results. The presence of the DNA barcoding gap proves the existence of major differences within and among the two species *B. lucorum* and *B. cryptarum*, and is confirmed by the very low intraspecific distance values and high interspecific distance values.

The clades of the phylogenetic NJ tree are reflected by the graph of haplotype network, which shows three distinct clades consisting of *B. lucorum*, *B. cryptarum* and *B. magnus*, which are separated from each other by several mutational steps represented by the network branches. Considering the geographical distribution, the network did not show any geographic differentiation among the haplotypes of *Bombus lucorum* or *Bombus cryptarum*. The Austrian haplotypes cluster with the northern European haplotypes. Interestingly, the haplotypes of Ireland and Denmark of *Bombus magnus* cluster separately and thus are geographically differentiated (Figure 19).

Haplotypes of the Austrian *B. lucorum* show that the major haplotype diversity consists of six single haplotypes connected to the main haplotype by single mutational steps. In contrast, the haplotypes of *B. cryptarum* show two single haplotypes connected to the remaining haplotypes (Figure 19). The sequences of the Austrian *B. lucorum* show one major haplotype and several single haplotypes, which differed by several mutational steps.

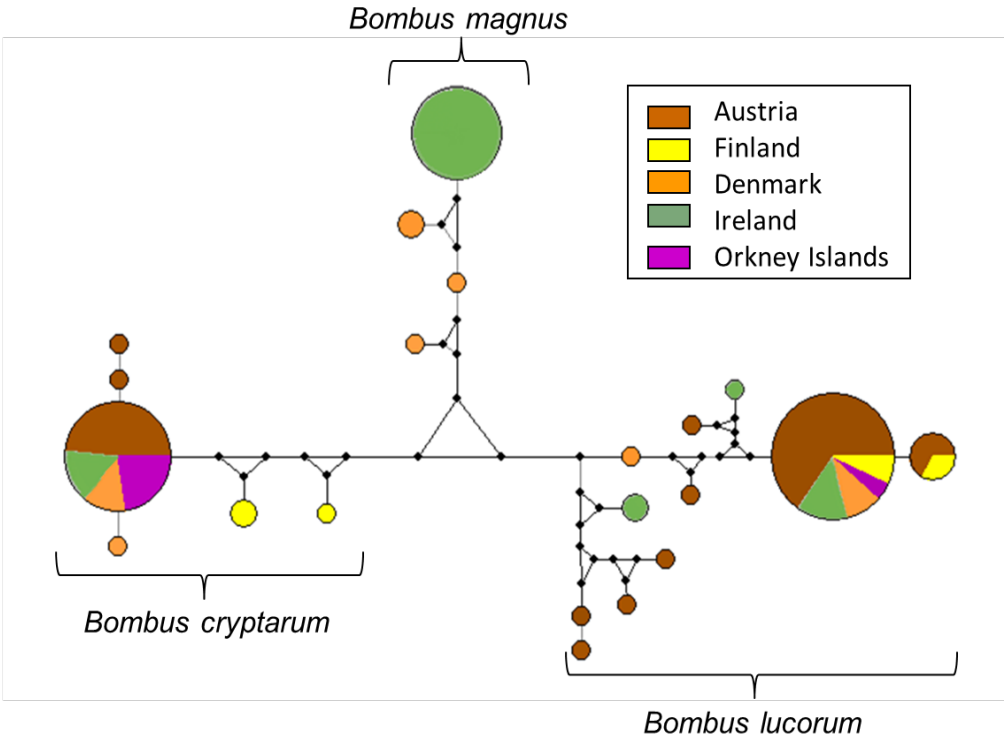


Figure 19. Haplotype network presenting three clades of the species of the *Bombus lucorum* - complex. The network consists of data from this study: Austria (55 sequences) and from previous studies: Finland (8), Denmark (14), Ireland (36) and Orkney Islands (9). Each branch represents one point mutation step and the black dots represent still missing haplotypes. Sizes of the coloured circles are proportional to the sample sizes.

5.2. Ecological differentiations, altitude and plant preferences of *Bombus lucorum* and *Bombus cryptarum*

The obtained molecular data of DNA barcodes yielded interesting facts about the ecological differentiation of the two species, *B. lucorum* and *B. cryptarum*, which occur sympatrically.

