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Barcoding cryptic bumblebee taxa: *B. lucorum*, *B. crytarum* and *B. magnus*, a case study

(Hymenoptera: Apidae: *Bombus*)

With 5 figures and 5 tables

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Zusammenfassung

Königinnen der fünf Taxa der Untergattung *Bombus* sensu stricto (*Bombus sporadicus, B. terrestris*, *B. lucorum*, *B. cryptarum* und *B. magnus*) wurden an verschiedenen Orten quer durch Europa im Frühjahr gefangen, um künstliche Kolonien zu züchten. Mitochondriale Cytochrome Oxidase Untereinheit I (COI) von 40 Proben wurde sequenziert (Teilsequenzen 1005 bp Länge). Die Divergenz der Sequenzen zwischen den Taxa beträgt etwa 30 bis 60 Basen-Substitutionen und die Tamura-Nei Genetische Distanz 0.05–0.25, während innerhalb der Taxa die Divergenz nur 1 bis 6 Basen-Substitutionen beträgt und die Tamura-Nei Genetische Distanz 0.002–0.007. Zusätzlich zu den Clustern für *B. sporadicus* und *B. terrestris* zeigt das Phylogramm drei weitere Cluster: den Cluster α für *B. lucorum*, den Cluster β für *B. cryptarum* und den Cluster γ für *B. magnus*. Die Cluster α, β und γ der Taxa des so genannten *lucorum*-Komplexes sind klar getrennt, mit geringer Variabilität, keiner Überlappung und keiner Endeinheit mit unklarer Position. Da die COI-Sequenzen keine Lücken aufweisen, können die einzelnen Nukleotide wie *homologe Positionen* verwendet werden. Jedes Taxon besitzt etwa 8–12 eigene Substitutionen, die als *diagnostische Positionen* verwendet werden können, um das Taxon zu charakterisieren. Mit den klassischen Werkzeugen der Kladistik wurde mittels dieser diagnostischen Positionen ein Stammbaum erarbeitet. Eine Barcode-Abfrage hat alle zweifelhaften Proben richtig bestimmt. Die topologische Position von GenBank-Sequenzen falsch bestimmter Proben und von Proben gealterter DNA wird diskutiert. Museumsproben dreier asiatischer Taxa mit unbekannter Zuordnung wurden sequenziert, um zu prüfen, inwieweit auch Museumsproben mit gealterter DNA mit Hilfe der diagnostischen Positionen zugeordnet werden können. Die Bestimmung mittels morphologischer und genetischer Merkmale wird diskutiert, und die Bestimmung kritischer Proben mittels Stammbaum (= genetischer Distanz) und mittels diagnostischen Positionen wird verglichen.

Summary

Spring queens of five taxa of the subgenus *Bombus* sensu stricto (*Bombus sporadicus, B. terrestris, B. lucorum, B. cryptarum* and *B. magnus*) were collected from different localities throughout Europe to rear artificial colonies. The mitochondrial cytochrome oxidase subunit I of 40 specimens was sequenced (partial sequence of 1005 bp length). Interspecific sequence divergence was about 30 to 60 base substitutions and the Tamura-Nei genetic distance was approximately 0.05 to 0.25, whereas the intraspecific sequence divergence was only 1 to 6 base substitutions and the Tamura-Nei genetic distance was about 0.002 to 0.007. In addition to the *B. sporadicus* and *B. terrestris* cluster, three clusters were obtained in the phylogenetic tree: cluster α for *B. lucorum*, cluster β for *B. cryptarum* and cluster γ for *B. magnus*. The three clusters α, β and γ, which represent taxa of the so-called *lucorum*-complex, were well separated, with low variability, no intergrading and no terminal units of unclear position. As there are no gaps in the alignments of the cytochrome oxidase subunit I sequences single nucleotide sites can be used as *positional homologies*. Each taxon is characterised by about 8 to 12 substitutions, which are unique ("private") and can be used as *diagnostic characters* to define and iden-

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tify that taxon. Using the classical tools of cladistics, a tree was built on the basis of these diagnostic characters. The Barcode engine successfully identified all critical specimens. The topological position of GenBank sequences of misidentified specimens and sequences with potentially degraded DNA is discussed. Museum specimens of three Asiatic taxa of the *lucorum*-complex with unknown relationships were sequenced to investigate the possibility of identifying specimens with degraded DNA by diagnostic positions. Identification based on morphological and molecular characters is discussed and the identification of critical specimens by tree building (= genetic distance) and by diagnostic characters is compared.

Introduction

With the recent publications of Pedersen (1996, 2002), Kawakita et al. (2004), Hines et al. (2006) and Cameron et al. (2007) we have, for the first time, a good general picture of the phylogenetic relationships of most bumblebee species. These molecular investigations show that the long-lasting work on the taxonomy of the bumblebee based on morphological characters has produced reliable results: At the level of subgenera only minor corrections are necessary (mainly New World subgenera), there are a few conflicting results, and there is more insight into the deeper nodes of phylogeny. However at the terminal units of the branches many questions remain. And we need more specimens from a broad range of geographical localities to investigate the genetic polymorphism of the taxa.

In recent years, the availability of genetic information has increased enormously. The inclusion of molecular information in taxonomic research can help to distinguish between species (equivalent to species identification or species diagnosis) and to discover new species (equivalent to species delimitation, species description). Species description and identification are among the most important tasks in biology, because biologists can neither report empirical results nor access published information on a study organism until it is correctly named. Hebert & Gregory (2005) described DNA barcoding as a novel system designed to provide rapid, accurate, and automated species identifications, by using short, standardized gene regions of cytochrome oxidase subunit I (COI) sequences as internal species tags. Munch et al. (2008) provided a statistical method for DNA barcoding based on a Bayesian phylogenetic approach, using automated database sequence retrieval. There is a heavy debate about the pros (e. g. Tautz et al. 2002, 2003; Hebert et al. 2003; Hebert & Gregory 2005;) and cons (e. g. Will & Rubinoff 2004; Will et al. 2005; Meier et al. 2006; Wheeler 2004, 2008) of these methods. Instead of comprehensive theoretical considerations, in this study the aim was to empirically test whether, despite all the theoretical challenges, DNA barcoding can deliver reliable species identifications, and to compare the results of the morphological and the molecular approach. The critical taxa *B. lucorum*, *B. cryptarum* and *B. magnus* of the so-called *Bombus lucorum*-complex were used as a case study. Initial reports demonstrated that these taxa can be safely separated by COI sequences (Pedersen 2002; Bertsch et al. 2005; Murray et al. 2008).

