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A phylogenetic framework for the bumblebee species of the subgenus *Bombus* sensu stricto based on mitochondrial DNA markers, with a short description of the neglected taxon *B. minshanicola* Bischoff, 1936 n. status.

(Hymenoptera: Apidae: *Bombus*)

With 4 figures and 5 tables

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Zusammenfassung

Königinnen von 12 Taxa der Untergattung *Bombus* sensu stricto (*Bombus affinis*, *B. albocinctus*, *B. cryptarum*, *B. lucorum*, *B. magnus*, *B. moderatus*, *B. occidentalis*, *B. patagiatus*, *B. sporadicus*, *B. terrestris*, *B. terricola* and *B. tunicatus*) wurden im Frühjahr gefangen, um künstliche Kolonien zu züchten. Zusätzlich wurden Männchen von *B. franklini* und *B. lucorum*/China gefangen. Mitochondriale Cytochrome Oxidase Untereinheit I (COI) von 53 Proben wurde sequenziert (Teilsequenzen der Länge 1257 bp). GenBank Sequenzen der Ostasiatischen Arten *B. hypocrita*, *B. ignitus* und *B. lucorum*/China wurden einbezogen. Der Unterschied zwischen den Arten beträgt 30 – 92 Basen-Substitutionen und die Tamura-Nei genetische Distanz 0.030-0.103, während der Unterschied innerhalb der Arten nur 1 – 3 Basensubstitution beträgt und die Tamura-Nei genetische Distanz nur 0.001-0.003. Dreizehn Taxa mit Artrang sowie eine neue Art *B. minshanicola* Bischoff 1936 (= *B. terrestris* ssp. *minshanicola* = *B. lucorum*/China) wurden nachgewiesen. In der Topologie des phylogenetischen Stammbaums gibt es 4 Cluster: ein Artenpaar *B. sporadicus* – *B. ignitus* und ein Artenpaar *B. terricola* – *B. occidentalis*, ein Cluster für *B. magnus*, *B. patagiatus* and *B. cryptarum* mit den Subspecies *B. cryptarum albocinctus* und *B. cryptarum moderatus* sowie ein Cluster für *B. hypocrita*, *B. lucorum*, *B. minshanicola*, *B. franklini* and *B. affinis*. Die Arten *B. terrestris* mit den Subspecies *B. terrestris canariensis* und *B. terrestris sassaricus* sowie *B. tunicatus* sind einzeln abgetrennt. Da sich im Alignment der COI Sequenzen keine Lücken finden, können die einzelnen Nucleotid-Positionen als homolog betrachtet werden. Jede Art besitzt eine Reihe einzigartiger ('privater') Positionen, die als diagnostische Merkmale benutzt werden können um die Art zu definieren und zu identifizieren.

Summary

Queens of 12 taxa of the subgenus *Bombus* sensu stricto (*Bombus affinis*, *B. albocinctus*, *B. cryptarum*, *B. lucorum*, *B. magnus*, *B. moderatus*, *B. occidentalis*, *B. patagiatus*, *B. sporadicus*, *B. terrestris*, *B. terricola* and *B. tunicatus*) were collected in spring to establish artificial colonies. Males of *B. franklini* and *B. lucorum*/China were also collected. Mitochondrial cytochrome oxidase subunit I (COI) was sequenced from 53 specimens (partial sequence length 1257 bp). GenBank sequences of the east Asiatic species *B. hypocrita* and *B. ignitus* were included in the investigation. The interspecific sequence diversity was about 30–92 base substitutions with a Tamura-Nei genetic distance of 0.030 – 0.103, whereas the intraspecific sequence diversity was only 1–3 base substitutions with a Tamura-Nei genetic distance of 0.001–0.003. Thirteen Taxa ranked as species were found and a new taxon ranked as species *B. minshanicola* Bischoff 1936 (= *B. terrestris* ssp. *minshanicola* = *B. lucorum* /China) was established. Four clusters were obtained in the topology of the

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phylogenetic tree: a species pair *B. ignitus* – *B. sporadicus*, a species pair *B. terricola* – *B. occidentalis*, a cluster including *B. magnus*, *B. patagiatus* and *B. cryptarum* with ssp. *B. cryptarum albocinctus* and *B. cryptarum moderatus*, and a cluster including *B. hypocrita*, *B. lucorum*, *B. minshanicola*, *B. franklini* and *B. affinis*. The species *B. terrestris* with ssp. *B. terrestris canariensis* and *B. terrestris sassaricus* and *B. tunicatus* were well separated. As there are no gaps in the alignment of the COI sequences, single nucleotide sites can be used as positional homologies. Each taxon is characterized by substitutions that are unique and can be used as diagnostic characters to define and identify the taxon.

Introduction

With the recent publications of Pedersen (1996, 2002), Kawakita et al. (2004) and Cameron et al. (2007) we have a good general picture of the phylogenetic relationships of most bumblebee species, and much insight into the deeper nodes of phylogeny. However, many questions remain at the terminal units of the branches, and we need more specimens from a broad range of geographical localities to investigate the genetic polymorphism of the taxa and to understand the phylogenetic relationships within subgenera. The mitochondrial DNA marker cytochrome oxidase I (COI) has proven to be useful because the genetic difference between the taxa is about one order of magnitude larger (Pedersen 2002; Bertsch 2009; Bertsch et al. 2010a, 2010b) than in nuclear genes (Kawakita et al. 2004; Cameron et al. 2007).

The use of single nucleotide positions as homologue characters is a useful tool for the construction of cladistic trees, and the use of nucleotide positions that are unique for critical taxa enables safe identification (Bertsch 2009). As these unique positions depend on the number of species included in the comparison it seems to be necessary to include all known species of a group. This approach was taken in this investigation of the subgenus *Bombus* sensu stricto, a subgenus that is well defined by the very special form of the male genital capsule, especially the funnel-shaped penis valves (Vogt 1909).

Materials and methods

Bumblebee samples

Table 1 shows the bumblebee samples used for DNA sequencing, their identification codes and the localities of their origin with geographical coordinates. For comparison GenBank sequences of East Asian taxa in particular were included (Table 2). As some of these GenBank sequences are shorter in length the sequences had to be clipped to equal length for some calculations.

Cameron et al. (2007) reported that *B. lucorum* is split into a European (*B. lucorum*) and a Chinese taxon (*B. lucorum*/China), with obviously different DNA in the 16S mitochondrial gene and the four nuclear genes investigated. An unpublished COI GenBank sequence GU085202 (*B. lucorum* from North China) confirmed this result. Therefore specimens of *B. lucorum* from China/Sichuan (collected by Dr. van Asperen de Boer, Amsterdam) and a male specimen of *B. lucorum* from China/Gansu (supplied by Dr. An, Beijing) were sequenced.