The distribution of these species in the sampling areas across the Austrian Alps showed a certain pattern. On the one hand, *B. cryptarum* tends to be more abundant in the western Alpine areas, in contrast to *B. lucorum*, which appears to be abundant in all sampling areas. This pattern of distribution is not surprising for *B. lucorum*, since it is known to be the most common and abundant species among the *B. lucorum* - complex, i.e., an ubiquist (Goulson 2010, Carolan et al. 2012). On the other hand, altitudinal preferences show that *B. cryptarum* occurs mainly in high altitude levels, while *B. lucorum* does not seem to have a preference for particular height levels. The latter results are confirmed by prior studies which indicate the presence of *B. cryptarum* at high altitude ranges (Hagen et al. 2003, Murray et al. 2008).

The altitudinal distributions of the bumblebees reflect the habitat types, i.e. since most individuals of both species were found in high altitude ranges in the krummholz zone. In addition, the examined species seem to occur in a wide range of habitat types; the majority of specimens were collected on meadows, Alpine grass and way sides.

The examined species tend to prefer heath and clover since most individuals visited flowers of the latter plant species. Based on the comparison between plant preferences of *B. lucorum* and *B. cryptarum*, it is evident that they are not specialised foragers (Brodie 1996) since many of the collected individuals had visited a various range of plant species.

Although the sampling was performed with the help of bumblebee specialists (Gereben-Krenn, Krenn, Neumayer & Gokcezade), very low numbers of bumblebees in general were recorded in the year 2012. In sum, it was not a rich season for bumblebees and in particular those of the *B. lucorum* - complex. These results reflect how necessary a species monitoring can be.

5.2. Reinvestigation of morphological characters in *Bombus lucorum* and *Bombus cryptarum*

The comparison of the morphological data and the data of DNA barcodes, based on a total of 55 examined specimens including *B. lucorum* and *B. cryptarum* revealed: 47 matches corresponding to the yellow collar character, 45 matches corresponding to the surface structure of the 2nd tergite character and 66 matches corresponding to the character of the shape of the lamella of the labrum. In short, it was ascertained that most morphological characters turned out to be unreliable when used to positively identify specimens of the *B. lucorum* - complex species. In addition, the measured body parts, which were previously believed, when taken together, to expose slight differences among the female individuals (Bertsch 2009), failed to result in any significant differences.

Interestingly, only the shape of the lamella of the labrum character turned out to be reliable in determination with morphological characters of *B. lucorum* and *B. cryptarum*. In contrast to the yellow collar extension and the surface structure of the 2nd tergite, differences of the narrow U-shape and V-shape of the lamella could be clearly seen and distinguished.

It was not astonishing that no significant differences in biometry were found between these species and that they could not be positively determined by their morphological characters since the cryptic nature of the species has long been suspected. In addition, it has been shown not only that the species are distinct, but that they do not hybridise despite similar morphological appearances (Jonghe et al. 1983).

6. Conclusion

Regarding the two investigated species of the *Bombus lucorum* - complex, namely *B. lucorum* and *B. cryptarum*, it can be concluded that they are very well separated at the genetic level, yet less distinguishable in the morphological level. The DNA barcoding is a good substitute method to discriminate these species. The existing DNA barcoding gap shows that there is a major difference between intraspecific variation and interspecific divergence within and between the investigated species. The morphological characters are not consistently reliable, only the lamella of the labrum character state could be a differentiating character. Furthermore, *B. lucorum* and *B. cryptarum* occur syntopically in krummholz zones and meadow habitats and both tend to prefer *Calluna vulgaris*, as well as diverse *Trifollium* species.

7. Literature

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9. Appendix

Zusammenfassung

Hummeln zählen zu den wichtigsten Bestäubern im heimischen Hochgebirge. Neben vielen Arten, die im Freiland sicher angesprochen werden können, gibt es schwer bestimmbare Arten bzw. kryptische Arten wie jene des *Bombus lucorum* - Komplexes.

Jüngste Untersuchungen mittels DNA-Barcoding haben gezeigt, dass die Differenzierung der zum Teil sehr häufig vorkommenden Arten des *Bombus lucorum* - Komplexes (*B. lucorum*, *B. cryptarum* und *B. magnus*) mit den bisher angewendeten morphologischen Merkmalen nicht möglich ist. Jedoch ergaben Analysen der mitochondrialen DNA einwandfrei drei Taxa (Carolan et al. 2012, Williams et al. 2012). Durch den Nachweis der Unzuverlässigkeit der Artbestimmung von Arbeiterinnen anhand phänotypischer Merkmale, sind auch die Ergebnisse aller bisherigen ökologischen Untersuchungen an dieser Artengruppe obsolet geworden.