The identification of many species of the subgenus *Bombus* sensu stricto (syn. *Terrestribombus* Vogt) is often difficult because most species share a similar general appearance in colour and morphology, and there is a long-standing discussion about which taxa of the subgenus *Bombus* have species status, and which taxa might be subspecies belonging to a broader species. In Europe, there are five known taxa in the subgenus *Bombus* s. str.: *Bombus (Bombus) terrestris* (Linnaeus, 1758), *B. (B.) lucorum* (Linnaeus, 1761), *B. (B.) cryptarum* (Fabricius, 1775), *B. (B.) sporadicus* Nylander, 1848, and *B. (B.) magnus* Vogt, 1911. Their taxonomical status has been extensively examined based on morphology (Krüger 1939, 1951, 1954, 1956, 1958; Løken 1973; Pekkarinen 1979; Rasmont 1984; Rasmont et al. 1986), enzyme electrophoretic data (Scholl

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& Obrecht 1983; Pamilo et al. 1984; Scholl et al. 1992), analyses of the compounds of the male labial glands (Pamilo et al. 1997; Bertsch 1997; Urabanová et al. 2001; Bertsch et al. 2004), and DNA data (Pedersen 1996, 2002; Bertsch et al. 2005; Hines et al. 2006; Cameron et al. 2007; Murray et al. 2008). The species status of *B. sporadicus*, *B. terrestris* and *B. lucorum* is generally accepted; however the taxonomic status of *B. magnus* and *B. cryptarum* is still in dispute. Whereas Rasmont (1983), Rasmont et al. (1984), Bertsch et al. (2004, 2005) and Murray et al. (2008) treated both taxa as separate species, Williams (1991, 1998) grouped them with *B. lucorum "*interpreted in the broadest sense, to include a complex of similar taxa*"* (see Williams 2008).

Materials and Methods

Bumblebee samples

Females of all five European taxa of the subgenus *Bombus* s. str. were collected in spring from different localities throughout Europe (see Table 1). After collection, bumblebees were kept alive in a cool-box. Sometimes the characters essential for identification, such as the tufts of hair on the thorax and abdomen, were soaked and stuck together, especially in wet weather. In such cases, the bees were kept in flight cages with some honey-water. They started to clean and brush their hair by themselves, which restored all the essential characters. Morphological details were studied using a stereo microscope (Wild M16, Planar 1.0, Oculars 10x/21). As previously reported by E. Krüger (1928, p. 363), hair details are best studied in diffuse light (use of diffuse filter and indirect light with Novoflex Macrolight Plus) at high magnification by stroking the hair with a fine artist's brush or an insect pin. In this way the distribution of hair on different parts of the thorax and especially at the end of the collare below the tegulae (border of pronothalobus and episternite) was carefully investigated.

Identification of specimens

The identification of females (♀♀) of the so-called *lucorum*-complex is still under debate, but most fresh specimens can be identified without any problems. Specimens ♀♀ Mag-01 - Mag-09, Cry-02 - Cry-09, and Luc-02 - Luc-08 were identified without problems by the characters described in Rasmont (1984) and Bertsch et al. (2004). Using the queens collected in the field artificial colonies were reared in an air-conditioned greenhouse and the morphological identification of the founder female was verified by investigation of the male labial glands in each case. Males of *B. lucorum*, *B. cryptarum* and *B. magnus* can be identified by their specific labial gland secretions (Bertsch 1997, Bertsch et al. 2004, 2005). In all cases, labial gland secretions from males of artificial colonies confirmed the identification of the founder queen. Only specimen Cry-03, which was identified by morphological characters as *B. magnus*, was a misidentification, as the male labial gland secretions (and the DNA sequences) identified this specimen as *B. cryptarum*.

Critical and unidentified specimens

To test the different methods of identification (by morphology, by male labial glands and by DNA) four females were included whose identification by morphological characters proved to be problematic (♀♀ Mag-10, Cry-01, Luc-09, and Luc-10). The specimen Mag-10 from Milde was identified as *B. magnus*, but because the parts of the collare below the tegulae were relatively short there was some uncertainty that the specimen might belong to *B. cryptarum*. Specimen Cry-01 from the Orkney Islands was a typical light form of *B. cryptarum* but as this species has not yet been identified from the Orkney Islands it was classified as uncertain. Specimen Luc-09 from Central Spain had a very broad collare reaching below the tegulae and habitually looked like

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Tab. 1: List of 40 *Bombus* samples (Mag = *magnus*, Cry = *cryptarum*, Luc = *lucorum*, Ter = *terrestris*, and Spo = *sporadicus*) used in the present analysis with identification codes, and collection locality information. Q = ♀♀, aC → M = artificial colonies with production of males. Shaded and ? mark specimens which could not be safely identified.

Code	Locality	Country	Region	Latitude	Longitude	Altitude
Mag-01	Glenmore Forest	UK	Scotland	57° 09.83' N	3° 41.31' W	334 m Q, aC → M
Mag-02	Glen Oykel	UK	Scotland	57° 59.74' N	4° 49.11' W	140 m Q, aC → M
Mag-03	Duncery Beacon	UK	England	51° 09.48' N	3° 34.64' W	417 m Q, aC → M
Mag-04	Luccombe	UK	England	51° 11.01' N	3° 34.05' W	220 m Q, aC → M
Mag-05	Milde/Bergen	Norway	Hordaland	60° 15.28' N	5° 16.36' E	16 m Q, aC → M
Mag-06	Klaistow	Germany	Brandenburg	52° 17.89' N	12° 51.60' E	50 m Q, aC → M
Mag-07	Roth	Germany	Bayern	49° 14.61' N	11° 08.95' E	372 m Q, aC → M
Mag-08	Marcinkonys	Lithuania	Alytus County	54° 21.04' N	24° 25.46' E	145 m Q, aC → M
Mag-09	Sestroretsk	Russia	St. Petersburg	60° 08.02' N	29° 57.76' E	15 m Q, aC → M
Mag-10	Milde/Bergen	Norway	Hordaland	60° 15.28' N	5° 16.36' E	16 m ?Q, aC → M
Cry-01	Loch Swannay	UK	Orkney Islands	59° 08.53' N	3° 12.24' E	51 m ?Q, aC → M
Cry-02	Hillside/Burray	UK	Orkney Islands	58° 51.29' N	2° 56.29' E	50 m Q, aC → M
Cry-03	Duncery Beacon	UK	England	51° 09.48' N	3° 34.64' W	417 m Q, aC → M
Cry-04	Rollag/Numedalen	Norway	Buskerud	59° 59.87' N	9° 17.03' E	231 m Q
Cry-05	Biesental	Germany	Brandenburg	52° 45.81' N	13° 36.66' E	39 m Q, aC → M
Cry-06	Kuopio	Finland	Northern Savonia	62° 54.56' N	27° 39.55' E	211 m Q, aC → M
Cry-07	Marcinkonys	Lithuania	Alytus County	54° 21.04' N	24° 25.46' E	145 m Q, aC → M
Cry-08	Strelna	Russia	St. Petersburg	59° 51.63' N	30° 05.33' E	3 m Q, aC → M
Cry-09	Nassfeld	Austria	Kärnten	46° 34.49' N	13° 06.26' E	1415 m Q, aC → M
Cry-10	Vent	Austria	Tirol	46° 52.07' N	10° 54.44' E	2320 m ?M
Luc-01	Kirkwall	UK	Orkney Islands	58° 59.05' N	2° 57.56' W	5 m ?M
Luc-02	Luccombe	UK	England	51° 11.01' N	3° 34.05' W	220 m Q, aC → M
Luc-03	Klaistow	Germany	Brandenburg	52° 17.89' N	12° 51.60' E	50 m Q, aC → M
Luc-04	Kuopio	Finland	Northern Savonia	62° 54.56' N	27° 39.55' E	211 m Q, aC → M
Luc-05	Marcinkonys	Lithuania	Alytis County	54° 21.04' N	24° 25.46' E	145 m Q, aC → M
Luc-06	Briancon, Col de Vars	France	Hautes Alpes	44° 32.25' N	6° 42.21' E	2112 m Q, aC → M
Luc-07	Moscow	Russia		55° 49.26' N	37° 36.57' E	159 m Q
Luc-08	Ascania Nova	Ukraine	Kherson Oblast	46° 28.00' N	33° 53.05' E	25 m Q
Luc-09	Noguera de Albarracin	Spain	Teruel	40° 26.17' N	1° 35.38' W	1392 m ?Q, aC → M
Luc-10	Chita	Russia	Chitinskaja Obl.	52° 00.86' N	113° 28.56' E	730 m ?Q, aC → M
Ter-01	Marburg	Germany	Hessen	50° 48.09' N	8° 48.57' E	320 m Q
Ter-02	Assergi	Italy	Abruzzo	42° 25.57' N	13° 30.43' E	972 m Q
Ter-03	Alcala de los Gazules	Spain	Andalusia	36° 31.25' N	5° 38.86' W	417 m Q
Ter-04	Ascania Nova	Ukraine	Kherson Oblast	46° 28.00' N	33° 53.05' E	25 m Q
Ter-05	St Andrews	UK	Scotland	56° 20.17' N	2° 48.45' W	20 m Q
Ter-06	Porlock Hill	UK	England	51° 11.79' N	3° 38.95' W	384 m Q
Spo-1	Kuopio	Finland	Northern Savonia	62° 54.56' N	27° 39.55' E	211 m Q
Spo-2	Kuopio, Research Garden	Finland	Northern Savonia	62° 54.55' N	27° 34.88' E	95 m Q
Spo-3	Puutossalmi	Finland	Northern Savonia	62° 44.01' N	27° 43.42' E	87 m Q
Spo-4	Geilo	Norway	Buskerud	60° 31.75' N	8° 12.92' E	770 m Q

