

The Cryptic *Bombus lucorum* Complex (Hymenoptera: Apidae) in Austria: Phylogeny, Distribution, Habitat Usage and a Climatic Characterization Based on COI Sequence Data

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(Received 22 January 2015; Accepted 15 December 2015)

Silas Bossert, Barbara-Amina Gereben-Krenn, Johann Neumayer, Bernhard Schneller, and Harald W. Krenn (2016) The *Bombus lucorum* complex represents a group of three distinct but cryptic bumblebee species in Europe. With the advent of DNA-based identification methods, their species status was confirmed and the use of COI barcoding proved to be an especially useful tool for species identification within the group. Meanwhile, the identification based on morphology remains difficult and recent studies challenged the general distinguishability by revealing an important character to be unreliable. This has consequences for our understanding of the distribution and ecology of the species in Europe and aggravates our patchy knowledge of the situation in Austria and the whole area of the European Alps. In this study, we investigate the exact species composition and distribution of the *Bombus lucorum* complex in Austria based on the reliable species identification with COI sequence data. The habitat usage is studied and the first extensive investigation of altitudinal and climatic differentiation is provided. The results support three distinct genotypic groups in the *Bombus lucorum* complex. *B. lucorum* and *B. cryptarum* co-occur in several areas across the country, with *B. lucorum* being the most common and most widespread species. The study provides no evidence for the presence of *B. magnus* in Austria. The less common species, *B. cryptarum*, mainly occurs in the high mountains and is the predominant species of the complex above altitudes of 2100 m a.s.l. Further, *B. cryptarum* is almost absent from woodlands and is relatively more abundant in habitats with colder climate than *B. lucorum* in Austria. Additionally, the results indicate a very low intraspecific genetic variation within *B. lucorum* and *B. cryptarum*. This study confirms previous findings of three distinct species within the species complex. Based on reliable COI identification, the first coherent overview of the species complex in Austria can be achieved. The climatic data allows us to explain the differences in the distribution patterns. Moreover, the low intraspecific variation may indicate past bottleneck conditions for *B. lucorum* and *B. cryptarum*.

Key words: *Bombus lucorum* complex, *Bombus cryptarum*, Cryptic species, Ecological differentiation, Distribution patterns, Austrian Alps.

BACKGROUND

The investigation of cryptic species is of

increasing interest and impacts our understanding of global species diversity and conservation biology (Bickford et al. 2006). Regarding bumblebees,

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the subgenus *Bombus* s. str. is especially rich in cryptic species (Williams et al. 2012a) and species recognition and delimitation has been traditionally difficult. At the same time, species of this subgenus are considered to be important pollinators of natural environments and are commercially reared for pollination services of agricultural crops (Velthuis and van Doorn 2006; Goulson 2010; Abrol 2012; Williams et al. 2012a, 2012b). In Middle Europe, the subgenus is considered to consist of four distinct species, *Bombus terrestris*, *B. lucorum*, *B. cryptarum* and *B. magnus*. Particularly the taxonomic status of the latter three species, namely the *Bombus lucorum* complex, was uncertain for a long period. However, new tools, notably the use of nucleotide sequence data, improved our understanding of the species complex and supported their species status (Bertsch et al. 2005; Murray et al. 2008; Bertsch 2009; Carolan et al. 2012; Williams et al. 2012a). Still, the possibilities of a morphological delineation remain a complicated issue. Identification keys were published and used before the advent of DNA-based delineation methods (Rasmont 1984; Rasmont et al. 1986) and recent DNA studies cast doubt on important distinguishing characters (Waters et al. 2011; Carolan et al. 2012). The question of a general distinguishability in the wider European context remains unresolved and a DNA-ensured key that covers males and both female castes is not available (see Bossert 2015 for a review). Queens were reported to be recognizable in most cases (Bertsch 1997; Bertsch et al. 2004), but it is reasonable that the vast majority of collectors are not able to properly distinguish the species, especially when the species identification is conducted in the field. This is reflected by the common merging of the *Bombus* s. str. taxa in ecological studies (e.g., Neumayer and Paulus 1999; Goulson and Darill 2014; Fitzpatrick et al. 2007; Schneller et al. 2014).

Besides the difficult morphological distinguishability, we face another major challenge regarding the ecological delineation. Due to the highly similar morphology and the late reintroduction of *B. cryptarum* in the scientific community (Rasmont 1981a, 1981b), many previous studies are based on insufficient data. Before the studies of Rasmont (1981a), prominent bumblebee experts disagreed on the treatment of *B. cryptarum* as a distinct species (e.g., Løken 1966; Tkalců 1969; Pekkarinen 1979). Further, the majority of studies are limited to small regions and no study provides a comparative sampling over an

extensive altitudinal range. This study represents the first countrywide attempt to investigate the ecology and distribution of the *Bombus lucorum* complex in Austria which is based on the accurate identification with COI sequence data.

MATERIALS AND METHODS

Taxon sampling

Specimens were collected from 18 areas across Austria (Table 1). To cover a broad altitudinal range, the collections were conducted in the Austrian Alps from the valley floors to the highest possible elevation of the respective areas. Additional sampling was conducted in the lower-lying Vienna Woods and in the wetlands of northern Austria.

The region of Hohe Tauern was subdivided into three distinct sites due to the different conditions of the sites on the main ridge of the Alps. For every collection area, altitudinal categories were defined. Each category covered 300 m in altitude and was assigned a unique letter: A (300-600 m asl.), B (600-900 m), C (900-1200 m), D (1200-1500 m), E (1500-1800 m), F (1800-2100 m), G (2100-2400 m) and H (2400-2700 m). A maximum of 13 specimens for one altitudinal category per area was collected. To avoid excessive sampling of several bumblebees per nest, a maximum of two specimens was collected at any location. Seven habitat categories were defined: bogs together with fens, buffer stripes such as green way- and roadsides, dwarf shrub and krummholz communities, scree areas, tall forb meadows and woodlands together with forest edges. The different kinds of grassland communities, such as lowland and alpine pastures, grasslands with poor and rich nutrient levels, and dry and wet meadows, were associated with the category of meadows. Specimens from all castes with an emphasis on workers were manually collected using nets, killed in 99% ethanol and stored at -20°C prior to DNA extraction. The following set of information was recorded for each specimen: GPS coordinates, elevation, collection date, potential flower visit with details such as pollen, nectar foraging or nectar robbery, and the habitat type of the collection site. In addition, 26 specimens of *B. terrestris* and one specimen each of *B. magnus* and *B. soroeensis* from Norway were collected and included into the phylogenetic analyses. A collection table with all examined

specimens can be found in the supplementary file (Additional file 1: Collection table).