In den Alpen kommen zumindest zwei der kryptischen Arten des *Bombus lucorum* - Komplexes (*B. lucorum*, *B. cryptarum*) vor, vielleicht ist auch die dritte Art *B. magnus* in Österreich vertreten. Die Vertreter der *Bombus lucorum* - Gruppe zählen zu den häufigsten Hummelarten mit großer Bedeutung für die Bestäubung heimischer Pflanzen und leben vermutlich sympatrisch in alpinen Lebensräumen (Neumayer & Paulus 1999).

Für diese Studie wurden *Bombus lucorum* - Komplex Hummeln in sieben verschiedenen Gebieten der Österreichischen Alpen in verschiedenen Höhenstufen eingesammelt.

Das Ziel dieser Arbeit war einerseits die Identifizierung der Individuen des *Bombus lucorum* - Komplexes auf molekularer Ebene mittels DNA-Barcoding. Basierend auf den molekularen Ergebnissen, Fragestellungen bezüglich der ökologischen Differenzierung, sowie der Verbreitung, Höhen- und Blütenpräferenzen der Hummeln konnten beantwortet werden.

Die Ergebnisse dieser Arbeit zeigten dass nur zwei Arten vom *Bombus lucorum* - Komplex genetisch identifiziert wurden nämlich *Bombus lucorum* und *Bombus cryptarum*, die sympatrisch miteinander vorkommen. Die beiden Arten *B. lucorum* und *B. cryptarum* sind gut getrennt. Die Höhenpräferenzen zeigten dass *B. lucorum* in allen Höhenstufen vorkommt während *B. cryptarum* Höhenstufen über 2000 Meter bevorzugt. Weiterhin zeigten die Ergebnisse, dass *B. lucorum* häufiger verbreitet ist als *B. cryptarum*, welche in den westlichen Alpen Gebieten vermehrt vorkommen. Einen Großteil der Individuen wurde in Krummholz Gebieten sowie in Weiden Gebieten gefunden. Die Hummeln besuchten eine weite Auswahl an Blüten, die meisten Individuen besuchten *Calluna vulgaris* und *Trifollium* spezies.

Im zweiten Teil der Arbeit wurden morphologische Merkmale der Artbestimmung mit den Ergebnissen der molekularen Bestimmungen verglichen. Die vergleichende morphologische Untersuchung zeigte, dass die meisten Merkmale keine zuverlässigen Bestimmungen der Individuen erlaubten. Nur die Form der Lamelle des Labrums stimmte zu 100 % mit den DNA-Barcoding Bestimmungen überein.

Lebenslauf

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09/1991 - 07/2003	Schulbildung im „Al Nour“ Wissenschaftliches Gymnasium Damaskus, Abschluss mit Reifezeugnis
09/2003 - 06/2005	Grundstudium in Biologie an der Universität Damaskus
04/2006 - 03/2008	Biologiestudium Technische Universität München, Abschluss mit Bachelor of Science
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05/2007- 08/2007	Werkstudentin am Lehrstuhl für „Organische Chemie und Biochemie“ an der Technischen Universität München
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05/2009- 01/2011	Technische Assistentin am „Institut für Garten-, Obst- und Weinbau“, Universität für Bodenkultur, Wien
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Besuch von wissenschaftlichen Kongressen:

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04/ 2013	Hymenopteren-Symposium Naturhistorisches Museum Wien, Poster Presentation

Poster Titel: Cryptic bumblebee species of the *Bombus lucorum* - complex in the Austrian Alps

Appendix 1. Sampling schedule of specimens of the *Bombus lucorum* - complex for the year 2012 from sampling areas in the Austrian Alps: (Kaunertal, Schneeberg, Glocknerstraße Nord and Süd, Silvretta, Gesäuße, Karwendel).

Specimen Code	State	Transect	Location	Sampling date (dd.mm.yyyy)	Altitude range (m)	Altitude (m)	Geographic data (latitude, longitude)	Exposition
ZT-B-1	Tirol	Kaunertal	no data	09.08.2012	B: 1300 - 1400	1311	N 47°04'0,34'' E 12°46'39,3''	no data
ZT-B-2	Tirol	Kaunertal	no data	09.08.2012	B: 1300 - 1400	1313 (according to Google Earth)	N 47°05'' E 12°44'40''	no data
ZT-B-3	Tirol	Kaunertal	no data	09.08.2012	B: 1300 - 1400	1386 (according to Google Earth)	N 47°05'' E 12°44'40''	no data
ZT-B-4	Tirol	Kaunertal	no data	09.08.2012	B: 1300 - 1400	1403	N 47°01'41,6'' E 10°44'26,5''	no data
ZT-B-5	Tirol	Kaunertal	no data	09.08.2012	B: 1300 - 1400	1313	N 47°01'10,6'' E 10°44'20,8''	no data
ZT-B-6	Tirol	Kaunertal	no data	09.08.2012	B: 1300 - 1400	1339	N 47°00'42,3'' E 10°44'24,8''	no data
ZT-D-1	Tirol	Kaunertal	Fernergries	08.08.2012	D: 1900 - 2000	1925	N 46°53'23,5'' E 10°44'04,9''	no data