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B. magnus, and specimen Luc-10 from the Russian Transbaikal could not be safely separated from similar-looking taxa like *B. mongolicus* or *B. burjaeticus*. The identification of males by morphological characters is unreliable; therefore, specimens of two males were also included (♂ Cry-10 and Luc-01).

GenBank data

GenBank data were included (Table 2) in order to enlarge the database and to compare DNA sequences from different laboratories (Belfast, Inuyama/Kyoto, and Copenhagen). The specimens of *B. magnus* (GenBank accession nos. EF362738, EF362736, AY530014, AY630015), *B. cryptarum* (AY530011, AY530012), and *B. lucorum* (AY694095, AY530010) were from the artificial colonies from which I had collected and identified the founder queens. These colonies produced males, and male labial gland secretions verified the morphological identification of these females.

Tab. 2: List of *Bombus* samples from Genbank data (Mu = Murray, Belfast, Ta = Tanaka, Kyoto, Pe = Pedersen, Copenhagen) used in the present analysis with Genbank numbers, and collection locality information, ? = misidentified specimens. Q = ♀♀, aC → M = artificial colonies with production of males.

Code		Locality	Country	Region	Latitude	Longitude	Altitude	
Mag EF362735	Mu	Benbulben	Ireland	Sligo	54° 18.87' N	8° 23.79' W	280 m	
Mag EF362738	Mu	Porlock Hill	UK	England	51° 11.79' N	3° 38.95' W	382 m	Q, aC → M
Mag EF362736	Mu	Craig y Fan Ddu	UK	Wales	51° 51.38' N	3° 22.47' W	642 m	Q, aC → M
Mag AY530014	Ta	Duncansby Head	UK	Scotland	58° 38.57' N	3° 01.77' W	47 m	Q, aC → M
Mag AY530015	Ta	Menz	Germany	Brandenburg	53° 06.84' N	12° 68.54' E	85 m	Q, aC → M
?Mag AY181123	Pe	Sölk Pass	Austria	Steiermark	47° 16.34' N	14° 04.79' E	1797 m	Q
?Mag AY181124	Pe	Julier Pass	Switzerland	Graubünden	46° 28.36' N	9° 43.76' E	2291 m	Q
Cry AY530011	Ta	Duncansby Head	UK	Scotland	58° 38.57' N	3° 01.77' W	47 m	Q, aC → M
Cry AY530012	Ta	Menz	Germany	Brandenburg	53° 06.84' N	12° 68.54' E	85 m	Q, aC → M
Cry EF362728	Mu	Benbulben	Ireland	Sligo	54° 18.87' N	8° 23.79' W	280 m	
Luc AY694095	Ta	Crail	UK	Scotland	56° 16.79' N	2° 35.39' W	7 m	Q, aC → M
Luc AY530010	Ta	Menz	Germany	Brandenburg	53° 06.84' N	12° 68.54' E	85 m	Q, aC → M
Luc AF279497	Ta	Yakutsk	Russia	Sakha Republic	62° 01.82' N	129° 44.09' E	115 m	
?Cry AY181119	Pe	Ekkodalen	Denmark	Bornholm	55° 38.98' N	12° 18.16' E	14 m	Q
?Cry AY181117	Pe	Kaprun	Austria	Vorarlberg	47° 16.34' N	12° 45.56' E	795 m	M
Ter EF362742	Mu	Cork	Ireland	Scotland	51° 53.83' N	8° 28.22' W	13 m	
Ter AY181122	Pe	Ponta Lourenco	Portugal	Madeira	32° 44.94' N	16° 41.67' W	52 m	
Spo AY181163	Pe	Skute	Norway	Oppland	60° 38.83' N	10° 19.82' E	171 m	
Spo AF279500	Ta	Primorsk territory	Russia					
AY181121	Pe	Edinburgh	UK	Scotland				
AY181116	Pe	Bøverdalen	Norway	Oppland				

DNA extraction, polymerase chain reaction (PCR) and 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, Germany). For sequence analysis overlapping fragments (a total of 1027 bp) of mitochondrial COI were amplified using primers specifically designed for *Bombus*. BO-1-fwd (5' TAGGATCACCAGATATAGC 3') and BO-K-rev (5' GAGCTCAAACAATAAATCC 3') amplified a 609 bp fragment, and BO-5-fwd (5' AATGAAAGAGGTAAAAAAGAAAC 3') and BO-A-rev (5' ATGTTGAGGGAAAAATGT

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TAT 3') amplified 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 $(NH_4)_2SO_2$, 0.01 % Tween20, 0.2 mM of each dNTP, 20 pmol of each primer, and 1.5 units *Taq*DNA polymerase (*Fermentas*, St. Leon-Rot, Germany). Conditions for PCR amplifications 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 61 °C (for BO-1-fwd and BO-K-rev) or 63 °C (for BO-5-fwd and BO-AA-rev), and final extension for 7 min. 10 µl of each reaction were checked on a 1 % agarose gel. PCR products were purified using 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 and then analyzed on an Abi® 3100 sequencer (*Applied Biosystems*).