Polymerase chain reaction (PCR) and DNA sequencing of mitochondrial COI

Total DNA was extracted from legs using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications for tissue, and eluted in 150 µl of highly purified water (Ampuwa®, Fresenius Kabi, Bad Homburg,

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Germany). For sequence analysis overlapping fragments (in all 1257 bp) of mitochondrial COI were amplified using primers specifically designed for *Bombus*. BO-0-fwd (5'GAATAATATATTTTATTTTTGC3') and BO-Z-rev (5'CCAAAAAATCAAAATAAATGTTG3') resulted in the amplification of a 671 bp fragment, BO-1-fwd (5'TAGGATCACCAGATATAGC3') and BO-K-rev (5' GAGCTCAAACAATAAATCC 3') resulted in the amplification of a 609 bp fragment, and BO-5-fwd (5' AATGAAAGAGGTAAAAAAGAAAC 3') and BO-A-rev (5' ATGTTGAGGGAAAAATGTTAT 3') resulted in the amplification of a 510 bp fragment. PCR amplifications were performed in 50 µl reactions containing 100 ng DNA template, 1.6 mM $MgCl_2$, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % Tween20, 0.2 mM of each dNTP, 20 pmol of each primer and 1.5 units TaqDNA polymerase (Fermentas, St. Leon-Rot, Germany). Conditions for PCR amplification were initial denaturation for 5 min at 94 °C, 40 cycles of 45 s denaturation at 94 °C, 1 min annealing at 46 °C, 3 min elongation at 62 °C (for BO-0-fwd and BO-Z-rev), at 61 °C (for BO-1-fwd and BO-K-rev) and 63 °C (for BO-5-fwd and BO-A-rev), and final extension for 7 min. Ten µl of each reaction was checked on a 1 % agarose gel. PCR products were purified using an AMPure® PCR Purification Kit (Agencourt, Beverly, MA, USA). Sequencing reactions were performed using ABI® BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, all fragments were sequenced from both strands and then analysed on an ABI 3100 sequencer (Applied Biosystems). Sequences were aligned manually using ClustalX. No gaps or poorly aligned regions occurred in the alignment, but missing characters were trimmed from the ends of the alignment to produce an equal sequence length of 1257 bp for all individuals (encoding 419 amino acids). Individual alignments were aligned against the complete COI gene sequence of *Bombus ignitus* (GenBank accession no. DQ870926; Cha et al. 2007) between positions 262 and 1519.

Analysis of sequence divergence of mitochondrial COI

The absolute numbers of substitutions were counted based on a pair wise comparison of COI sequences. The analysis for the sequences investigated was performed using the maximum composite likelihood method of Mega 4.0 (Tamura et al. 2007; Kumar et al. 2008), a program distributed by K. Tamura, J. Dudley, M. Nei and S. Kumar. Because the distribution of nucleotides in the COI of Hymenoptera is known to be heterogeneous, with a strong A + T bias, we selected the Tamura-Nei (TN) model of base substitution (Tamura and Nei 1993), which corrects this bias in its assumption of sequence evolution. The nucleotide frequencies and the parameters necessary for this model were estimated from the sequence data, and TN genetic distances were calculated. The best maximum likelihood model was selected using the jModelTest (Posada 2008). The tree topology was inferred by a maximum likelihood tree based on the general time-reversible model (GTR plus gamma) calculated by Bayesian analysis (Huelsenbeck and Ronquist 2001) using MrBayes, a program distributed by J. P. Huelsenbeck and F. Ronquist. Tree topology was also calculated as a Minimum Evolution tree (ME) with bootstrap sampling, using Mega 4.0 (Kumar et al. 2008). Geneious Pro 4.5 (Biomatters Ltd.) was used to analyse the alignment and detect diagnostic positions, and the GreenButton plugin (InterGrid) was used to perform the time-consuming MrBayes calculations on a supercomputer cluster. The nucleotide changes along the branches of cladograms were examined with MacClade 3.04. The COI sequence data of *B. soroeensis* (GenBank accession no. AY181159; Pedersen 2002) were used as an out-group.

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Tab. 1: List of *Bombus* samples (Aff = affinis, Cry = *cryptarum*, Bur = *cryptarum burjaeticus*, Mod = *cryptarum moderatus*, Luc = *lucorum*, Mag = *magnus*, Min = *minshanicola*, Occ = occidentalis, Pat = *patagiatus*, Spo = *sporadicus*, Terre = *terrestris*, Sas = *terrestris sassaricus*, Can = *terrestris canariensis*, Terri = *terricola* and Tun = *tunicatus*) used in the present analysis with identification codes, and collection locality information. Q = ♀♀, aC → M = artificial colonies with production of males.