DNA extraction, PCR, sequencing and data analyses

DNA extraction was conducted using two different methods. In general, a single crushed midleg was used. If the extraction had to be repeated or if the specimens were very small, a second leg or additional legs were clipped. For most specimens, a Proteinase K digestion was used, and the DNA was extracted following a standard phenol-chloroform protocol (Sambrook et al. 1989). For the remaining samples, the extraction was performed using a DNeasy® Blood & Tissue Kit according to the manufacturer's manual. Polymerase chain reactions (PCR) of a partial COI sequence were conducted using the universal primers LCO1490 and HC02198 (Folmer et al. 1994). The DreamTaq™ PCR Mastermix (2x) was used to include the remaining PCR components. Reaction mixtures and the amplification profiles were implemented according to the manufacturer's protocol. Purified PCR amplicons were sent to

VBC-Biotech Service GmbH (Vienna, Austria) for sequencing. Sequences were manually checked and aligned using BioEdit 7.2.5 (Hall 1999). Uncorrected mean genetic distances (p -distances) between and within the species were computed using Mega 6 (Tamura et al. 2013) and 1000 bootstrap replications. To calculate the trees, a more comprehensive dataset was used: identical sequences were removed, resulting in one individual sequence for every haplotype shown in Table 2. Therefore, a haplotype was defined as an individual with unique partial COI sequence. Additional sequence data from GenBank was included, representing three additional samples for *B. lucorum* ([GenBank: JN872604], [GenBank: JN872597], [GenBank: JN872589]), three for *B. cryptarum* ([GenBank: JN872574], [GenBank: JN872581], [GenBank: JN872566]), three for *B. magnus* ([GenBank: JN872620], [GenBank: JQ692958], [GenBank: JN872607]), and three for *B. terrestris* ([GenBank: KJ837297], [GenBank: KJ838879], [GenBank: JQ843670]). jModeltest 2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012) was used to identify the best-fit nucleotide substitution model, which was GTR+I

Table 1. Comprehensive collection table

Collection site	Altitudinal range categories	Number of females			Number of males			Total
		<i>B. lucorum</i>	<i>B. cryptarum</i>	<i>B. terrestris</i>	<i>B. lucorum</i>	<i>B. cryptarum</i>	<i>B. terrestris</i>	
Vienna Woods (V)	300 - 600 m a.s.l.	17	0	10	1	0	1	29
Blockheide (LA)	300 - 600 m a.s.l.	9	3	0	0	0	0	12
Lunz am See (LA)	600 - 1500 m a.s.l.	27	0	0	1	0	0	28
Raxalpe and Schneeberg (LA)	300 - 2100 m a.s.l.	50	3	0	6	0	0	59
Rottalmoos (LA)	300 - 600 m a.s.l.	2	3	0	0	0	0	5
Dachstein (ST)	600 - 2100 m a.s.l.	0	2	0	2	0	0	4
Gesäuse (ST)	300 - 1800 m a.s.l.	8 (1 queen)	0	1	29	0	3	41
Tannermoor (UA)	600 - 900 m a.s.l.	11	2	0	0	0	0	13
Tennen Mountains (SA)	1200 - 2100 m a.s.l.	17	0	1	0	0	0	18
Hohe Tauern, main ridge (SA)	2400 - 2700 m a.s.l.	4	7	0	0	0	0	11
Hohe Tauern, northern slope (SA)	900 - 2400 m a.s.l.	2	17	0	0	0	0	19
Hohe Tauern, southern slope (C)	900 - 2400 m a.s.l.	6	21	0	0	5	0	32
Karawanks (C)	600 - 2100 m a.s.l.	34	1	0	0	0	0	35
Karwendel (T)	1200 - 2400 m a.s.l.	2	2	10	1	1	0	16
Ötztal Alps (T)	900 - 2400 m a.s.l.	17	13	0	0	2	0	32
Zillertal Alps (T)	600 - 1500 m a.s.l.	2	1 queen	0	8	0	0	11
Silvretta Alps (VA)	900 - 2100 m a.s.l.	19 (3 queens)	3	0	0	0	0	22
Total		227	78	22	48	8	4	387

The abbreviations in the brackets refer to the respective federal states within Austria: V = Vienna, LA = Lower Austria, ST = Styria, UA = Upper Austria, SA = Salzburg, C = Carinthia, T = Tyrol and VA = Vorarlberg.

according to both the corrected Akaike Information Criterion (AICc) and Bayesian Information Criterion (BIC). Bayesian phylogenetic inference based on the Markov Chain Monte Carlo method (MCMC) was completed with MrBayes 3.2.2 (Ronquist et al. 2012) using the generalised time reversible model with proportion of invariable sites (GTR+I). 10000000 generations with a sample frequency of 1000 were calculated. The consensus tree with the respective Bayesian posterior probabilities values was produced with the default burn-in of 25%. Further, a maximum likelihood analyses (ML) was performed using RAxML (Stamatakis 2014) as implemented in CIPRES (Miller et al. 2010), with 1000 rapid bootstrap replicates. A third tree was calculated using a neighbor joining (NJ) cluster analysis which was carried out in Mega 6 using gamma distribution and 10 000 bootstraps. All trees were visualized with TreeGraph 2.0.50 (Stöver and Müller 2010). The specimens were identified based on the relationships of the Bayesian phylogram (Fig. 1).

Biogeographical and ecological analyses

To decipher the ecological differences between the species, the data collected on their

distribution and foraging were compared against the determination based on the COI analyses. Therefore, the respective proportions of the species in every habitat category, altitudinal category and area were calculated. To test if the proportion of the species was effected by habitat, an analysis of variance (ANOVA) was performed. Additionally, a likelihood ratio test of independence (G test) was computed to compare the proportions of the two species in the category "Woodland/Forest edge". As in Murray et al. (2008), a G test was used to examine the species composition below and above an altitudinal threshold, which was set to 1200 m in the study at hand. Since crucial climatic factors, such as precipitation and temperature, change considerably with increasing altitude, these factors were also included in the analyses. Given the importance of dependable data concerning the altitudinal climate changes, high resolution Austrian climate maps for 1971-2000 (Hiebl et al. 2011) were used. The grids consider altitudinal changes by a digital elevation model. On this basis, together with the collected GPS data, it was possible to assign the mean annual precipitation sum and the mean annual air temperature of this period for each collection site where at least one specimen was collected.