ZT-D-2	Tirol	Kaunertal	Fernergries	08.08.2012	D: 1900 - 2000	1926	N 46°53'24,1'' E 10°44'05,2''	no data
ZT-D-3	Tirol	Kaunertal	Fernergries	08.08.2012	D: 1900 - 2000	1935	N 46°53'16,4'' E 10°44'04,4''	no data
ZT-D-4	Tirol	Kaunertal	Fernergries	08.08.2012	D: 1900 - 2000	1930	N 46°53'16,5'' E 10°44'05,1''	no data
ZT-D-5	Tirol	Kaunertal	Fernergries	08.08.2012	D: 1900 - 2000	1988 (according to Google Earth)	N 46°53'09,3'' E 10°44'04,4''	no data
ZT-D-6	Tirol	Kaunertal	Fernergries	08.08.2012	D: 1900 - 2000	1963 (according to Google Earth)	N 46°53'11,5'' E 10°44'05,1''	ENE
ZT-E-1	Tirol	Kaunertal	Gletscherstraße	08.08.2012	E: 2200 - 2300	2255	N 46°53'24,1'' E 10°43'14,0''	SE
ZT-E-2	Tirol	Kaunertal	Gletscherstraße	08.08.2012	E: 2200 - 2300	2287	N 46°53'24,5'' E 10°43'14,2''	SE
ZT-E-3	Tirol	Kaunertal	Gletscherstraße	08.08.2012	E: 2200 - 2300	2265 (according to Google Earth)	N 46°53'21,6'' E 10°43'10,6''	SE
ZT-E-4	Tirol	Kaunertal	Gletscherstraße	08.08.2012	E: 2200 - 2300	2275	N 46°53'21,6'' E 10°43'09,1''	SE
ZT-E-5	Tirol	Kaunertal	Gletscherstraße	08.08.2012	E: 2200 - 2300	2301 (according to Google Earth)	N 46°53'18,7'' E 10°42'58,8''	SE

SB-A-1	Nieder-Österreich	Schneeberg	no data	31.07.2012	below 1000	608	N 47°46'45,0'' E 15°53'55,6''	0
SB-B-1	Nieder-Österreich	Schneeberg	no data	31.07.2012	B: 1300 - 1400	1358	N 47°45'11,5'' E 15°51'14,7''	0
SB-B-2	Nieder-Österreich	Schneeberg	no data	31.07.2012	B: 1300 - 1400	1297	N 47°45'15,6'' E 15°51'27,1''	0
SB-C-1	Nieder-Österreich	Schneeberg	no data	31.07.2012	C: 1600 - 1700	1770 (according to Google Earth)	N 47°45'26,4'' E 15°50'08,4''	SE
SB-C-2	Nieder-Österreich	Schneeberg	no data	31.07.2012	C: 1600 - 1700	1770 (according to Google Earth)	N 47°45'26,4'' E 15°50'08,4''	SE
HB-A-1	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	1000	N 46°59'40,1'' E 10°01'03,4''	no data
HB-A-2	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	1000	N 46°59'36,4'' E 10°01'05,2''	no data
HB-A-3	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	968 (according to Google Earth)	N 46°59'04,6'' E 10°01'30,0''	no data
HB-A-4	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	972 (according to Google Earth)	N 46°59'01,2'' E 10°01'29,3''	no data
HB-A-5	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	999 (according to Google Earth)	N 46°58'34,9'' E 10°02'08,9''	no data