Code	Locality	Country	Region	Latitude	Longitude	Altitude	
Aff-01	Boston	USA	Massachusetts	42° 17.87' N	71° 07.35' W	19 m	Q, aC → M
Aff-02	Marconi Beach	USA	Massachusetts	41° 53.45' N	69° 57.75' W	8 m	Q
Aff-03	Bridgewater	USA	Massachusetts	41° 59.05' N	70° 59.16' W	30 m	Q, aC → M
Aff-04	New Hampton	USA	New Hampshire	43° 36.48' N	71° 39.26' W	175 m	Q, aC → M
Cry-01	Duncery Beacon	UK	England	51° 09.48' N	3° 34.64' W	417 m	Q, aC → M
Cry-02	Marcinkonys	Lithuania	Alytus County	54° 21.04' N	24° 25.46' E	145 m	Q, aC → M
Cry-03	Strelna	Russia	St. Petersburg	59° 51.63' N	30° 05.33' E	3 m	Q, aC → M
Cry-04	Nassfeld	Austria	Kärnten	46° 34.49' N	13° 06.26' E	1415 m	Q, aC → M
Bur-01	Kadachta	Russia	Karymski District	51° 37.22' N	114° 14.56' E	650 m	Q, aC → M
Bur-02	Chita	Russia	Zabaikalsky Krai	52° 00.54' N	113° 28.35' E	750 m	Q, aC → M
Mod-01	Isabel Pass	USA	Alaska	63° 11.77' N	145° 33.64' W	1095 m	Q, aC → M
Mod-02	Broad Pass	USA	Alaska	63° 19.31' N	149° 09.21' W	725 m	Q, aC → M
Mod-03	Ya-Ha-Tinda Ranch	Canada	Alberta	51° 44.57' N	115° 32.52' W	1615 m	Q
Mod-04	Sheep River	Canada	Alberta	50° 39.11' N	114° 21.85' W	1315 m	M
Fra-01	Gold Hill	USA	Oregon	42° 25.81' N	123° 02.69' W	330 m	worker
Luc-01	Marcinkonys	Lithuania	Alytus County	54° 21.04' N	24° 25.46' E	145 m	Q, aC → M
Luc-02	Col de Vars	France	Hautes Alpes	44° 32.25' N	6° 42.21' E	2112 m	Q, aC → M
Luc-03	Chita	Russia	Chitinskaja Obl.	52° 00.86' N	113° 28.56' E	730 m	Q, aC → M
Mag-01	Glen Oykel	UK	Scotland	57° 59.74' N	4° 49.11' W	140 m	Q, aC → M
Mag-02	Roth	Germany	Bayern	49° 14.61' N	11° 08.95' E	372 m	Q, aC → M
Mag-03	Marcinkonys	Lithuania	Alytus County	54° 21.04' N	24° 25.46' E	145 m	Q, aC → M
Mag-04	Sestroretsk	Russia	St.Petersburg	60° 08.02' N	29° 57.76' E	15 m	Q, aC → M
Min-01	North of Zoigê	China	Sichuan	33° 59.26' N	102° 44.77' E	3450 m	worker
Min-02	Northwest of Songpan	China	Sichuan	33° 13.13' N	103° 44.50' E	3320 m	worker
Min-03	West of Yongdeng	China	Gansu	36° 41.26' N	102° 42.77' E	2260 m	Q, aC → M
Occ-01	Mt. Ashland	USA	Oregon	42° 04.41' N	122° 43.41' W	2050 m	Q, aC → M
Occ-02	Okotoks	Canada	Alberta	50° 43.25' N	113° 53.98' W	1050 m	Q, aC → M
Occ-03	Kitwanga	Canada	British Columbia	55° 06.12' N	128° 04.32' W	175 m	M
Occ-04	Smithers	Canada	British Columbia	54° 48.48' N	127° 04.51' W	685 m	M
Pat-01	Listvyanka	Russia	Irkutsk Oblast	51° 52.25' N	104° 50.15' E	620 m	Q, aC → M
Pat-02	Chita	Russia	Zabaikalsky Krai	52° 00.54' N	113° 28.35' E	750 m	Q, aC → M
Spo-01	Kuopio	Finland	Northern Savonia	62° 54.56' N	27° 39.55' E	211 m	Q
Spo-02	Kuopio, Research Garden	Finland	Northern Savonia	62° 54.55' N	27° 34.88' E	95 m	Q
Terre-01	Assergi	Italy	Abruzzo	42° 25.57' N	13° 30.43' E	972 m	Q
Terre-02	Alcala de los Gazules	Spain	Andalusia	36° 31.25' N	05° 38.86' W	417 m	Q
Terre-03	Marburg	Germany	Hessen	50° 48.13' N	08° 48.59' W	273 m	Q, aC → M
Terre-04	St Andrews	UK	Scotland	56° 20.17' N	02° 48.45' W	20 m	Q, aC → M
Sas-01	Passo Tascusi	Italy	Sardinia	40° 01.82' N	09° 13.79' E	870 m	Q, aC → M
Sas-02	Desulo	Italy	Sardinia	40° 00.53' N	09° 13.62' E	898 m	Q, aC → M
Sas-03	Passo di Caravai	Italy	Sardinia	40° 08.50' N	09° 20.10' E	1120 m	Q
Sas-04	Cappannacia	Italy	Sardinia	41° 10.23' N	09° 19.17' E	34 m	Q

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Code	Locality	Country	Region	Latitude	Longitude	Altitude	
Can-01	Tacaronte	Spain	Tenerife	28° 28.73' N	16° 24.91' W	492 m	Q, aC → M
Can-02	Santiago el Teide	Spain	Tenerife	28° 18.23' N	16° 48.89' W	948 m	Q
Can-03	Mogan	Spain	Gran Canaria	27° 52.79' N	15° 43.77' W	248 m	Q, aC → M
Can-04	Tafira Alta	Spain	Gran Canaria	28° 03.92' N	15° 27.61' W	311 m	Q
Terri-01	Winchester	USA	New Hampshire	42° 46.19' N	72° 22.21' W	140 m	Q, aC → M
Terri-02	Weld	USA	Maine	44° 41.02' N	70° 21.45' W	375 m	worker
Terri-03	Sturgeon Lake	Canada	Alberta	55° 14.58' N	117° 24.11' W	715 m	Q
Terri-04	Smithers	Canada	British Columbia	54° 48.48' N	127° 04.51' W	685 m	M
Tun-01	Theog, Shimla Hills	India	Himachal Pradesh	31° 15.37' N	77° 27.34' E	2740 m	Q, aC → M
Tun-02	Solang, Kullu valley	India	Himachal Pradesh	32° 18.50' N	77° 08.81' E	2610 m	Q, aC → M
Tun-03	Vashisht, Kullu valley	India	Himachal Pradesh	32° 16.23' N	77° 11.38' E	2730 m	Q
Tun-04	Shogran, Naran valley	Pakistan	Northwest Frontier	34° 38.22' N	73° 26.85' E	2360 m	M

Tab. 2: List of *Bombus* samples (Cha = Cha, Gwanju, Hon = Hong, Gwanju, Pe = Pedersen, Copenhagen, Tan = Tanaka, Kyoto, Wu = Wu, Beijing,) used in the present analysis with Genbank numbers, and collection locality information.

Species	Kam	GenBank #	Locality	Region	Country
albocinctus	Tan	AF279482	??	Kamschatka Krai	Russia
	Tan	AF279483	Paramushir	Sakhalin Obl.	Russia
franklini	Tan	AY694097	Mt. Ashland	Oregon	USA
hypocrita	Tan	AF385804	??	Sakhalin	Russia
	Tan	AF279492	??	Primorsk Distr.	Russia
	Hon	EU401918	??	--	South Korea
	Tan	AF279489	Kyoto	Honshu	Japan
ignitus	Wu	GU085201	??	North China	China
	Cha	DQ870926	??	--	South Korea
	Tan	AF279495	??	Oita	Japan
	Tan	AF279494	Kyoto	Honshu	Japan
lucorum	Tan	AF279497	Yakutsk	Sakha Republic	Russia
minshanicola	Wu	GU085202	??	North China	China
patagiatus	Wu	GU085203	??	North China	China
	Tan	AF279499	Yakutsk	Sakha Republic	Russia
sporadicus	Ped	AY181163	Skute	Oppland	Norway
	Tan	AF279500	??	Primorsk Distr.	Russia

Tab. 3: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution. The transition/transversion rate ratios are k_1 = 4.51 (purines) and k_2 = 7.51 (pyrimidines), the overall transition/transversion bias is R= 1.566.