Table 2. The estimated haplotypes, their frequency and accession numbers

Species	Haplotype	Number of specimens	Sequence corresponds to ENA acc. no.
<i>Bombus lucorum</i>	L1	231	LN714024
	L2	1	LN714025
	L3	1	LN714026
	L4	1	LN714027
	L5	3	LN714028
	L6	1	LN714029
	L7	1	LN714030
	L8	2	LN714031
	L9	1	LN714032
	L10	1	LN714033
	L11	7	LN714034
	L12	1	LN714035
	L13	16	LN714036
	L14	3	LN714037
	L15	2	LN714038
	L16	1	LN714039
	L17	2	LN714040
<i>Bombus cryptarum</i>	C1	2	KJ787691
	C2	67	LN714041
	C3	16	LN714042
	C4	1	LN714043
<i>Bombus terrestris</i>	T1	1	LN714044
	T2	25	LN714045

In addition, we examined whether these assigned values differ between the investigated species. Since both the mean annual precipitation sum and the mean annual air temperature data do not follow a normal distribution, differences were tested with two-sample Wilcoxon rank sum tests. The GIS grids were kindly provided by Alexander Orlik from the Zentralanstalt für Meteorologie und Geodynamik (ZAMG) and processed with QGIS 2.2 (QGIS Development Team 2014). Statistical analyses were performed using R (R Core Team

2013). For the *G* tests, the Deducer package (Fellows 2012) was used.

RESULTS

A total of 387 bumblebees was sampled from July 17, 2012 to August 22, 2013 by the collectors listed in the collection table (Additional file 1: Collection Table). Overall, the sample consisted of 275 specimens of *Bombus lucorum*, 86 *B.*

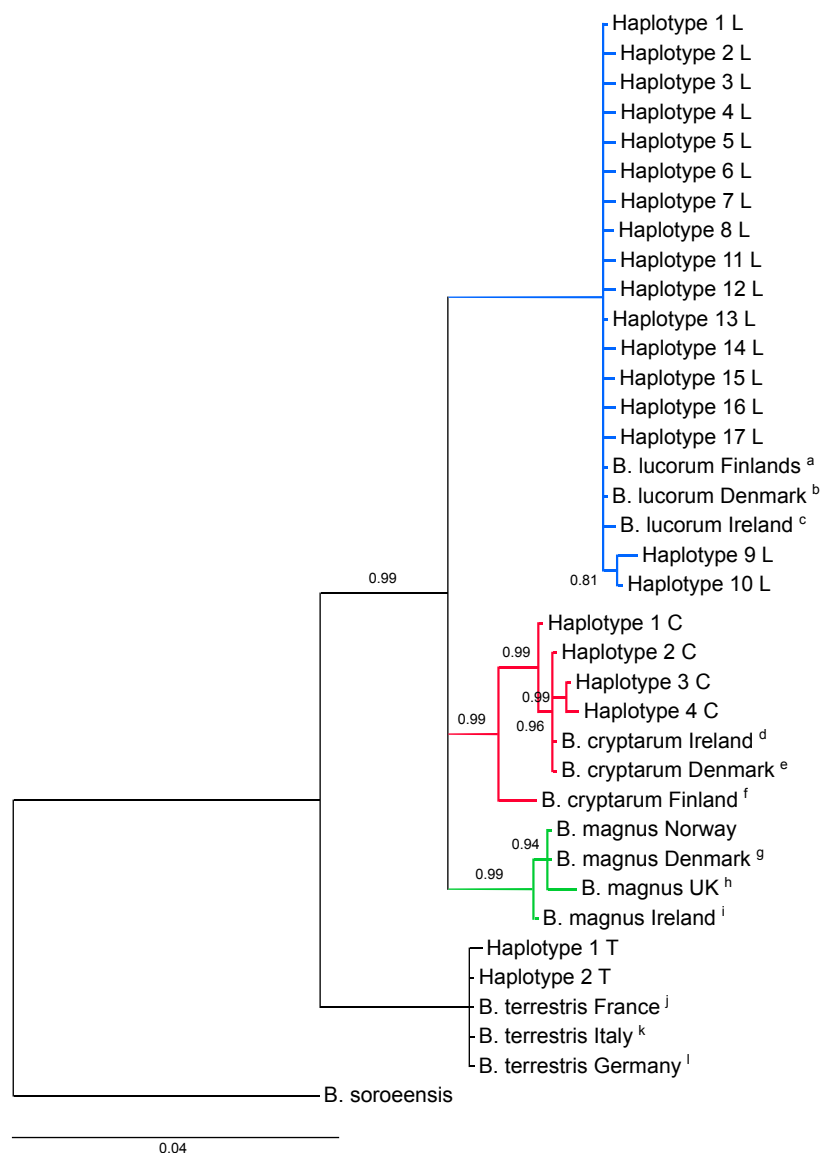


Fig. 1. Bayesian phylogram. Values at branches show Bayesian posterior probabilities (PP). Only values that are below 1.00 are shown. Samples with superscript letters are imported from GenBank: a [GenBank:JN872589], b [GenBank:JN872597], c [GenBank:JN872604], d [GenBank:JN872574], e [GenBank:JN872581], f [GenBank:JN872566], g [GenBank:JN872620], h [GenBank:JQ692958], i [GenBank:JN872607], j [GenBank:JQ843670], k [GenBank:KJ837297] and l [GenBank:KJ838879]. The *B. magnus* sample from Norway corresponds to voucher #388, and *B. soroeensis* to #389.

cryptarum and 26 *B. terrestris* (Table 1). There is presently no evidence for the presence of *B. magnus* in Austria.

Haplotypes, genetic distances and phylogeny

Partial COI sequences for 387 samples were obtained and successfully aligned. Overall, the 609 comparable positions revealed 67 polymorphic sites, 40 sites with transitions and 29 sites with transversions. 23 different haplotypes were found consisting of 17 haplotypes for *B. lucorum*, four haplotypes for *B. cryptarum* and two for *B. terrestris* (Table 2). The haplotype L1 was by far the most common for *B. lucorum*, and the sequences of all other haplotypes were highly similar. Only two haplotypes differed in more than one substitution, namely L9 with three and L14 with two substitutions. The most common haplotype of *B. cryptarum*, C2, shared all except one position with C1 and C3 and the rare haplotype C4 differed in two positions (see Additional file 2: Diagnostic positions).

Both the Bayesian (Fig. 1) and the ML tree (Additional file 3: Maximum Likelihood tree) show comparable results of three distinct and well-supported monophyletic clades in the *B. lucorum* group. This is also reflected by the similarity tree (Additional file 4: Neighbor-Joining tree). The relationships within the species complex are unresolved and no relationship between *B. lucorum*, *B. cryptarum* and *B. magnus* can be inferred due to the tree polytomies. *Bombus terrestris* appears as the sister-group in all trees.

As shown in Table 3, the interspecific *p*-distances between *B. lucorum*, *B. cryptarum* and *B. terrestris* ranged from 0.041 to 0.071 and the distances within the species were considerably smaller with 0.0001 to 0.0006, revealing a clear “barcoding gap” locally within Austria.