Abi Sequences were edited with 4Peaks (by A. Griekspoor and T. Groothuis, mekentisj.com) and aligned manually using ClustalX. To produce an equal sequence length for all individuals, sequences were trimmed to 1005 bp (encoding 335 amino acids). Individual alignments were aligned against the complete COI gene sequence of *Bombus ignitus* between positions 262 and 1267 (GenBank accession no. DQ870926, Cha et al. 2007).

In living organisms, DNA damage is repaired by various enzymatic mechanisms. However, once the metabolic pathways of a cell cease to operate the DNA molecules progressively decay. The decay rate is influenced by a variety of factors related to the storage conditions. Biochemical processes subsequent to cell death may alter nucleotide sequence information in many ways. Several of these post-mortem DNA modifications can block amplification during the polymerase chain reaction (PCR), whereas others allow PCR products to be obtained, but with incorrect bases incorporated and maintained in the amplification products. These kinds of PCR artefacts, termed miscoding lesions, are commonly represented by two types of transitions: type I (A → G) (T → C) and type II (C → T) (G → A). Miscoding lesions can lead to higher estimated substitution rates at the degraded sites and consequent overestimates of levels of polymorphism.

The general number of transitions attributed to damage processes is suspected to be inflated because it may include some errors caused by the PCR technique itself (Gilbert et al. 2007). The amplification of DNA consists of iterative steps, which form the chain reaction. Because these biochemical processes produce errors the PCR is not a deterministic process. Known experimental parameters that influence PCR performance are the quality of the *polymerase*, the *buffer* composition and the *temperature* of the primer annealing. A critical step in the procedure is also the *purification* of the PCR products. Therefore, great care was taken to obtain high quality sequences by adjusting buffer composition and annealing temperature, and only sequences were used where both the forward and the backward primer delivered flawless and identical sequences. Two independent PCR products were investigated where necessary. All positions were checked by carefully inspecting the original ABI traces, because sometimes the software used to analyze and edit trace files (4Peaks) produces erroneous results. In the investigation and interpretation of sequences from museum specimens this thorough inspection of the ABI traces proved to be essential to detect miscoding lesions and to interpret doubtful positions of degraded DNA (Hofreiter et al. 2001; Hajibabei et al. 2006; Junqueira et al. 2002; Sefc et al. 2007).

Analysis of sequence divergence and tree topology of mitochondrial COI

The absolute numbers of substitutions were counted based on pairwise comparison of COI sequences. The nucleotide frequencies and the parameters necessary for computer models were estimated from the sequence data and Tamura-Nei genetic distances were calculated. The tree topology was inferred by a maximum likelihood tree based on the general time reversible mod-

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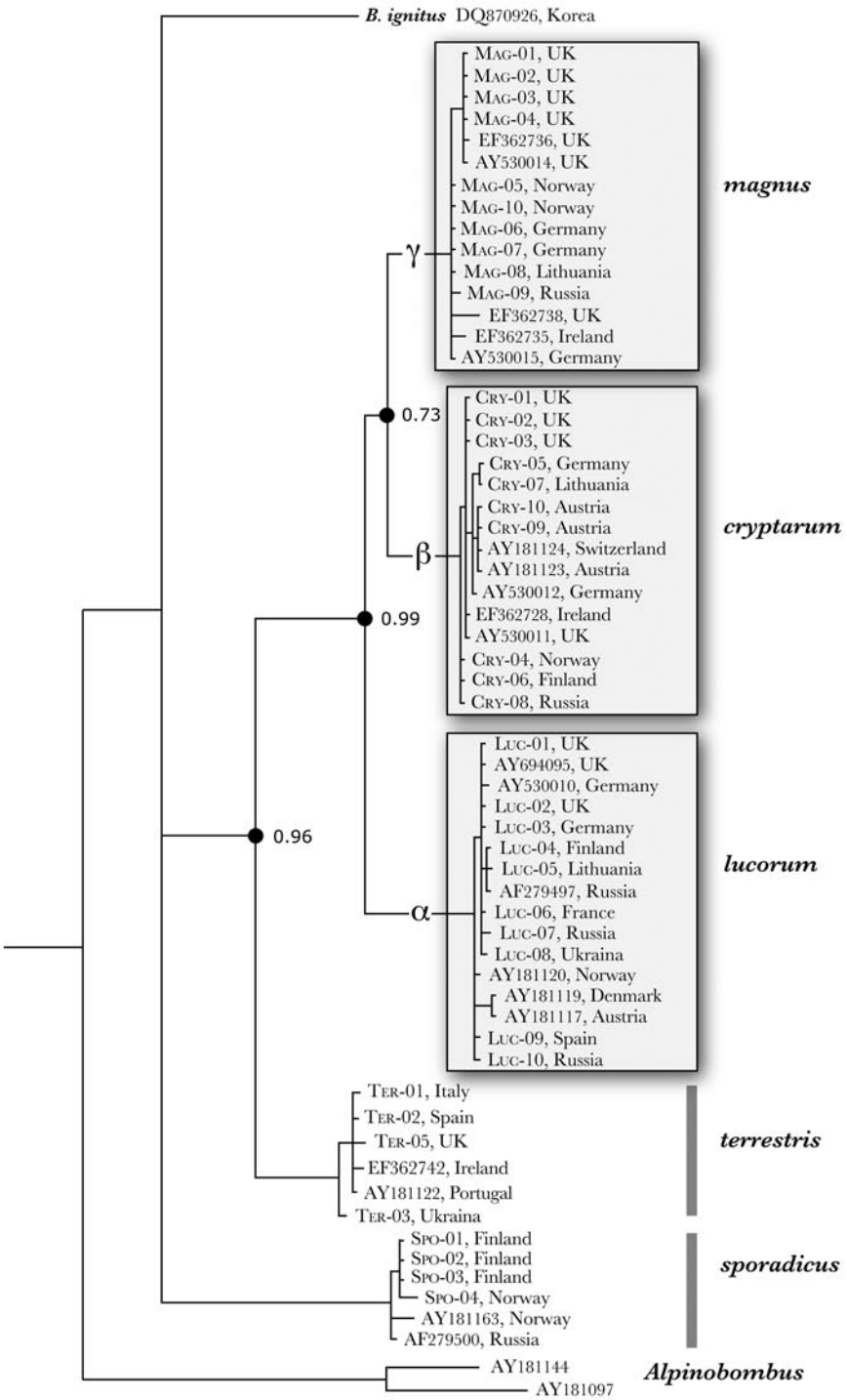

Fig. 1: Tree topology calculated as Maximum-Likelihood tree using Bayesian MCMC analysis with the general time reversal model of base substitutions, gamma distribution and 5 000 000 generations.