HB-A-6	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	999 (according to Google Earth)	N 46°58'34,9'' E 10°02'08,9''	no data
HB-A-7	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	1079	N 46°57'58,1'' E 10°04'18,9''	no data
HB-A-8	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	1078	N 46°57'59,1'' E 10°04'16,7''	no data
HB-A-9	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	1119	N 46°57'43,5'' E 10°04'35,5''	no data
HB-A-10	Vorarlberg	Silvretta	Partenen, Mautstelle	05.08.2012	A: 1000 - 1100	1118	N 46°57'45,2'' E 10°04'37,2''	no data
HB-C-1	Vorarlberg	Silvretta	Vermunt, Stausee	06.08.2012	C: 1600 - 1700	1770 (according to Google Earth)	N 46°56'06,9'' E 10°03'15,5''	no data
HB-C-2	Vorarlberg	Silvretta	Vermunt, Stausee	06.08.2012	C: 1600 - 1700	1740	N 46°56'05,4'' E 10°03'13,2''	no data
HB-C-3	Vorarlberg	Silvretta	Vermunt, Stausee	06.08.2012	C: 1600 - 1700	1766 (according to Google Earth)	N 46°56'02,1'' E 10°03'11,3''	no data
HB-C-4	Vorarlberg	Silvretta	Vermunt, Stausee	06.08.2012	C: 1600 - 1700	1766 (according to Google Earth)	N 46°56'02,1'' E 10°03'11,3''	no data
HB-D-1	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	2030	N 46°55'08,0'' E 10°05'22,2''	no data

HB-D-2	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	2012 (according to Google Earth)	N 46°55'11,7'' E 10°05'09,0''	no data
HB-D-3	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	2007 (according to Google Earth)	N 46°55'12,1'' E 10°05'06,7''	no data
HB-D-4	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	1999 (according to Google Earth)	N 46°55'13,9'' E 10°04'54,8''	no data
HB-D-5	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	1998	N 46°55'13,0'' E 10°04'56,5''	no data
HB-D-6	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	1945	N 46°55'00,4'' E 10°04'51,7''	no data
HB-D-7	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	1958 (according to Google Earth)	N 46°55'00,5'' E 10°04'51,1''	no data
HB-D-8	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	1958 (according to Google Earth)	N 46°55'00,5'' E 10°04'51,1''	no data
HB-D-9	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	1956 (according to Google Earth)	N 46°55'01,5'' E 10°04'44,4''	0
HB-D-10	Vorarlberg	Silvretta	Bielerhöhe	04.08.2012	D: 1900 - 2000	2012	N 46°55'03,1'' E 10°05'24,8''	0

GE-A-1	Steiermark	Gesäuse	Hieflau-Waaggraben	25.08.2012	below 1000	770	N 47°35'18,0'' E 14°44´16,0''	NW
GE-A-2	Steiermark	Gesäuse	Hieflau-Waaggraben	25.08.2012	below 1000	690	N 47°35'26,0'' E 14°44´29,0''	NW
GS-A-1	Kärnten	Glocknerstraße Süd	near Heiligenblut	no data	A: 1000 - 1100	no data	no data	no data
GS-A-2	Kärnten	Glocknerstraße Süd	near Heiligenblut	no data	A: 1000 - 1100	no data	no data	no data
GS-A-3	Kärnten	Glocknerstraße Süd	near Heiligenblut	no data	A: 1000 - 1100	no data	no data	no data
GS-C-1	Kärnten	Glocknerstraße Süd	near Mautstelle	28.08.2012	C: 1600 - 1700	1650	N 47°02'35,5'' E 12°51'13,2''	SW
GS-D-1	Kärnten	Glocknerstraße Süd	Kasereck	17.07.2012	D: 1900 - 2000	1910	N 47°03'15'' E 12°50'00''	SW
GS-D-2	Kärnten	Glocknerstraße Süd	NW Kasereck	17.07.2012	D: 1900 - 2000	1910	N 47°03'40,3'' E 12°49'38,2''	SSW
GS-D-3	Kärnten	Glocknerstraße Süd	Schöneck	17.07.2012	D: 1900 - 2000	1910	N 47°03'24,8'' E 12°48'20,5''	SE
GS-D-4	Kärnten	Glocknerstraße Süd	Wernischalm	18.07.2012	D: 1900 - 2000	1910	N 47°03'51,8'' E 12°49'37,7''	SW
GS-D-5	Kärnten	Glocknerstraße Süd	Wernischalm	18.07.2012	D: 1900 - 2000	1989	N 47°03'51,5'' E 12°49'34,3''	SW

GS-D-6	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2020	N 47°03'53,9'' E 12°49'30,8''	SW
GS-D-7	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-8	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-9	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-10	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-11	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-12	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-13	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-14	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-15	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S