Ecological implications

Bombus lucorum and *Bombus cryptarum* are widespread in Austria, whereas no evidence

for *B. magnus* could be found. *B. lucorum* and *B. cryptarum* co-occur in 13 of 18 study areas, in the remaining five areas only *B. lucorum* was found. *B. lucorum* is by far the most common species, followed by *B. cryptarum* with 86 specimens. *B. lucorum* was more abundant in the majority of study areas, only the three areas in the Hohe Tauern and the small sample from Litschau show greater proportions of *B. cryptarum*. Most specimens of *B. cryptarum* were collected in the Central Eastern Alps, located in western Austria, especially in the Hohe Tauern and the Ötztal Alps. *B. cryptarum* seems to be very rare in eastern Austria, whereas there are remarkable findings of this species in wetlands in the north. Both species occurred in all seven habitat categories (Fig. 2).

Neither the number of collected specimens of *B. lucorum* ($F = 0.276$; $p = 0.606$), nor that of *B. cryptarum* ($F = 0.734$; $p = 0.402$) was significantly affected by the habitat. However, most individuals of both species were captured in meadow habitats, and *B. cryptarum* seems to be relatively more abundant in scree areas. Except for one collected individual, it is conspicuous that *B. cryptarum* is almost completely absent from woodlands, whereas *B. lucorum* was found there frequently. The proportion of the numbers of these two species found in woodlands, compared to all other habitats, shows that *B. lucorum* is highly significantly more abundant in woodlands than *B. cryptarum* ($G = 30.225$; $p < 0.0001$). As shown in figure 3, both species occur along the full altitudinal range, whereas the proportion of *B. cryptarum* clearly rises with increasing altitude. *B. lucorum* is more abundant in all altitudinal categories except the two highest categories in which *B. cryptarum* occurred more often. Comparing the species composition below and above the altitudinal threshold of 1200 m reveals a significant inhomogeneity ($G = 40.193$; $p < 0.0001$). Based on the mean annual air temperature for 1971–2000 (Fig. 4), the mean annual air temperature for collection sites was 2.53°C for sites of *B. cryptarum* and 4.49°C for sites of *B. lucorum*. The Wilcoxon rank sum test demonstrates that the difference between these

Table 3. Inter- & intraspecific *p*-distances of the COI gene $\pm$ S.E.

	<i>B. lucorum</i>	<i>B. cryptarum</i>	<i>B. terrestris</i>
<i>B. lucorum</i>	0.0006 $\pm$ 0.0002		
<i>B. cryptarum</i>	0.0411 $\pm$ 0.0073	0.0002 $\pm$ 0.0001	
<i>B. terrestris</i>	0.0707 $\pm$ 0.0096	0.0625 $\pm$ 0.0092	0.0001 $\pm$ 0.0001

groups is statistically significant ($W = 6273.5$; $p < 0.0001$).

Bombus lucorum occurs in the warmest and coldest study areas, whereas *B. cryptarum* is absent from regions with a mean annual air temperature higher than 7.1°C . This includes great parts of eastern Austria. In addition, most *B. cryptarum* in areas with a mean annual air temperature above 5.6°C are from the northern wetlands. The mean annual precipitation seems to have no detectable effect on the species composition ($W = 10897$; $p = 0.2722$). Both species occurred in areas with high and low precipitation (Fig. 5), resulting in a mean precipitation of 1356 mm per year for the collection localities of *B. cryptarum* and 1410 mm per year for the sites of *B. lucorum* (Fig. 6).

Both *B. lucorum* and *B. cryptarum* could be found foraging on a variety of plant species (Additional file 5: Flower visits). A total of 216 flower visits were recorded for *B. lucorum* and

74 flower visits were observed for *B. cryptarum*. All in all, *B. lucorum* visited 59 plant species. 53 species from 19 different plant families were used for collecting pollen. *Bombus cryptarum* was found on flowers of 30 plant species, of which 18 species from 11 families were used for collecting pollen. The majority of plant species were observed to be visited by both bumblebee species. The two plant species with the highest number of visits are shared along the two species, namely *Calluna vulgaris* and *Rhinanthus glacialis*. Other plants that were frequently used by *B. lucorum* and *B. cryptarum* are species of *Vaccinium* and *Trifolium*. Rather uncommon pollen collection could be observed on species of *Rumex* (Polygonaceae) and *Dactylis glomerata* (Poaceae). However, due to the great number of the plant species visited, there are many for which only one visit could be observed. Both bumblebee species could be observed performing nectar robbery, especially on flowers of *Rhinanthus*. Consulting the table

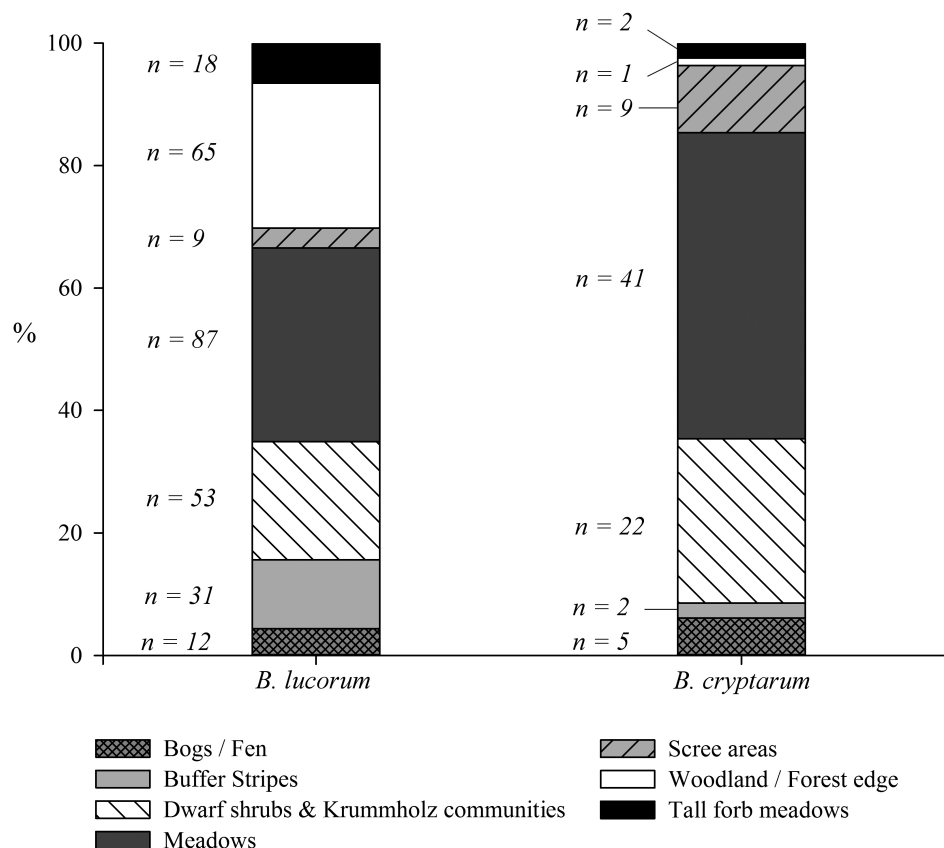


Fig. 2. The distribution of *Bombus lucorum* and *Bombus cryptarum* along the examined habitats. Both species occurred in every habitat type and most often on the various meadow types. *B. lucorum* seems to occur often in woodlands and forest edges whereas *B. cryptarum* appears to avoid these habitats. The figure shows the respective proportion of both species and is designed after Fig. 3 from Waters et al. (2011).