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el (GTR) of base substitution with gamma distribution, calculated by Bayesian analysis using MrBayes (Huelsenbeck & Ronquist 2001). Tree topology was also calculated as a neighbour-joining tree (NJ) and as a most parsimonious tree (MP) with bootstrap sampling, using Mega 4.0 (Tamura et al. 2007). Geneious Pro 4.5 (Biomatters Ltd.) was used to analyze the alignment, to detect diagnostic positions and the GreenButton plugin (InterGrid) to do the time consuming MrBayes calculations on a supercomputer cluster. MacClade 3.04 was used to examine the nucleotide changes on cladograms. The COI sequence of *B. soroeensis* was used as the outgroup (GenBank accession no. AY181159, Pedersen 2002), and a few sequences from the genetically nearest subgenus *Alpinobombus* (Pedersen 2002; Cameron et al. 2005) were also included.

Results

Nucleotide frequencies, substitution parameters and COI divergence

The aligned data matrix of 1005 bp of 40 sequences (Table 1) included 134 variable sites. Of these variable positions five were uninformative (singleton substitutions = noise), and 129 informative (= signal). However, most of these informative sites were at silent positions, and translation resulted in amino acid sequences of 335 amino acids with only 11 variable sites. Most of the amino acid sequence variability was in *B. sporadicus* (7 variable sites out of 11); within the *lucorum*-complex only three sites out of 335 amino acids were variable. The nucleotide frequencies were pi(A) = 34.0 %, pi(C) = 12.2 %, pi(G) = 11.7 % and pi(T) = 42.2 %, demonstrating the known strong A + T bias typical for sequences of Hymenoptera. Therefore the Tamura-Nei model of base substitution was used (Tamura and Nei 1993), which corrects this bias in its assumption of sequence evolution. Gamma-distributed rates ($\alpha = 0.16$) were used as a model for rate heterogeneity.

The 1005 base-pair sequences of COI were used in analyses of sequence divergence among the five European taxa. Table 3 presents the matrix of genetic distances estimated by the Maximum composite Likelihood model (MEGA) with rates among sites gamma-distributed. The intraspecific genetic variability was low for all taxa (1–6 nucleotid substitutions, genetic distance 0.002–0.007), even when the specimens of each taxon were collected in geographically distant localities (Table 4). In contrast, the interspecific genetic variability was approximately one order of magnitude larger (30–65 nucleotid substitutions, genetic distance 0.046–0.266).

Tab. 3: Calculated genetic distance within and between taxa. (MEGA, model: Maximum Composite Likelihood, rates among sites gamma distributed).

	Mag	Cry	Luc	Ter	Spo
magnus	*0.0020*				
cryptarum	0.0467	*0.0018*			
lucorum	0.0677	0.0642	*0.0039*		
terrestris	0.1076	0.1148	0.1352	*0.0068*	
sporadicus	0.2663	0.2415	0.2606	0.2105	*0.0115*

Tree building by maximum likelihood models

The maximum likelihood tree (Fig. 1) generated using the Bayesian MCMC (Markov Chain Monte Carlo method) analysis was based on the general time reversible model (GTR) of base substitution, gamma distribution, and 5 000 000 generations to achieve equilibrium, sampling

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every 50 generations and a "burn-in" of 5 000 generations. Phylogenetic trees were also generated using the neighbour-joining (NJ) and the most-parsimonious (MP) model with a bootstrap value of 1 000. As expected (Suzuki et al. 2002; Douady et al. 2003) the reliability of nodes measured by bootstrap percentages (BP) was slightly smaller than Bayesian posterior probabilities (PP). However the data were quite robust and irrespective of the model used, we obtained five distinct clusters, one cluster for *B. sporadicus*, one cluster for *B. terrestris,* cluster α for operational taxonomic units (OTU) *lucorum*, cluster β for OTUs *cryptarum* and cluster γ for OTUs *magnus*. The three clusters α, β and γ, representing OTUs of the so-called *lucorum*-complex, were well separated, with low variability, no intergrading and no terminal units of unclear position.

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). The alignment file (Fig. 2) shows quite clearly that each taxon is characterized by about 8 to 12 substitutions, which are unique ("private") and can be used as *diagnostic characters* to define and identify that taxon. In MacClade the changes at the nodes and the diagnostic characters at the last branch of the terminal units can be investigated in detail and a tree can be built with the classical tools for morphological characters (Fig. 3). With the large number of diagnostic characters available it is normal that not all of these changes are unambiguous. However each of the three taxa of the so-called *lucorum*-complex is characterized by about 8 to 12 unambiguous diagnostic characters.

All specimens of *B. cryptarum* from alpine habitats (Cry-09, Cry-10, AY181123 and AY181124) differed from the rest of the *cryptarum* sequences by diagnostic position 1101 with a (T → C) replacement, and all specimens of *B. magnus* from the UK differed from the rest of the *magnus* sequences by the diagnostic positions 409 (T → C), position 579 (A → G) and position 603 (C → T). More material is needed but as the sequences were obtained from different laboratories the possibility of stochastic variability is very low. Diagnostic position 409 in *B. magnus* was one of the three sites within the *lucorum*-complex that results in amino acid sequence replacement; all specimens of *B. magnus* from the UK differed from the rest by the amino acid proline instead of serine at amino acid position 137.

Morphologically problematic specimens and misidentifications

Specimen ♀♀ Cry-03 was identified by morphological characters as *B. magnus*, but the labial gland secretions from males reared in an artificial colony from this queen identified this specimen as *B. cryptarum.* This *identification by labial gland secretions* was confirmed by the DNA data; specimen Cry-03 was integrated into the *cryptarum*-cluster β. As discussed in Bertsch et al. (2005), the specimens AY181117 (from Austria) and AY181119 (from Denmark) identified by Pedersen (2002) as *B. cryptarum* were morphological misidentifications, and both specimens cluster with the *lucorum*-cluster α. The observed differences of 4 bp were within the observed infraspecific variability of *B. lucorum* (Table 4). Specimens AY181123 and AY181124 from alpine habitats, which were identified by Pedersen (2002) as *B. magnus*, clustered with the *cryptarum*-

Tab. 4: Calculated geographic distances (km) and sequence differences (base pairs) for *B. lucorum.* (Luc-01, Luc-04, Luc-08 and AF279497).

		Kirk	Brian	Chit	Yak
Luc-01	Kirkwall	--	2	3	2
Luc_04	Briancon	1600	--	2	1
Luc-08	Chita	6400	7200	--	2
AF279497	Yakutsk	5900	7100	1500	--