	A	T	C	G
A	-	5.15	1.48	6.68
T	4.20	-	11.11	1.48
C	4.20	38.64	-	1.48
G	18.96	5.15	1.48	-

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Results

Nucleotide frequencies and substitution parameters

The aligned data matrix of 1257 bp length of 53 sequences included 984 invariant and 273 variable sites. Of these positions, 7 were parsimony uninformative (noise) and 266 parsimony informative (signal). However, differences in this pattern were evident in the codon positions. The first position comprised 51 informative positions; in the second position 7 were informative, in the third position 208 positions were informative. Table 3 gives the pattern of nucleotide substitutions estimated from the data with the maximum composite likelihood model (Mega). The nucleotide frequencies were 34.1 (A), 41.8 (T), 12.0 (C) and 12.0 (G), which proves the known strong A + T bias typical for sequences of Hymenoptera.

COI sequence divergence between and within subgenera in Bombus

Investigations with the genetic marker COI produce a very detailed picture of genetic distances; some taxa show large genetic differences, some are smaller. Therefore it seems to be useful to 'calibrate' these differences: What is a typical genetic difference between species in the genus *Bombus* and what are the typical genetic differences between subgenera? Fig. 1 shows: estimates for genetic p-distances (1) *between subgenera* of the genus *Bombus* (median = 0.095, interquartile range = 0.019), (2) *between species* of the subgenus *Bombus* s. str. (median = 0.059, interquartile range 0.018) and (3) *within species* of the subgenus *Bombus* s. str. (median = 0.002, interquartile range = 0.003, with two outliers for *B. cryptarum* (with ssp. *albocinctus* and ssp. *moderatus)* and *B. terrestris* (with ssp. *canariensis* and ssp. *sassaricus*)). Details of the subgenera and taxa included in this statistic are summarized in Appendix I.

'Bombus lucorum China'

The genetic p-distances for the sequences *B. lucorum*/China included in the analysis as *B. minshanicola* are separated from *B. lucorum* by a p-distance of 0.035, which far exceeds the p-distances of variable species such as *B. terrestris* and *B. cryptarum*. The mean p-distance to all *Bombus* s. str. species included in this investigation is 0.049 (range 0.035–0.086) and fits completely into the p-distances found for species differences. Therefore the taxon *B. minshanicola* should be treated as a taxon separate from *B. lucorum*, most probably in the rank of a good species. For further information see Appendix II.

COI sequence divergence between and within species Bombus sensu stricto

Table 4 presents the matrix of genetic distances estimated by the TN model and as p-distances within and between the 14 taxa investigated. The infraspecific genetic variability was low for all taxa (TN distance 0.001–0.004). The infraspecific genetic variability was only larger (TN distance 0.013–0.014) in taxa with well-differentiated subspecies such as *B. terrestris* (with ssp. *canariensis* and ssp. *sassaricus*) and *B. cryptarum* (with ssp. *albocinctus* and ssp. *moderatus*). In contrast, the interspecific genetic variability was approximately one order of magnitude larger (p-distance 0.030–0,092, TN distance 0.030–0.103).

Tree building by maximum likelihood models

The maximum likelihood tree (Fig. 2) generated using the Bayesian MCMC (Markov Chain Monte Carlo) analysis with two 1257 bp full-length sequences for each taxon was based on the

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Tab. 4: Mean genetic distance within and between taxa. Diagonal and lower left: Tamura-Nei model, rates among sites gamma distributed. Upper right: p-distance. [Sequences of *B. franklini* are shorter, distance data in italics].

	Aff	Fra	Min	Luc	Hyp	Cry	Pat	Mag	Occ	Terri	Tun	Terre	Ign	Spo
affinis	**0.002**	0.049	0.041	0.049	0.061	0.059	0.062	0.054	0.062	0.057	0.065	0.069	0.071	0.081
franklini	*0.046*	**0.001**	*0.037*	*0.044*	*0.069*	*0.054*	*0.057*	*0.047*	*0.051*	*0.048*	*0.063*	*0.069*	*0.083*	*0.093*
minshanicola	*0.049*	*0.041*	**0.003**	0.033	0.044	0.031	0.036	0.036	0.051	0.038	0.047	0.053	0.063	0.076
lucorum	0.051	*0.044*	0.035	**0.002**	0.052	0.040	0.041	0.041	0.048	0.046	0.062	0.062	0.064	0.078
hypocrita	0.076	*0.071*	0.050	0.066	**0.002**	0.053	0.048	0.052	0.057	0.049	0.067	0.067	0.076	0.084
cryptarum	0.064	*0.055*	0.033	0.044	0.067	**0.013**	0.032	0.034	0.040	0.045	0.054	0.058	0.070	0.077
patagiatus	0.072	*0.060*	0.038	0.050	0.060	0.038	**0.001**	0.037	0.047	0.041	0.053	0.064	0.072	0.083
magnus	0.051	*0.046*	0.039	0.039	0.062	0.034	0.038	**0.004**	0.042	0.041	0.057	0.057	0.070	0.079
occidentalis	0.069	*0.063*	0.057	0.054	0.073	0.048	0.058	0.043	**0.002**	0.035	0.055	0.063	0.074	0.081
terricola	0.069	*0.052*	0.041	0.055	0.066	0.057	0.053	0.044	0.040	**0.001**	0.049	0.055	0.062	0.068
tunicatus	0.081	*0.070*	0.052	0.075	0.085	0.066	0.062	0.062	0.067	0.062	**0.001**	0.063	0.064	0.081
terrestris	0.083	*0.072*	0.059	0.066	0.085	0.067	0.072	0.061	0.078	0.067	0.079	**0.016**	0.067	0.072
ignitus	0.082	*0.078*	0.071	0.071	0.091	0.083	0.084	0.078	0.089	0.071	0.076	0.073	**0.004**	0.068
sporadicus	0.091	*0.092*	0.086	0.089	0.101	0.090	0.096	0.087	0.103	0.080	0.100	0.086	0.078	**0.003**

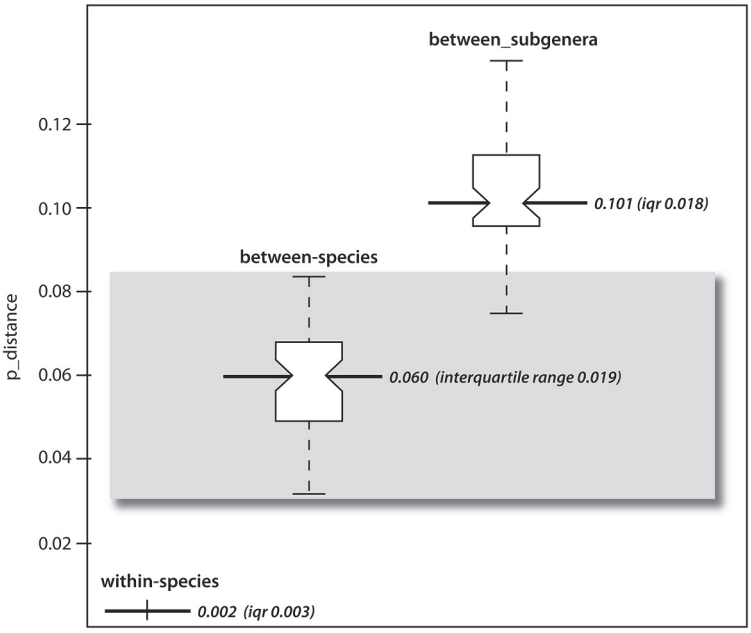

Fig. 1: Boxplots for *between species* and *between subgenera* median p-differences of COI sequences (849 bp length). The top and bottom of each box are the 25th and 75th percentiles of the samples, the distance is the interquartile range (iqr = middle 50 % of samples), the whiskers (broken lines) the range of the samples. Notches display the variability of the median between samples; the width of a notch is computed so that boxplots whose notches do not overlap have different medians at the 5 % significance level.