GS-D-16	Kärnten	Glocknerstraße Süd	Tauerneck	28.08.2012	D: 1900 - 2000	2100	N 47°03'50,7'' E 12°49'50,3''	S
GS-D-20	Kärnten	Glocknerstraße Süd	NW Kasereck	17.07.2012	D: 1900 - 2000	1862	N 47°03'40,4'' E 12°49'34,4''	SSW
GS-D-21	Kärnten	Glocknerstraße Süd	NW Kasereck	17.07.2012	D: 1900 - 2000	1863	N 47°03'40,4'' E 12°49'34,4''	SSW
GS-D-22	Kärnten	Glocknerstraße Süd	Schöneck	17.07.2012	D: 1900 - 2000	1937	N 47°03'26,8'' E 12°48'22,4''	ESE
GS-D-23	Kärnten	Glocknerstraße Süd	Schöneck	17.07.2012	D: 1900 - 2000	1935	N 47°03'24,9'' E 12°48'20,5''	SE
GS-D-24	Kärnten	Glocknerstraße Süd	Schöneck	18.07.2012	D: 1900 - 2000	1965	N 47°03'24,2'' E 12°48'19,3''	S
GS-D-25	Kärnten	Glocknerstraße Süd	Schöneck	18.07.2012	D: 1900 - 2000	1965	N 47°03'24,2'' E 12°48'19,3''	S
GS-D-26	Kärnten	Glocknerstraße Süd	Albitzen	18.07.2012	D: 1900 - 2000	2126	N 47°04'0,34'' E 12°46'39,3''	SSW
GS-E-20	Kärnten	Glocknerstraße Süd	Pasterzenweg	18.07.2012	E: 2200 - 2300	approx. 2420	N 47°05'' E 12°44'40''	SW
GS-E-21	Kärnten	Glocknerstraße Süd	Pasterzenweg	18.07.2012	E: 2200 - 2300	approx. 2420	N 47°05'' E 12°44'40''	SW

GN-B-1	Salzburg	Glocknerstraße Nord	Between reversement 1 and 2	17.07.2012	B: 1300 - 1400	1400	N 47°09'06,0'' E 12°48'42,0''	WNW
GN-C-1	Salzburg	Glocknerstraße Nord	GH Piffkar	18.07.2012	C: 1600 - 1700	1631	N 47°09'39,0'' E 12°48'55,0''	WNW
GN-E-1	Salzburg	Glocknerstraße Nord	Scientific station 300 m E	17.07.2012	E: 2200 - 2300	2265	N 47°07'22,0'' E 12°49'32,0''	0
GN-E-2	Salzburg	Glocknerstraße Nord	Scientific station 300 m E	17.07.2012	E: 2200 - 2300	2265	N 47°07'22,0'' E 12°49'32,0''	0
KW-B1	Tirol	Karwendel	Scharnitz	11.08.2012	B: 1300 - 1400	1480	N 47°20´15“ E 11°23´23“	0
KW-C1	Tirol	Karwendel	Innsbruck	10.08.2012	C: 1600 - 1700	1600	N 47°18´05“ E 11°23´00“	SSE
KW-C2	Tirol	Karwendel	Innsbruck	10.08.2012	C: 1600 - 1700	1690	N 47°18´13" E 11°23´18"	SSE
KW-C3	Tirol	Karwendel	Scharnitz	11.08.2012	C: 1600 - 1700	1540	N 47°20´13" E 11°24´15"	0
KW-D1	Tirol	Karwendel	Innsbruck	10.08.2012	D: 1900 - 2000	1905	N 47°18´29" E 11°23´10"	SSE
KW-D2	Tirol	Karwendel	Scharnitz	11.08.2012	D: 1900 - 2000	1840	N 47°20´08" E 11°25´32"	SW

KW-E1	Tirol	Karwendel	Scharnitz	11.08.2012	E: 2200 - 2300	2150	N 47°19´36" E 11°26´22"	S

Appendix 2. Sampling data providing details of habitat types and activities of the collected specimens on the determined plants.

Individual	**Habitat type**	**Forage plant species**	**Activity on forage plant**
ZT-B-1	wayside, ruderal	*Vicia sp.*	nectar
ZT-B-2	wayside, ruderal	*Epilobium angustifolium*	nectar
ZT-B-3	wayside, ruderal	*Knautia arvensis*	nectar
ZT-B-4	avalanche burial, dry slope	*Galeopsis sp.*	pollen
ZT-B-5	fertile meadow	*Trifolium pratense*	nectar
ZT-B-6	wayside	*Galeopsis sp.*	pollen
ZT-D-1	krummholz zone, east exposed slope	*Calluna sp.*	nectar
ZT-D-2	krummholz zone, east exposed slope	*Calluna sp.*	nectar
ZT-D-3	krummholz zone, east exposed slope	*Calluna sp.*	pollen
ZT-D-4	krummholz zone, east exposed slope	*Calluna sp.*	pollen
ZT-D-5	krummholz zone, east exposed slope	*Calluna sp.*	nectar