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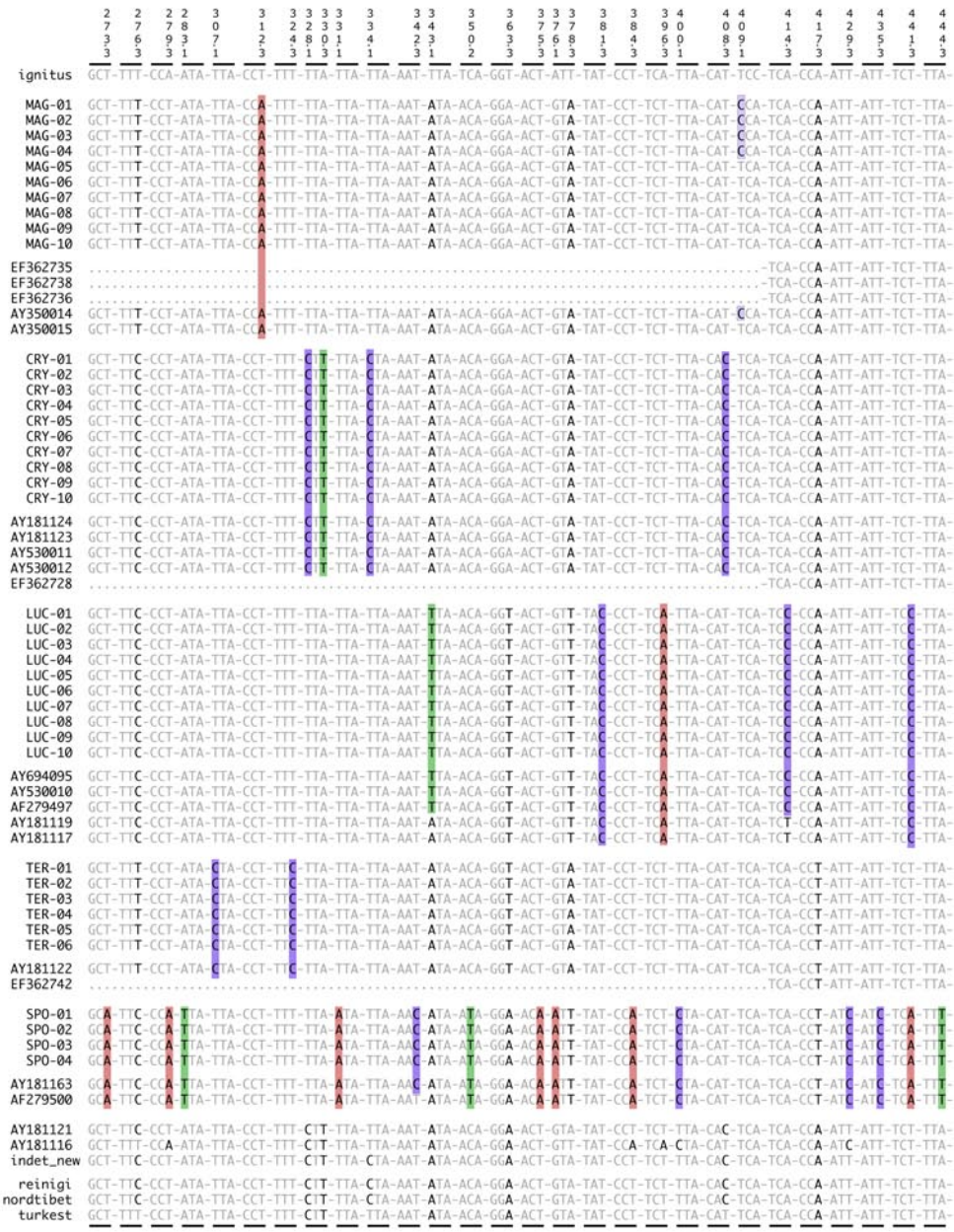

Fig. 2.1-4: Alignment of all parsimonious informative triplets (with uninformative sites deleted -), and with a pointer for position number (numbered for total COI) and codon position. Diagnostic (= private) positions marked with colour: green = Thymine, violet = Cytosine, red = Adenine and yellow = Guanine.

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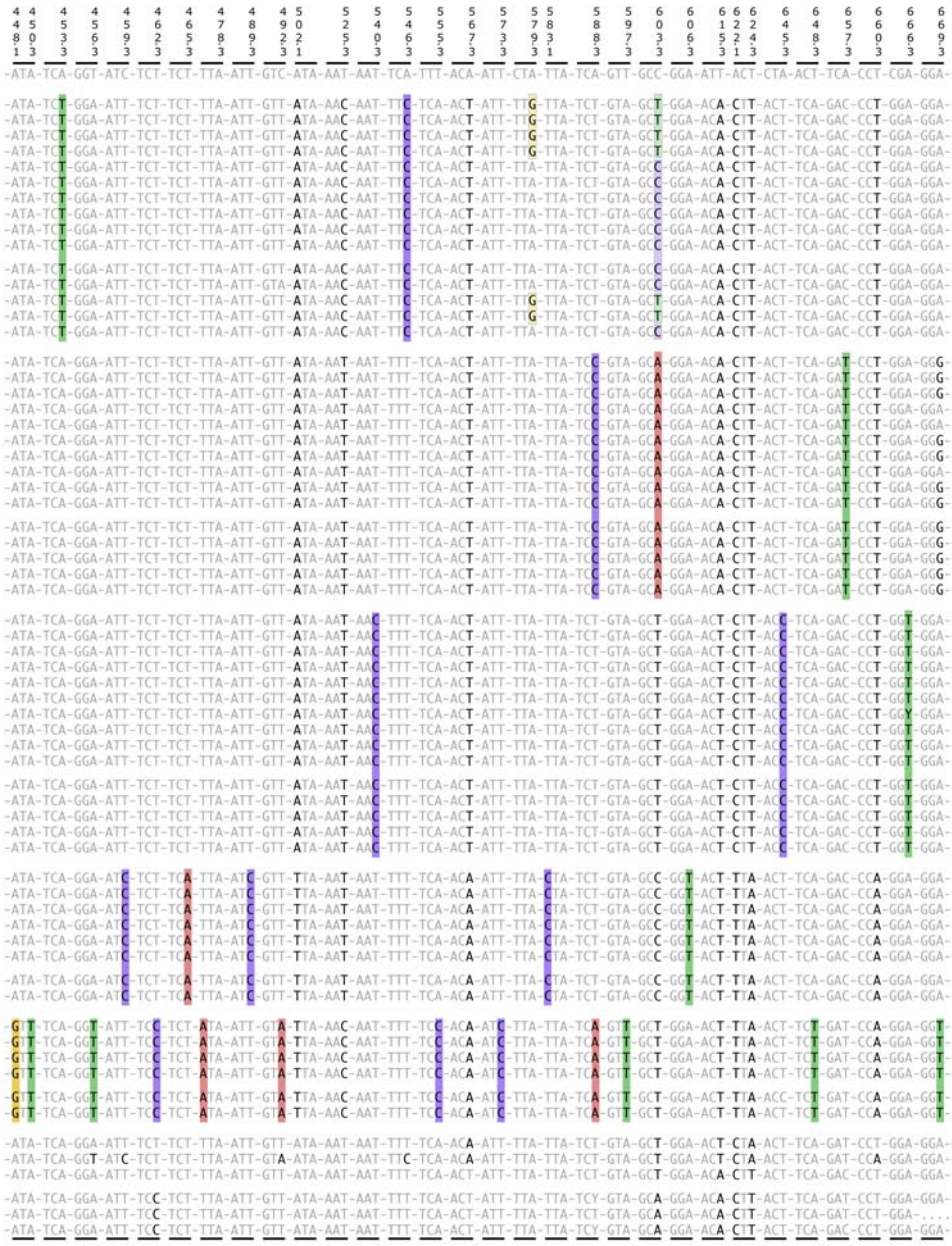

cluster β; these represent the light coloured form of *B. cryptarum* that is typically found in alpine habitats (Amiet 1996), and may easily be mistaken for *B. magnus*. The grouping of both alpine specimens from Pedersen with the alpine *B. cryptarum* specimens CRY-09 and CRY-10 was confirmed by diagnostic position 1101 (T → C).