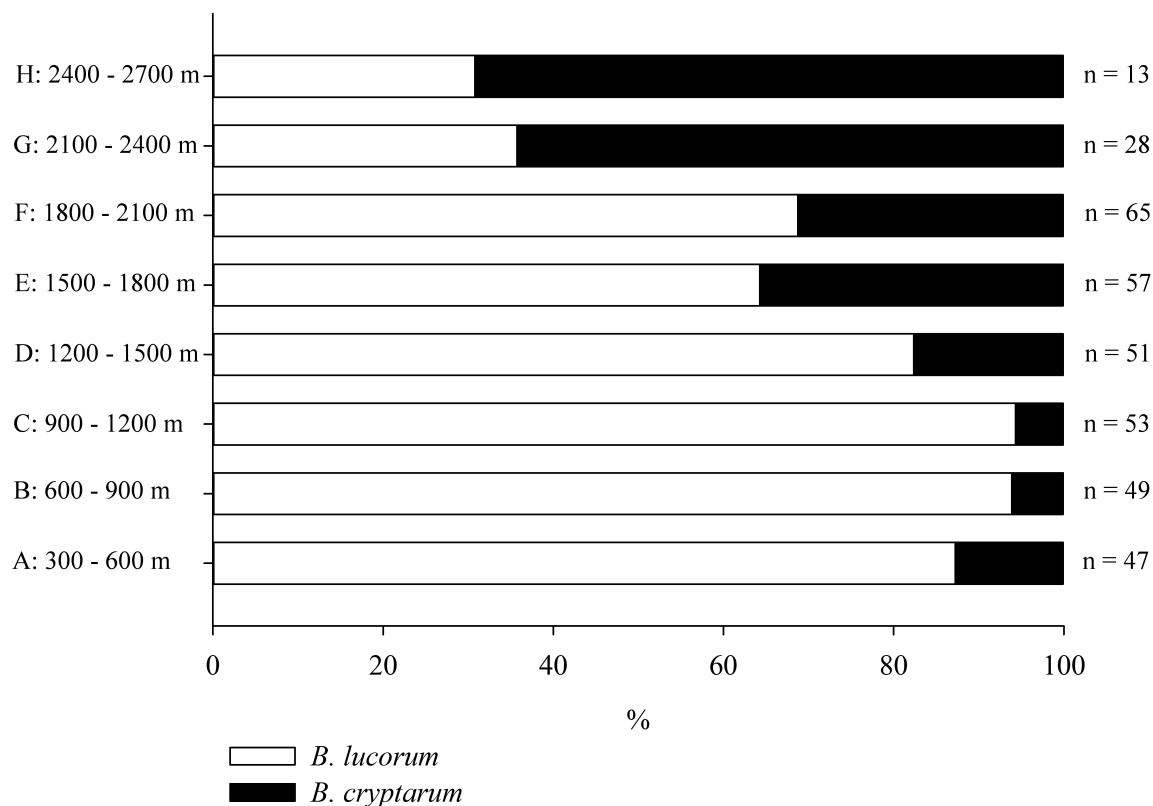


Fig. 3. The proportions of *Bombus lucorum* and *Bombus cryptarum* in the respective altitudinal categories. The proportions reveal a shift in the species abundances along the altitudinal gradient and show that the abundance of *B. cryptarum* rises with increasing altitude.

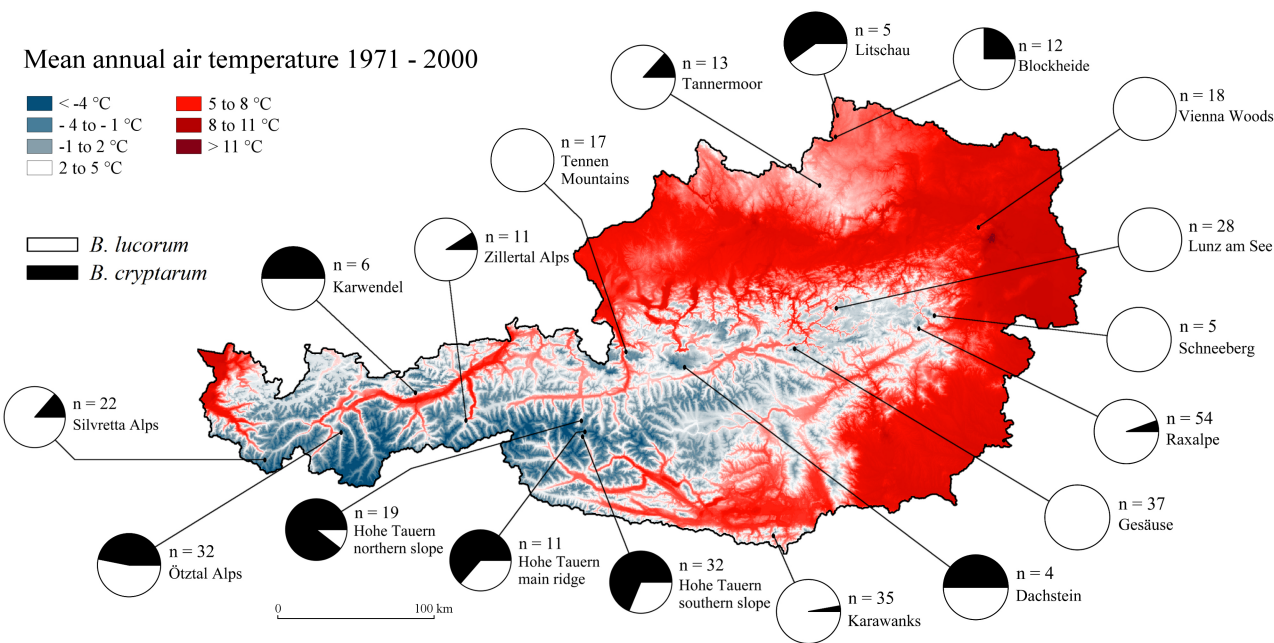


Fig. 4. The distribution of *Bombus lucorum* and *Bombus cryptarum* in Austria on a temperature climate map. The pie charts show the respective proportions of the species in the study areas. The climate map shows the mean annual air temperature for the period 1971 - 2000 with linearized color interpolation and is based on the data of Hiebl et al. (2011).