general time-reversible model of base substitution (GTR plus gamma), and was simulated for 5 000 000 generations to achieve stationary with sampling every 10 generations and a "burn-in" of 5000 generations. For comparison and to increase the geographical range, GenBank sequences

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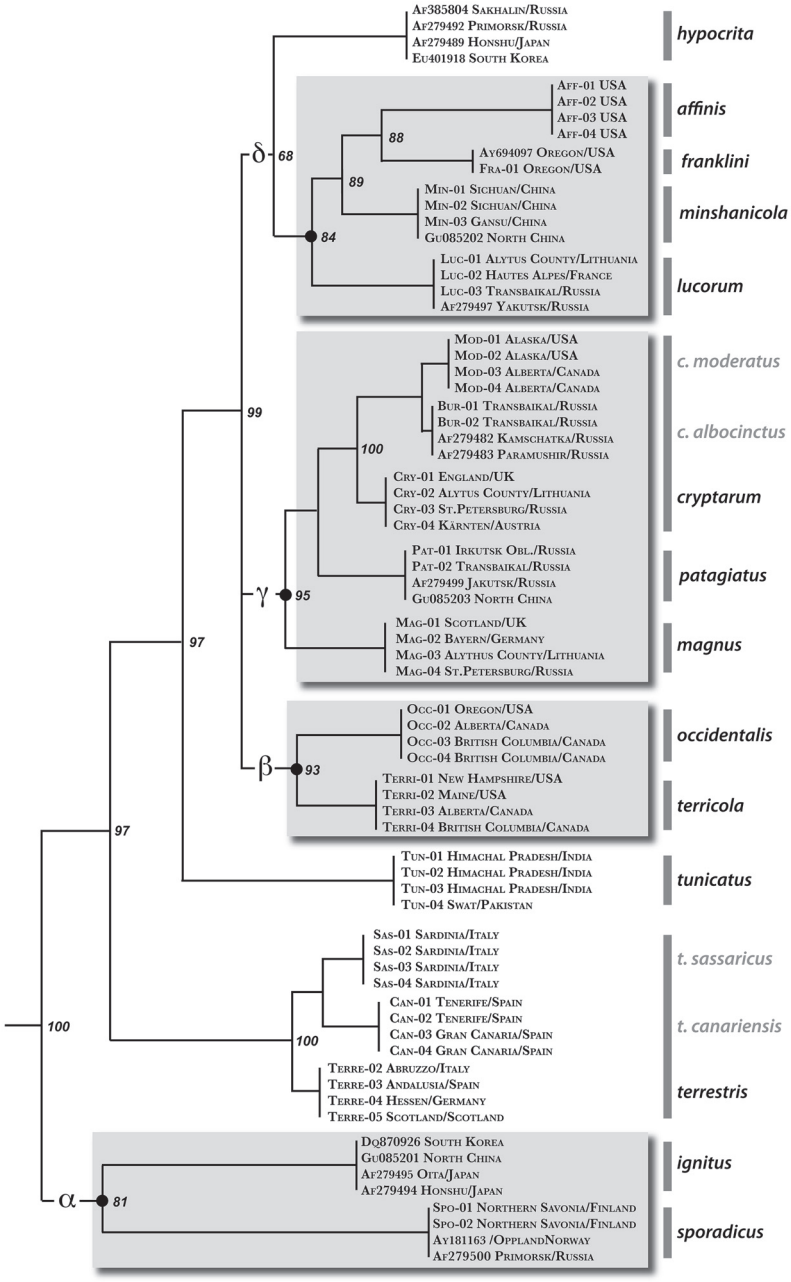

Fig. 2: Tree topology calculated as Maximum-Likelihood tree using Bayesian MCMC analysis with the general time reversal model of base substitutions with gamma distribution.

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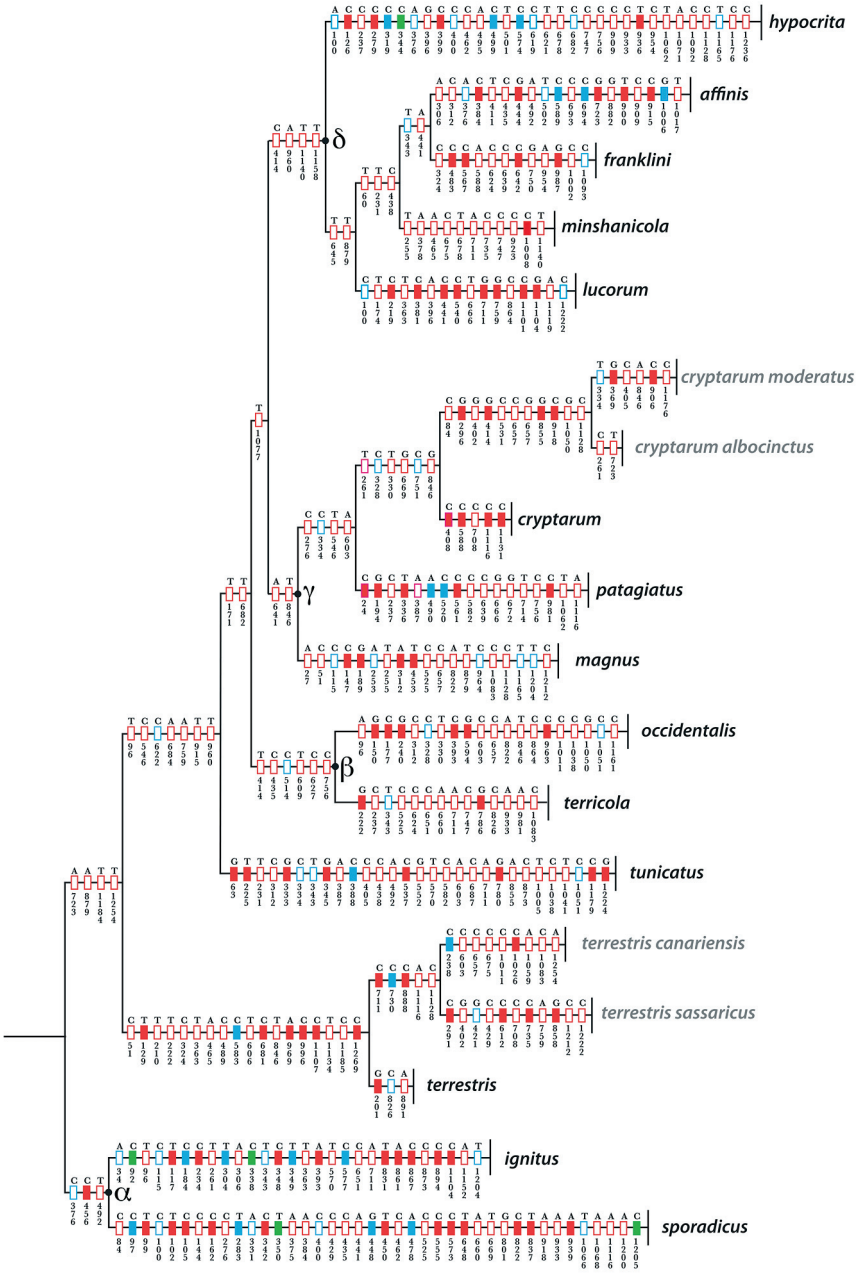