ZT-D-6	krummholz zone, east exposed slope	*Calluna sp.*	no data
ZT-E-1	krummholz zone	*Calluna sp.*, juniper	nectar
ZT-E-2	krummholz zone	*Calluna sp.*, juniper	nectar
ZT-E-3	krummholz zone	*Calluna sp.*, juniper	pollen
ZT-E-4	krummholz zone	*Calluna sp.*, juniper	nectar
ZT-E-5	krummholz zone	*Calluna sp.*, juniper	nectar
SB-A-1	edge of a forest	*Centaurea scabiosa*	pollen
SB-B-1	edge of a coniferous forest	*Vicia tenuifolia*	pollen
SB-B-2	meadow slope	*Trifolium pratense*	nectar
SB-C-1	krummholz zone	*Leontodon hispidus*	nectar & pollen
SB-C-2	krummholz zone	*Rhinanthus sp.*	pollen
HB-A-1	fertile meadow, roadside	*Trifolium pratense*	pollen
HB-A-2	fertile meadow, roadside	*Trifolium pratense*	no data
HB-A-3	fertile meadow	*Trifolium pratense*	no data
HB-A-4	fertile meadow	*Trifolium pratense*	no data
HB-A-5	fertile meadow	*Trifolium pratense*	nectar
HB-A-6	fertile meadow	*Trifolium pratense*	nectar
HB-A-7	fertile meadow	*Trifolium pratense*	nectar
HB-A-8	fertile meadow	*Trifolium pratense*	nectar
HB-A-9	fertile meadow	*Trifolium pratense*	nectar
HB-A-10	fertile meadow	*Trifolium pratense*	nectar

HB-C-1	alpine grass, dwarf shrubs	*Thymus sp.*	no data
HB-C-2	alpine grass, dwarf shrubs	*Thymus sp.*	no data
HB-C-3	alpine grass, dwarf shrubs	*Thymus sp.*	nectar
HB-C-4	alpine grass, dwarf shrubs	*Thymus sp.*	nectar
HB-D-1	wayside, parking place	no data	no data
HB-D-2	krummholz zone	Juniper, *Calluna vulgaris*, *Nardus sp.*	nectar
HB-D-3	krummholz zone	Juniper, *Calluna vulgaris*, *Nardus sp.*	no data
HB-D-4	krummholz zone	Juniper, *Calluna vulgaris*, *Nardus sp.*	nectar
HB-D-5	krummholz zone, source field	*Calluna sp.*	nectar
HB-D-6	riverbank	*Calluna sp.*	nectar
HB-D-7	riverbank	*Calluna sp.*	nectar
HB-D-8	riverbank	*Calluna sp.*	nectar
HB-D-9	riverbank	*Calluna sp.*	nectar
HB-D-10	road embankment	*Calluna vulgaris*, *Rhinanthus sp., Vaccinium myrtillus*	pollen
GE-A-1	tall forbs in a deciduous forest	*Cirsium oleraceum*	nectar & pollen
GE-A-2	tall forbs in a deciduous forest	*Cirsium oleraceum*	nectar
GS-A-1	edge of a forest	*Vicia cracca*	nectar robbing & pollen

GS-A-2	edge of a forest	*Vicia cracca*	nectar robbing & pollen
GS-A-3	edge of a forest	*Vicia cracca*	no data
GS-C-1	meadow	*Centaurea scabiosa*	nectar robbing & pollen
GS-D-1	mowed wayside	*Vicia cracca*	nectar robbing
GS-D-2	mountain mowing	*Trifolium montanum*	nectar
GS-D-3	mountain mowing	*Trifolium pratense*	nectar & pollen
GS-D-4	mountain mowing	*Rhinanthus glacialis*	nectar robbing & pollen
GS-D-5	pasture	*Trifolium pratense*	nectar robbing
GS-D-6	krummholz zone	*Calluna vulgaris*	nectar
GS-D-7	krummholz zone	*Calluna vulgaris*	nectar
GS-D-8	krummholz zone	*Calluna vulgaris*	nectar
GS-D-9	krummholz zone	*Calluna vulgaris*	nectar
GS-D-10	krummholz zone	*Calluna vulgaris*	nectar
GS-D-11	krummholz zone	*Calluna vulgaris*	nectar
GS-D-12	krummholz zone	*Calluna vulgaris*	nectar
GS-D-13	krummholz zone	*Calluna vulgaris*	nectar
GS-D-14	krummholz zone	*Calluna vulgaris*	nectar
GS-D-15	krummholz zone	*Calluna vulgaris*	nectar
GS-D-16	krummholz zone	*Calluna vulgaris*	nectar