(see Additional file 5: Flower visits), it seems that *B. lucorum* performs buzz pollination more frequently with 35 observed flower visits involving buzzing, in contrast to *B. cryptarum*, with only a single observation. However, it should be noted that the majority of plant species on which *B. lucorum* performed buzzing were not visited by *B. cryptarum* in this study.

DISCUSSION

New insights into the species ecology and distribution of *Bombus lucorum* and *Bombus cryptarum*

Overall, *Bombus lucorum* is the most abundant species of the *Bombus lucorum* complex

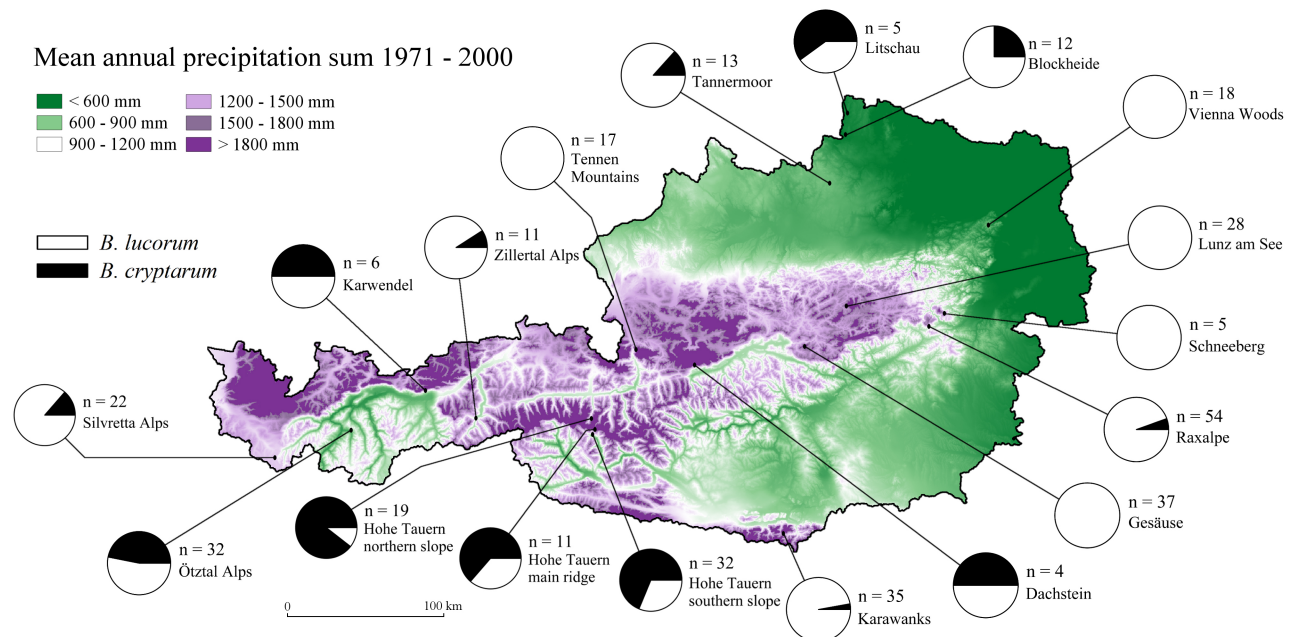


Fig. 5. The distribution of *Bombus lucorum* and *Bombus cryptarum* based on a precipitation climate map. The map shows the mean annual precipitation sum for the period 1971-2000 and is based on the data of Hiebl et al. (2011). The map has linearized color interpolation.

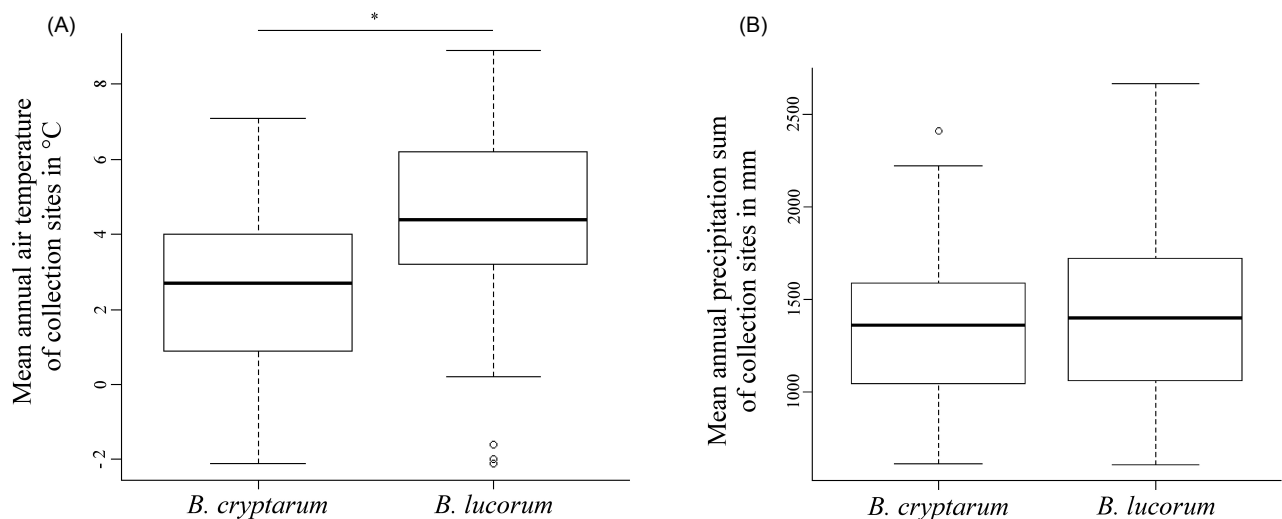


Fig. 6. (A) The mean annual air temperature and (B) the mean annual precipitation sum of the collection sites of all collected *B. cryptarum* and *B. lucorum* for the period 1971-2000. Box-Whisker-Plot, range = 1.5 * IQR. The asterisk indicates a significant difference between the groups based on a Wilcoxon rank sum test ($W = 6273.5$; $p < 0.0001$).