Fig. 3: Observed diagnostic character changes with position numbers mapped onto the Maximum-Likelihood tree. Box filled = unambiguous *diagnostic* character change, box open = unambiguous character change, first position = blue, second position = green and third position = red.

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were included. Two of the 14 taxa were well separated (*B. terrestris* & *B. tunicatus*), and the rest formed distinct clusters: cluster α with *B. sporadicus* and *B. ignitus* and a posterior probability of 0.81, cluster β with *B. occidentalis* and *B. terricola* and a posterior probability of 0.93, cluster γ with *B. magnus*, *B. patagiatus*, and *B. cryptarum* and a posterior probability of 0.95, and cluster δ with *B. hypocrita*, *B. lucorum*, *B. minshanicola*, *B. franklini* and *B. affinis* and a posterior probability of 0.68. Because the support for cluster δ has only a 0.68 probability, *B. hypocrita* is also probably completely separate.

Tree building by diagnostic characters

As there are no gaps in the alignments of the COI sequences, single nucleotide sites can be used as positional homologies (Hillis 1994). In MacClade, the changes at the nodes and the diagnostic characters at the last branch of the terminal units can be investigated in detail (Maddison & Maddison 2002), and a tree with the classical tools for morphological characters can be built (Fig. 3). With the large number of diagnostic characters available it is normal that not all of these changes are unambiguous, but each of the taxa investigated is characterized by between 11–40 unambiguous characters and with the exception of *B. minshanicola* 5–12 'unique' diagnostic positions. Clusters α to δ of the MrBayes maximum likelihood tree are also distinctly separate in this cladistic tree, and the larger infraspecific variability of *B. terrestris* and *B. cryptarum* is 'explained' by the differentiation of these species into genetically isolated subspecies.

Discussion

'B. lucorum China', the neglected taxon B. minshanicola Bischoff, 1936

Cameron et al. (2007) reported a sequence referred to as '*B. lucorum*/China' that differs from the sequences of *B. lucorum* from Europe, a result confirmed by an unpublished COI sequence of *B. lucorum* from North China, which also proves that this *B. lucorum*/China is very different from the typical European *B. lucorum*, defined by its morphology, male labial gland secretions and mitochondrial DNA markers (COI). This '*B. lucorum*/China' is thus not identical to the taxon *B. lucorum*.

As the subgenus *Bombus* s. str. has been thoroughly studied and has already delivered a huge number of colour variants and names (Krüger 1951, 1954, 1956, 1958), it seems improbable that this *B. lucorum*/China does not already have a name. *B. lucorum lanchouensis* Vogt 1908 was synonymized by Tkalců (1967) with *B. patagiatus* as *B. patagiatus* ssp. *lanchouensis*. Therefore only one taxon remains from the area (Northern Sichuan & Gansu/China) from which this unknown *B. lucorum* China has been described: *B. minshanicola* Bischoff, 1936, described as *B. terrestris* ssp. *minshanicola* and transferred to *B. lucorum* by Krüger (1951, p. 196).

The morphological characters in the *Bombus lucorum* complex are not well understood, mainly because the variability in the characters has not been thoroughly investigated. Without good morphological characters identification is unreliable. The species-specific male labial gland secretions and the species-specific 'unique' positions of the COI sequences are the only tools available so far for the safe identification of specimens. However, the case of *B. minshanicola* may be less problematic, because the description of Bischoff (1936) and the type material showed clear differences in colouration compared to the European *B. lucorum*, which might be used to characterize specimens of *B. minshanicola* and most probably prove its identity with '*B. lucorum*/China'. For further information see Appendix II.

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Genetic variability in B. cryptarum and B. terrestris

Bombus terrestris is one of the species in the subgenus *Bombus* s. str. that is known for its geographical variation, and besides colour forms also includes taxa in the rank of subspecies (Krüger 1951, 1954; Rasmont et al. 2008). Some isolated island populations, such as *B. terrestris* ssp. *sassaricus* (Tournier 1890) from Italy/Sardinia and *B. terrestris* ssp. *canariensis* (Pérez 1895) from Spain/Canary Islands are distinctly different in colouration. The separation of these island populations from specimens of the European continent was confirmed by a genetic p-distance of 0.014 and a difference of 11 unambiguous positions (including 4 'unique' positions) for *B. terrestris sassaricus*, and a genetic p-distance of 0.022 and a difference of 9 unambiguous positions (including 2 'unique' positions) for *B. terrestris canariensis*. Whereas more or less invariable species have a genetic p-distance of 0.001 to 0.003, in the variable *B. terrestris* the mean genetic p-distance is 0.016.

It has been shown that the North American taxon *B. moderatus* (Cresson 1863) and the East Asian taxon *B. albocinctus* (Smith 1854) are most probably subspecies of the species *B. cryptarum* (Bertsch et al. 2010a), which results in a mean genetic p-distance of 0.013 within *B. cryptarum*, about the same order of magnitude as within *B. terrestris*. As long as *B. terrestris sassaricus* and *B. terrestris canariensis* are treated as subspecies, the same should apply for *B. cryptarum albocinctus* and *B. cryptarum moderatus*.

Which Bombus sensu stricto taxa are good species?