GS-D-20	no data	no data	no data
GS-D-21	no data	no data	no data
GS-D-22	mountain mowing	*Scabiosa lucida*	nectar
GS-D-23	mountain mowing	*Thymus sp.*	nectar
GS-D-24	mountain mowing	*Silene vulgaris*	nectar
GS-D-25	mountain mowing	*Geranium sylvaticum*	nectar
GS-D-26	rock heaps	*Anthyllis vulneraria*	nectar
GS-E-20	tall forbs	*Cirsium spinosissimum*	nectar
GS-E-21	tall forbs	*Cirsium spinosissimum*	nectar
GN-B-1	edge of a forest, tall forbs	*Trifolium pratense*	nectar robbing & pollen
GN-C-1	wayside of a meadow	*Rhinanthus glacialis*	nectar robbing & pollen
GN-E-1	alpine meadow,	*Trifolium pratense (nivalis)*, *Sesleria sp.*	nectar robbing & pollen
GN-E-2	alpine meadow,	*Trifolium pratense (nivalis)* , *Sesleria sp.*	nectar robbing
KW-B1	wayside	*Carduus defloratus*	resting
KW-C1	tall forbs	*Cirsium erisithales*	nectar
KW-C2	Pasture	*Prunella grandiflora*	nectar robbing & pollen
KW-C3	pasture	*Hypericum maculatum*	pollen
KW-D1	meadow grass	*Cirsium spinosissimum*	nectar
KW-D2	krummholz zone, wayside	*Carduus defloratus*	nectar
KW-E1	alpine grass	*Thymus sp.*	nectar

Appendix 3. Comparison of determinations made using morphological characters and DNA barcodes of the examined individuals of *B. lucorum* and *B. cryptarum*. Species names written in bold indicate mismatches for the respective comparisons.

	Comparison 1		**Comparison 2**		**Comparison 3**	
Individual specimen code	„Yellow collar“ morphological determination	“Yellow collar” DNA barcoding determination	“Surface structure of T2” morphological determination	“Surface structure of T2” DNA barcoding determination	“Shape of labrum” morphological determination	“Shape of lamella” DNA barcoding determination
ZT-B-2	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
ZT-D-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-D-2	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-D-4	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-E-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-E-2	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-E-3	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-E-4	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
ZT-E-5	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
SB-A-1	*B. lucorum*	*B. lucorum*	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
SB-B-2	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
SB-C-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
SB-C-2	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-A-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*

HB-A-2	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-A-3	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-A-4	*B. lucorum*	*B. lucorum*	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
HB-A-5	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-A-6	***B. cryptarum***	***B. lucorum***	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
HB-A-7	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-A-8	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-A-9	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-C-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-C-2	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-C-3	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-D-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-D-3	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
HB-D-4	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
HB-D-5	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-D-6	*B. lucorum*	*B. lucorum*	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
HB-D-8	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
HB-D-9	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
HB-D-10	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
GE-A-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
GS-A-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
GS-A-2	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-A-3	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
GS-D-1	***B. lucorum***	***B. cryptarum***	***B. lucorum***	***B. cryptarum***	*B. cryptarum*	*B. cryptarum*
GS-D-2	***B. lucorum***	***B. cryptarum***	***B. lucorum***	***B. cryptarum***	*B. cryptarum*	*B. cryptarum*
GS-D-3	***B. lucorum***	***B. cryptarum***	***B. lucorum***	***B. cryptarum***	*B. cryptarum*	*B. cryptarum*
GS-D-4	*B. lucorum*	*B. lucorum*	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
GS-D-5	*B. lucorum*	*B. lucorum*	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
GS-D-6	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*

GS-D-8	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-D-9	*B. lucorum*	*B. lucorum*	***B. cryptarum***	***B. lucorum***	*B. lucorum*	*B. lucorum*
GS-D-12	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-D-15	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-D-16	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-D-20	***B. lucorum***	***B. cryptarum***	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-D-21	***B. lucorum***	***B. cryptarum***	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GS-D-24	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
GS-D-26	***B. lucorum***	***B. cryptarum***	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
GN-B-1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*
KW-C3	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*	*B. cryptarum*
KW-E1	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*	*B. lucorum*