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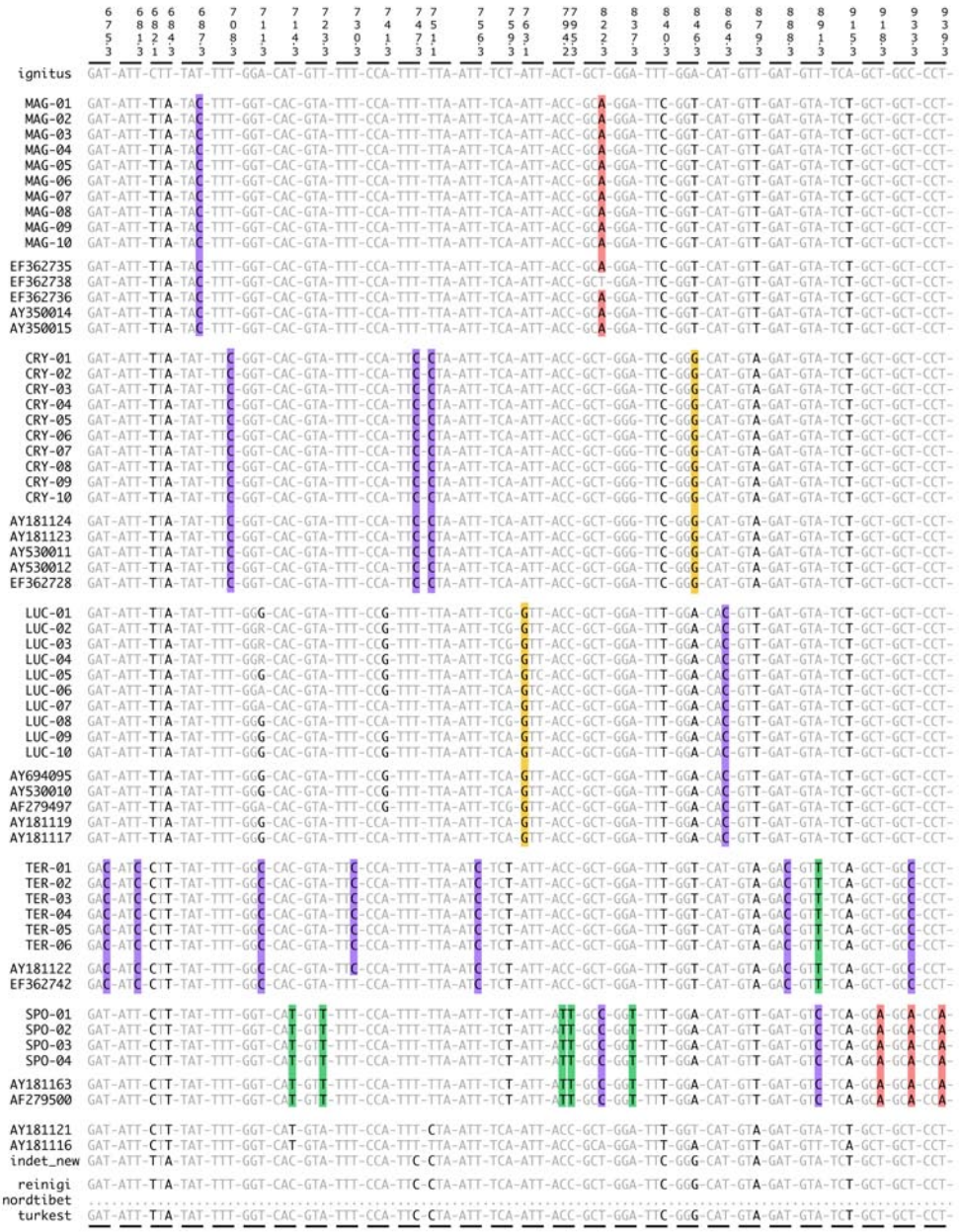

Identification by similarity

To check the "promises" in HEBERT & GREGORY (2005) of a *novel system for rapid and accurate species identification* the sequences of all critical or doubtful specimens were submitted to the Barcode identification engine. The species identity of a *query* (unknown) sequence is assigned on the basis of its similarity to a set of *reference* (identified) sequences. All three doubtful specimens of *B. lucorum* were identified as *B. lucorum*: the male LUC-01 from the Orkney Islands with

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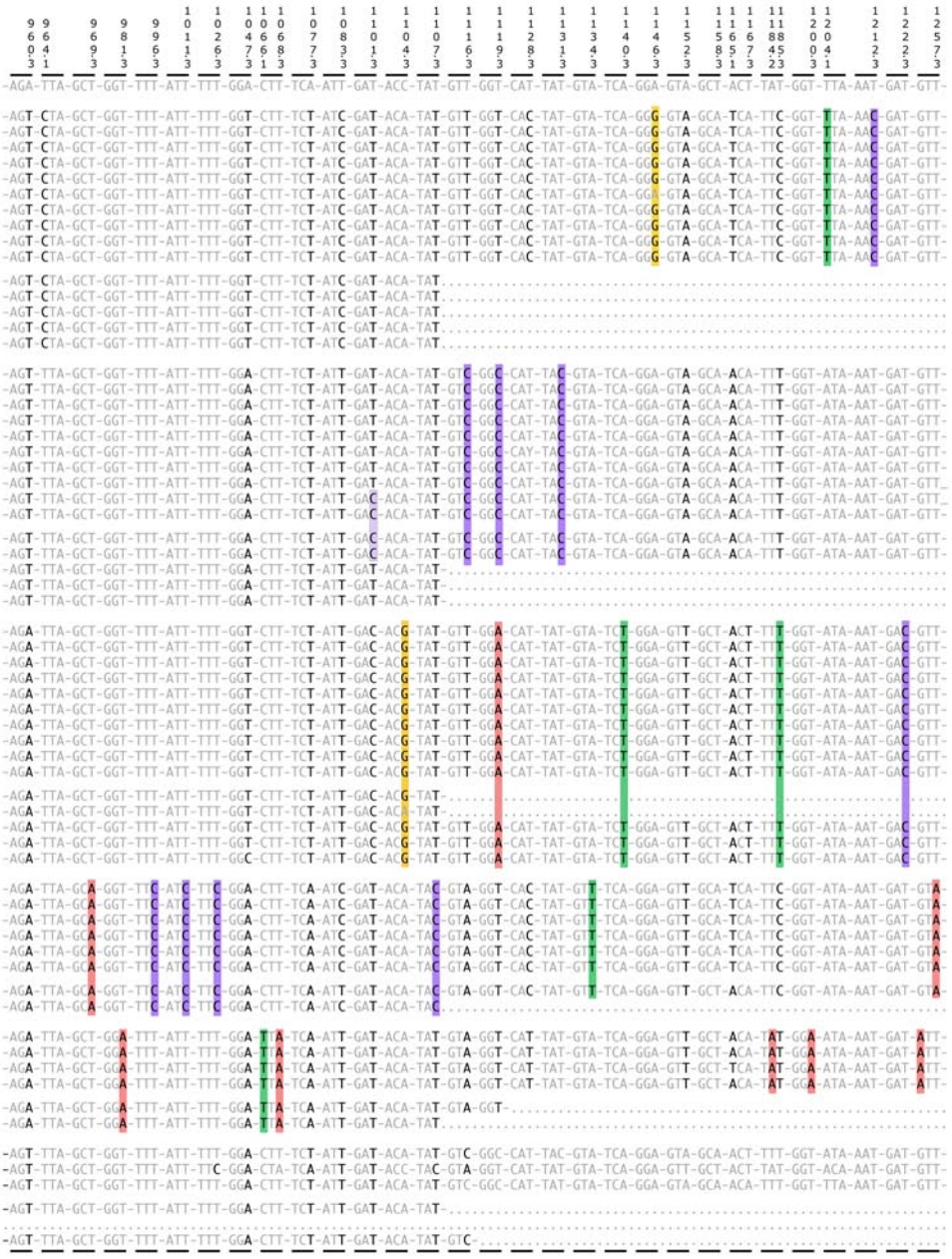