This question has been in dispute for a very long time. So far in most cases the assignment is based on colouration and geographical provenance but in some cases the assignment of specimens is in dispute. As long as objective criteria are lacking the question of lumping or splitting taxa remains a more or less subjective decision, based on different 'species concepts'. The latest checklists (Williams 1998; Williams et al. 2008) recognize 10 species; Williams (2010) treats *B. terricola* and *B. occidentalis* as separate species and therefore recognizes 11 species. With the mitochondrial DNA markers we have for the first time an objective measure of the genetic distance between the taxa of the subgenus *Bombus* s. str., with the result that 14 taxa might be 'good' species: the North American *B. terricola* and *B. occidentalis* are split into two species and *B. cryptarum*, *B. magnus* and *B. minshanicola* are added to the 10 species of Williams et al. (2008). Specimens of the subgenus *Bombus* s. str. are well represented in museum collections and in most parts of the world the taxa of the subgenus *Bombus* s. str. have been thoroughly investigated. It is possible that a few more taxa with the rank of species may be detected in parts of the Himalayas and China, but this should be done by genetic markers and labial gland secretions, not just by differences in colouration or doubtful morphological characters. The question as to how far these 14 'species' can also be established as morphological species is still open, and obviously the morphological differences are small (at least in some of these taxa). Thus it is only after safe assignment that an investigation of the morphological characters and their variability can be carried out.

Uniting *B. occidentalis* and *B. terricola* into a broader unit *B. terricola*, or separating *B. albocinctus* from *B. cryptarum* and *B. canariensis* from *B. terrestris*, is more a matter of taste rather than scientific insight. For further field studies and experimental work it is essential to give a proper name to the specimens investigated, but the specific rank of the taxon is not really important. The name is just a means to make sure that everybody understands which taxon has been under investigation. In times when the investigation of biodiversity is one of the central themes in biology and the definition of the rank 'species' is in dispute, the 'lumping' of taxa is not really helpful. It is an unnecessary loss of information.

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Phylogenetic relationships based on a single gene?

The trees presented in this investigation were built with programs simulating phylogenetic relationships, but they should be read as a graphic display of genetic distances rather than a real phylogeny. More sequences and more genes will have to be investigated before a reliable phylogeny can be constructed. The phylogeny of Cameron et al. (2007) is based on the 16S mitochondrial gene and four nuclear genes, but all the information on these genes was 'lumped' into one MrBayes simulation. A closer look at the GenBank data reveals that in some of these genes the genetic difference between the taxa of the subgenus *Bombus* s. str. is so small that the trees are poorly resolved. If the phylogenies for the different genes are calculated separately, we obtain trees with contradictory phylogenies (see Cameron et al. 2007, supplementary materials). This result is not unexpected; evolution is a complicated procedure and different characters evolve at different speeds and sometimes in different directions. A simulation of phylogenetic evolution based on maximum likelihood models is always a simulation; a process that took place a long time ago can never be reconstructed with certainty, and will always remain a hypothesis.

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Prof. R. Thorp (Davis/California) gave valuable advice and helped to collect the male *B. franklini* (AY694097), Prof. J.R.J. van Asperen de Boer (Amsterdam/Netherlands) supplied the specimens of *B. franklini* (FRA-01) and *B. minshanicola* (MIN-01, MIN-02) from his collection. Prof. B. Heinrich (Burlington/Vermont) collected the specimen of *B. terricola* (TERRI-02) from Maine/USA. Dr. M. Ito (Sapporo) confirmed the identity of the Far East GenBank sequences *B. cryptarum* (AF279482/83) with *B. albocinctus*. Dr. An Jian-dong (Beijing/China) collected males of '*B. lucorum*/China' for labial gland investigations. Prof. M. Hrabé de Angelis (Institute of Experimental Genetics, Helmholtz Zentrum München, Germany) kindly allowed me to work in his institute and laboratories, and Dr. G. K.-H. Przemeck (Institute of Experimental Genetics, Helmholtz Zentrum München, Germany) designed the primers, and gave, with much patience, initial instructions on how to extract DNA and perform a PCR, and always helped to obtain reliable ABI sequences. I would like to thank them all for their valuable help.

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Appendix I

Sequences without GenBank numbers are unpublished sequences from Bertsch et al. Statistics calculated and plotted with the Statistics Toolbox™ of Matlab. Sequence length 848 bp, COI positions 262–1110.

(01) **Alpinobombus:**

alpinus, *balteatus* AY181097, *hyperboreus* AY181107, *polaris* AY181144.

(02) **Bombus s. str.:**

albocinctus, *cryptarum*, *hypocrita* AF279489, *ignitus* AF279496, *magnus*, *lucorum*, *patagiatus*, *terrestris*, *tunicatus*.

(03) **Confusobombus:** *confusus* AY181098.

(04) **Kallobombus:** *soroeensis* AY181159.

(05) **Megabombus:**

argillaceus, *consobrinus*, *gerstaeckeri*, *hortorum*, *ruderatus*.

(06) **Melanobombus:**

lapidarius AY181114, *sicheli* AY181157, *incertus*.

(07) **Pyrobombus:**

cingulatus AF279534, *haematurus*, *hypnorum* AY181110, *jonellus* AY181113, *lapponicus*, *monticola* AY181130, *pratorum* AY181147, *pyrenaeus* AY181151.

(08) **Sibiricobombus:** *niveatus*.

(09) **Subterraneobombus:**

distinguendus, *fragrans*, *melanurus* AF385818, *subterraneus*.

(10) **Thoracobombus:**

deuteronymus AF279553, *humilis* AY181106, *mucidus*, *muscorum* AY181135, *pascuorum* AY181139, *ruderarius* AY181154, *schreberi*, *sylvarum* AY181166, *veteranus*, *zonatus*.

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Appendix II

Description by H. Bischoff, 1936, Arkiv för Zoologie vol. 27A, pp. 2-3.

B. terrestris minshanicola n. subsp.

Eine verhältnismäßig kleine Form, die ausgezeichnet ist durch schmale, gelbe Binden und geringe Ausdehnung der weißen Behaarung an der Hinterleibsspitze. Die weiße Behaarung beginnt erst am Hinterrand des 4. Abdomen Tergits. Die schwarze Zone des Abdomens erscheint dadurch fast doppelt so breit wie die gelbe Querbinde. Vorn am Clypeus und Labrum ist die Behaarung zu einem erheblichen Teil gebräunt. Die Behaarung der Unterseite ist vorherrschend schwarz.

Beim Männchen beginnt die weiße Endbehaarung am Hinterrand des fünften Tergits.

H. Bischoff describes the typical colouration of the abdomen of this taxon, which makes the specimen of the type series and the specimens that I have seen (from the North of Sichuan & Gansu) characteristically different from typical *B. lucorum*. By the colouration alone it could easily be taken for a small *B. terrestris*, especially as the yellow of the museum material is not 'lemon yellow' but more or less brownish.