in Austria. This is in accordance with the results from other geographic regions in Europe, e.g., Czech Republic (Urbanová et al. 2001), Poland (Banaszak and Rasmont 1994), Ireland (Murray et al. 2008), Great Britain (Scriven et al. 2015) and Finland (Pamilo et al. 1997; Vesterlund et al. 2014). In contrast, *B. cryptarum* is more abundant in a study from north-western Scotland (Waters et al. 2011). In Austria, *B. lucorum* and *B. cryptarum* are not geographically evenly distributed. One factor that is presumably of importance is altitude: *B. cryptarum* was reported to be a high mountain species (Neumayer and Paulus 1999). Also, Scholl and Obrecht (1983) concluded that one taxa of *Bombus lucorum* s.l. is primarily found in alpine habitats. Applying the color-based distinction between *B. cryptarum* and *B. lucorum* in the keys of Amiet (1996) and Gokcezade et al. (2010), the specimens used by Scholl and Obrecht (1983) should actually be assigned to *B. cryptarum*. The results from this study reveal a gradual increase in the proportion of *B. cryptarum* along an altitudinal gradient (Fig. 3), showing a higher abundance of *B. cryptarum* in the high-lying habitats of the Austrian Alps. *Bombus cryptarum* reaches altitudes up to 2570 m a.s.l. but is not restricted to high mountains as the records in northern Austria reveal. Therefore the classification as a high mountain species cannot be entirely upheld, corroborating a finding described in Dorow (2004). Murray et al. (2008) revealed altitudinal distribution patterns with similar tendencies as in this study, but their altitudinal range was restricted from 150 m to 550 m a.s.l. However, the fact that *B. cryptarum* and *B. lucorum* co-occur in all examined altitudinal categories and that both species are sympatric in various lowland habitats all over Europe (e.g., Bertsch 1997; Bertsch et al. 2004; Murray et al. 2008; Scriven et al. 2015) contradicts the notion that altitude is the determining factor for species distribution. This suggests the existence of other influencing factors that change along the examined altitudinal gradient, e.g., the changing climatic conditions. Two climatic parameters were examined in this study that are revealing. (i) The mean annual precipitation sum does not seem to have an effect on the abundance of the species (Fig. 6). Both species occur in areas with high and low annual precipitation, e.g., the Hohe Tauern and the Ötztal Alps (Fig. 5). (ii) In contrast, the mean annual air temperature reveals distributional differences between the species: as a trend, the localities that *B. cryptarum* inhabits are colder than those where *B. lucorum* is found. This

pattern can be traced along the colder habitats in the Alps (Fig. 4): *B. cryptarum* occurs mainly in western Austria along the main ridge of the Alps, whereas it is very rare or absent in the lower mountains and foothills of the Alps in the eastern regions. Exceptions are findings of *B. cryptarum* in the wetlands in northern Austria. In these cases, the mean air temperature of the collection sites are considerably higher than those in the Alps. However, the special climatic conditions of wetlands are not considered in the climate maps. Peatlands in Middle Europe can reveal significantly cooler air temperature than surroundings areas (Schmeidl 1965; Frahm et al. 2012). Moreover, the soil temperature can be lower and the temperature deviation during the diurnal rhythm is different from other soils (Biebl 1951; Bachmann et al. 2014). Especially drained peatlands can exhibit great temperature fluctuations and ground frost events can occur even in summer (E. Frahm, personal communications; Blüthgen and Weischet 1980). Therefore, the findings of *B. cryptarum* in the respective areas suit well the pattern and support the view that *B. cryptarum* is a species inhabiting areas with a cooler climate.

The collection sites of this study show that both *B. lucorum* and *B. cryptarum* may appear in all specified habitat categories. However, the species show different abundances: *B. cryptarum* is more abundant in habitats that occur mainly above the treeline, such as scree areas, krummholz and dwarfshrub communities. These findings are linked to the altitudinal patterns: several high-lying collection sites where *B. cryptarum* was abundant are located above the treeline, and scree areas are typical environments of the alpine level. In contrast, *B. cryptarum* could not be found in regions that were dominated by woodlands, such as the Gesäuse or Lunz am See. In a study in northwestern Scotland, Waters et al. (2011) also showed that *B. cryptarum* rarely occurs in woodlands; however, the same applies to *B. lucorum*. Nonetheless, it remains unclear how extensive the woodlands were sampled, since they provide a small number of specimens from that category. Additionally, they reported that both species occur frequently in heathlands, a habitat type that is not relevant in Austria. In contrast, Bertsch et al. (2004) collected queens from all three species of the *Bombus lucorum* complex in a pine forest. However, many bumblebees are probably opportunistic and rarely restricted to a single habitat type: a radio-tracking study showed that bumblebees can travel great distances for

food resources and frequently change habitats (Hagen et al. 2011).

With very limited available data, Rasmont (1984) attempted the first ecological characterization of *B. cryptarum* as an stenoeccious ericophilous species. Against the background of the currently available data, this categorization is too narrow. The species is not restricted to habitats dominated by Ericaceae nor does it rely on Ericaceae for flower visits. The results of this study, as well as from Waters et al. (2011), reveal that *B. cryptarum* occurs in many habitats and forages on a wide range of plant species from various families. Due to its widespread distribution in the Palaearctic and even in parts of the Nearctic (Williams et al. 2014), a distinct definition does not contribute significantly to the ecology of the species at our current state of knowledge. Therefore, further ecological data from other regions of the Palaearctic is indispensable.

As described in the literature and in previous studies, both species clearly show polylectic foraging (Waters et al. 2011; Scriven et al. 2015). The many flower visits on a large number of plant species from several families indicate a wide breadth of diet. Both species show similar preferences, however, the present study design does not allow calculating reliable diet breadths of the respective species, such as by a rarefaction procedure (Williams 2005).

The third species of the *Bombus lucorum* complex, *Bombus magnus*, is either extremely rare or absent in Austria. Even if the literature provides records of *B. magnus* in Austria, the findings must be confirmed with COI. The *Bombus magnus* records from W. F. Reinig in Aistleitner (2000) and those from B. Tkalců in Neumayer and Kofler (2005) are, in fact, *Bombus cryptarum* based on morphological determination (Bossert 2015). For Austria, no specimens of *B. magnus* are available that could be used for species identification and ensured by a genetic or pheromonal evidence.

Genetic variation and the haplotypes in the European context

In Europe, members of the cryptic *Bombus lucorum* complex can clearly be separated with the phylogenetic analysis of the COI gene. Therefore, the results based on the Austrian specimens are in accordance with the results of the recently published studies from Europe (Bertsch et al. 2005; Bertsch 2009; Murray et al. 2008; Carolan et al. 2012). The data on inter- and intraspecific

distances from these studies and from the present study (Table 3) reveal sufficient divergences to identify genetic discontinuities between species.