The males do not show the usual 'blonde' colouration of *B. lucorum*, and the scutellum and the first tergite are black not yellow as in typical *B. lucorum*. I cannot confirm that the white colouration at the end of the abdomen starts at the posterior border of the fifth tergite (see description by Bischoff). In the fresh males I have seen from Gansu the white hairs start at the posterior border of the fourth tergite, but the white hairs are so sparse that the black exoskeleton remains visible and the end of the abdomen looks more black than white.

Type series: The specimens of the type series Riksmuseet Stockholm (Minshan Southern Gansu/ China) could not be found (E-mail from Hymenoptera curator, Dr. Hege Vårdal).

Exact localities after Sjöstedt & Hummel 1932:

1 ♀, 29.07.1930, camp 50, [Drakana], 3100 m; 13 worker, 25.07.1930, camp 48, [Shi-men], 3500 m; 28./29.07.& 12.08.1930, camp 50, [Drakana], 3100 m; 02.08.1930, camp 53, [Jango], 3100-3600 m; 19.10.1930, camp 74, [Liang-chia-pa], 1300 m; 1 ♂, 03.09.1930, camp 64, [Tjeggala], Passhöhe Min-Shan 3700 m, alpine meadow.

Specimens seen:

(1) paratype specimens Museum für Naturkunde Berlin:

2 worker, 27.07.1930, camp 49 Kuan-ki-shan, valley south of the pass, ravine, shrubbery, 3600 m, 1 ♀, 29.07.1930, camp 50 Drakana, valley south of the Minshan main ridge, 3100 m.

(2) Collection Dr. van Asperen de Boer, Amsterdam: All specimens China/ Northern Sichuan:

1 worker, 15.08.2009, Rd Jiuzhaigo 53 km N Songpan, 3320 m, *Epilobium*; 2 worker, 11.08.2009, N. Zoige Rd Baxi 55 km NE, 3450 m, *Cirsium*; 1 worker, 14.08.2009, Rd Huanglong 51 km NE Singpan, 3560 m, *Cirsium*; 1 ♂, 03.08.2009, 5 km N Miyalo, 2830 m, *Vicia*.

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(3) Fresh material:

10 ♂♂, 23.08.2010, Yongdeng, Gansu Prov. Forest Border, 36° 41.26' N, 102° 42.77' E, 2260 m, *Cirsium leo.*

The localities *Southern Gansu* (Dr. H. Hummel) and *Northern Sichuan* (Dr. van Asperen de Boer) are very close (60 - 100 km distance). As the specimens inspected resemble the description given by Dr. H. Bischoff, and as the taxon is quite abundant in this area (Williams et al., 2009, Fig. 271 and 275 for Sichuan, and 'distributed widely in Gansu' according to information from Dr. An) *B. lucorum*/China and *B. minshanicola* Bischoff probably belong to the same taxon. An attempt to verify this assumption by sequencing one of the paratype specimens from the Museum für Naturkunde, Berlin was not successful; no reasonable DNA could be extracted.

Morphology & morphometry

Remains to be studied using more material.

Colouration

Should be investigated in fresh material; according to Williams et al. (2009) the yellow colouration is 'lemon yellow', but that is not the case in the museum material seen. Tkalců (1961) reported characteristic colour changes in museum specimens, which become brownish. The fresh males I have seen from Gansu/China show a bright yellow colouration with some black hair interspersed into the collare. The long hairs of the head (vertex, between the antennae & at clypeus) are black, with shorter feathered white-greyish hair intermixed. Similar to *B. lucorum* the tips of the black hairs are somehow whitened (pulverulence), not as much as in 'blonde' males of *B. lucorum* but still quite obvious and characteristic. The best characters of the colouration as described by Bischoff (1936) are the *narrow* bands (collare & 2. tergite) and the *broad black section* between the yellow band of the second tergite and the white end of the abdomen.

Distribution & ecology

Distributed in the higher mountains of Sichuan (Williams et al. 2009; Fig. 271 & 275) and Gansu provinces ('widely distributed' according to an e-mail from An), rare in Shanxi province and North China (Peng et al. 2009, Fig. 3). Restricted to the higher altitudes (upper montane zone), lower border of subalpine forest zone in Sichuan at about 3500 m (Winkler 1997, Abb. 3).

DNA sequences

B. lucorum, *B. hypocrita*, *B. minshanicola*, *B. franklini* & *B. affinis* form a separate cluster (Fig. 4), *B. minshanicola* is connected to the North American *B. franklini* & *B. affinis* with 96 % posterior probability.

Male labial gland secretions

The male labial gland secretions of *B. lucorum* are characterised by the main component Ethyl 9-tetradecenoate (58 %), which is found only in traces in *B. minshanicola*, whereas the main component in *B. minshanicola* is Ethyl dodecanoate (35 %). In *B. minshanicola* 2,3-Dihydro-farnesol is found in large quantities (26.6 %), a substance that is completely absent in *B. lucorum* (Table 5, Bertsch & Schweer, unpublished).

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Conclusion

B. mishanicola is essentially different from typical *B. lucorum* in (1) distribution, (2) colouration, (3) DNA marker (COI) and (4) male labial gland secretions. The genetic distance to the next neighbour taxa and the difference in compounds of the male labial glands prove *B. minshanicola* to be a separate species.

Tab. 5: Characteristic compounds of the male labial glands of *B. lucorum* and *B. minshanicola*. Compounds with more than 10 % in bold.

	MG	*lucorum*	*minshanicola*
Ethyl dodecanoate	228	9,56	**34,61**
Tetradecanal	212	--	1,86
2,3-Dihydrofarnesal	222	--	1,76
2,3-Dihydrofarnesol	224	--	**26,60**
Ethyl 9-tetradecenoate	254	**58,40**	0.02
Hexadecan-1-ol	242	3,54	3,60
9,12-Octadecadien-1-ol	266	1,64	0,54
9,12,15-Octadecatrien-1-ol	264	4,66	3,68
Ethyl 9-octadecenoate	310	0,82	**16,36**
15-Eicosen-1-ol	296	0,10	1,54

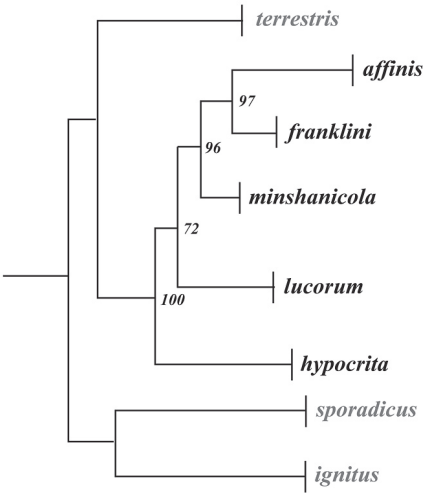

Fig. 4: MrBayes maximum likelihood tree calculated for cluster δ: *B. affinis*, *B. franklini*, *B. minshanicola*, *B. lucorum* and *B. hypocrita* with outgroup *B. soroeensis*.

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