When considering our results in the context of other studies, it is apparent that a single haplotype group for *B. lucorum* emerges that consists of relatively few and highly similar haplotypes indicating a low genetic diversity in Europe. The sequence overlap of L1 from this study is identical with the most common haplotype 1 [GenBank: JN872605] from Carolan et al. (2012) and with three of four haplotypes from Bertsch et al. (2005), namely luc-menz-1 [GenBank:AY530009], luc-menz-2 [GenBank:AY530010] and luc-yakutsk [GenBank:AF279497]. The fourth haplotype [GenBank:AY181117] is from Austria and differs only in a single substitution. Further, the haplotype L1 is predominant in the study of Williams et al. (2012a), among others it occurs in Turkey [GenBank: JQ843510], Sweden [GenBank:JQ843547], Iceland [GenBank:JQ843513] and Mongolia [GenBank:JQ843504]. In conclusion, this means that the most common haplotype from this study, L1, belongs to the same haplotype group that is predominant in Austria, Ireland, Scotland, Germany, Denmark, Sweden and Finland and even occurs in Iceland, Turkey, Mongolia, China and large parts of Russia. Low nucleotide diversity, together with low haplotype diversity, may characterize populations that suffered strong genetic bottleneck conditions or drastic population decreases (Avis 2000). In contrast, low diversity in mitochondrial DNA can also be caused by selective sweeps rather than bottleneck events (Hurst and Jiggins 2005). However, one can at least speculate that the dramatic climatic changes in the Pleistocene and the related multiple glaciations of great parts of Western Palaearctic may have caused bottleneck situations. All European areas mentioned above were presumably recolonized by *B. lucorum* after the last deglaciation, which began approx. 14 kyr ago (Randi 2007). The low diversity indices might be a consequence of a postglacial recolonization event by a single genealogical lineage that expanded towards central and northern Europe. In such a case, the high frequency of haplotypes that differ exclusively in one, or rarely two positions, suggests a sudden and rapid expansion of a more or less small population. During the period of postglacial proliferation, an increase in the number of slightly different haplotypes could have occurred, yet the length of time was too short to develop great nucleotide diversity. However, since

a selective sweep cannot be excluded based on the available data, the above mentioned scenario remains uncertain. Further, it remains unclear where the recolonization event began. A south European refugium seems plausible because of the short geographic distance. However, bumblebees are very mobile organisms and can spread quickly over great distances as revealed by the recent spreading of *Bombus haematurus* (e.g., Sárospataki et al. 2005; Šima and Smetana 2009; Jenič et al. 2010; Bossert and Schneller 2014) and *Bombus semenoviellus* (e.g., Rasmont et al. 2005; Streinzer 2010; Šima and Smetana 2012). Therefore, potential non-Mediterranean refugia, as mentioned by Bilton et al. (1998) for mammals, should be taken into account. A postglacial expansion originating from the hills of Crimea or the southwestern Ural Mountains might explain the presence of the haplotype in great parts of Russia and the same applies to the Altai Mountains and its geographical proximity to China and Mongolia.

The situation for *B. cryptarum* is different. Several studies revealed greater intraspecific divergences than in *B. lucorum* (Murray et al. 2008; Carolan et al. 2012; Williams et al. 2012a). This could not be confirmed in the present study, but a closer look at the haplotypes reveals the reason for this inconsistency. Recent studies (Carolan et al. 2012; Williams et al. 2012a) revealed two major haplotype groups with distinct COI sequences and diagnostic positions of substitutions. The extensive sampling of Williams et al. (2012a) allowed the authors to draw distributional patterns of the haplotype groups: haplotype 1 occurs on the British Isles, Central Europe, Turkey and further east reaching central Asia, whereas haplotype 2 has a more widespread boreal distribution in Scandinavia, Russia, Mongolia, China and even reaches western North America. Interestingly, both haplotypes co-occur in Scotland (Williams et al. 2012a). In line with these patterns, all haplotypes of *B. cryptarum* from this study belong to group 1, indicating that our sampling does not encompass the full known genetic variation, which leads to reduced intraspecific divergence values. Due to the stable diagnostic positions, it can safely be assumed that both haplotype groups represent separate genealogical lineages that recolonized great parts of the Palaearctic on different dispersal routes. As discussed for *B. lucorum*, potential refugia remain unclear. Significant inroads into the topic were conducted by Bertsch et al. (2010; 2014). Based on the present-day distribution patterns, it can be assumed that the

ancestral lineage of the haplotype 2 group was not contracted in a Mediterranean refugium and originated from a refugium in eastern Europe or Asia. Additionally, cryptic northern refugia as suggested by Stewart and Lister (2001), should be considered. In contrast, the present distribution of haplotype group 1 indicates a southeastern refugium. However, the current data does not allow us to clarify the phylogeography of the species with certainty.

CONCLUSIONS

Bumblebees represent pollinators of great ecological and economic importance, yet many species are in decline (Goulson et al. 2008; Williams and Osborne 2009). To effectively address conservation efforts, it is of fundamental significance to have knowledge about the species distribution and ecology. This study is the first contribution to clarify the distribution of the *Bombus lucorum* complex in Austria and allows us to estimate where the respective species occur. Further, the combination of faunistic records with reliable climatic data represents a powerful approach to characterize the ability of species to handle abiotic factors in their habitats, especially in respect to temperature and precipitation.

Acknowledgment: We are greatly indebted to S. Bardakji, J. F. Gokcezade and G. Timelthaler for their significant contributions to the topic and for being part of the coordinated effort to investigate the *Bombus lucorum* complex. Great thanks go also to Elizabeth Murray, Enrico Frahm and Elisabeth Haring for providing valuable information. Lastly, we are particularly grateful to J. Plant for the very helpful suggestions to the text. This work was supported by the Naturpark Kaunergrat, the Hochgebirgs-Naturpark Zillertaler Alpen, the Alpenpark Karwendel, the Amt der Tiroler Landesregierung Abteilung Umweltschutz, Naturschutzförderungen, the National Park Gesäuse and the Department of Integrative Zoology of the University of Vienna.

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Additional Material:

1. Title of data: Additional file 1: Collection table
File name: collection_table.xlsx
Description of Data: This table contains the collection data of all specimens used in the study.
2. Title of data: Additional file 2: Diagnostic positions
File name: diagnostic_positions.xlsx
Description of Data: This table provides the diagnostic positions that differ in the COI sequence of the treated haplotypes.
3. Title of data: Additional file 3: Maximum Likelihood tree
File name: ML_Tree.pdf
Description of Data: The best-scoring Maximum Likelihood tree with the respective bootstrap support. Support values below 70 are not shown in the analyses.
4. Title of data: Additional file 4: Neighbor-Joining tree
File name: NJ_Tree.pdf
Description of Data: The bootstrap consensus tree for the NJ analyses. Numbers show the bootstrap values, whereby the cutoff value is 70.
5. Title of data: Additional file 5: Flower visits
File name: flower_visits.xlsx
Description of Data: This table provides all observed flower visits by the collected specimens. Details, such as pollen or nectar collection are included.