100 % similarity to *B. lucorum* reference sequences, the female Luc-07 from Spain with 99.8 % similarity and the female Luc-08 from Transbaikal with 99.8 % similarity. There was a clear gap (barcoding gap) between those and the next similar sequences with 96 % similarity. Specimen Mag-06, the most *cryptarum*-like of all *B. magnus* specimens from Milde/Bergen, was identified with 100 % similarity as *B. magnus* and again there was clear gap between that and the next similar sequences with 97 % similarity. The male Cry-01 from the Orkney Islands was identified

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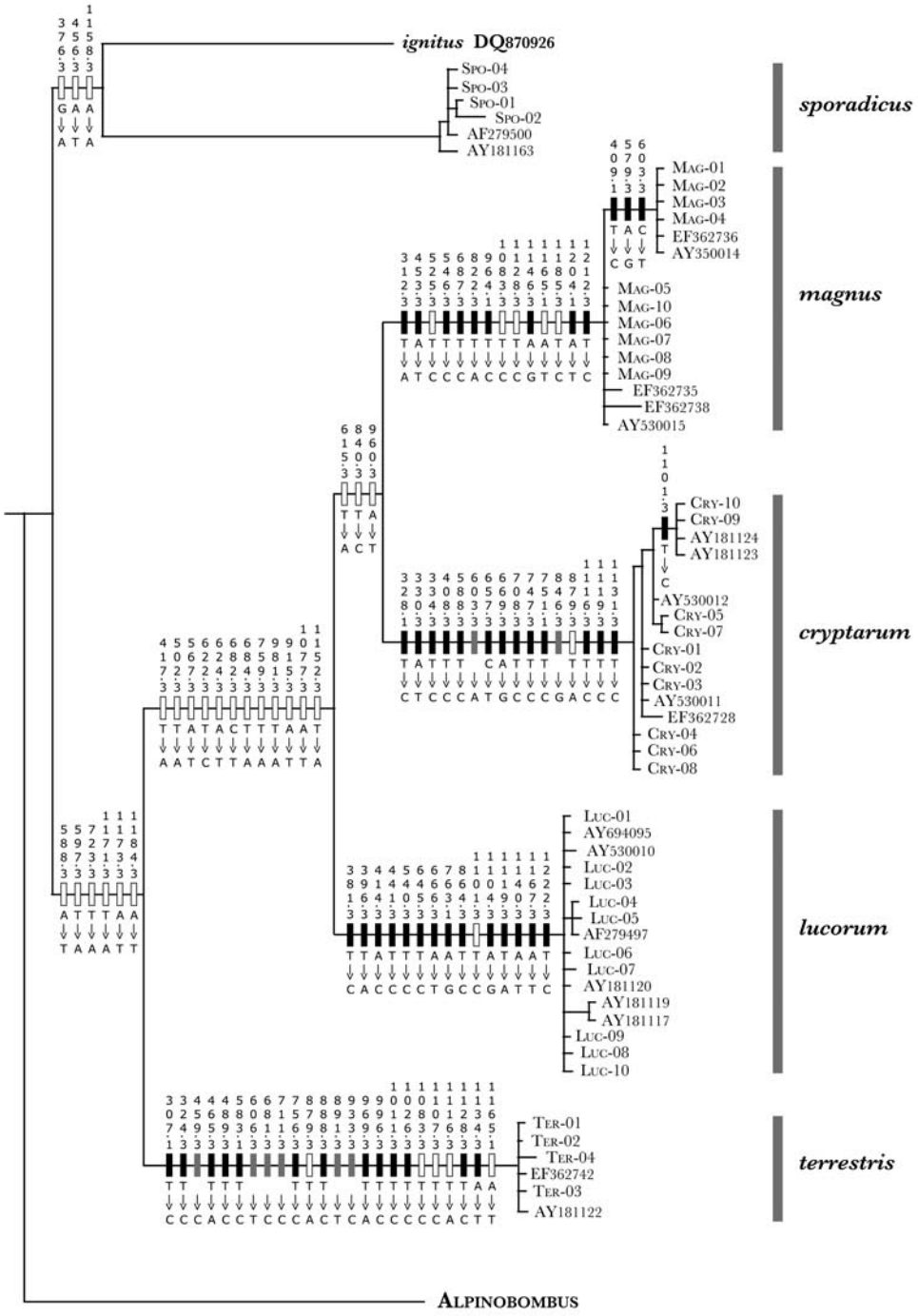

Fig. 3: Observed diagnostic character changes with position numbers mapped onto the Maximum-Likelihood tree. Black box = unambiguous *diagnostic* character change, grey box = ambiguous *diagnostic* character change, and white box = unambiguous character change.

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as 100 % *B. cryptarum* and 99.8 % *B. magnus*. The male Cry-10 from Austria was identified as 100 % *B. cryptarum* and 100 % *B. magnus*, which is an astonishing result considering the mean sequence difference between *B. cryptarum* and *B. magnus* of about 30 base pairs. But we will see that this is the result of misidentified specimens within the reference database.

The Barcode identification engine also delivers a tree (Kimura 2 parameter) and the results of the different barcode engine identification requests together with this Kimura 2 parameter tree have been summarized in Fig. 4. The three clusters α, β and γ for the three taxa of the *lucorum*-complex are immediately obvious. But a closer look reveals that all three taxa are spread all over the tree. The *lucorum*-cluster α contains two specimens of *B. cryptarum*, which we know already to be the specimens *B. lucorum* AY181117 and AY181119, which were misidentified as *B. cryptarum* by Pedersen (2002). *B. magnus* is divided into two clusters, each of two specimens. The *magnus*-cluster γ is the real *B. magnus* represented by the two specimens AY530014 and AY530015 from Bertsch et al. (2005) (and the critical specimen Mag-06 from this study). The two specimens of *B. magnus* within the *cryptarum*-cluster β_1 are the specimens *B. cryptarum* AY181123

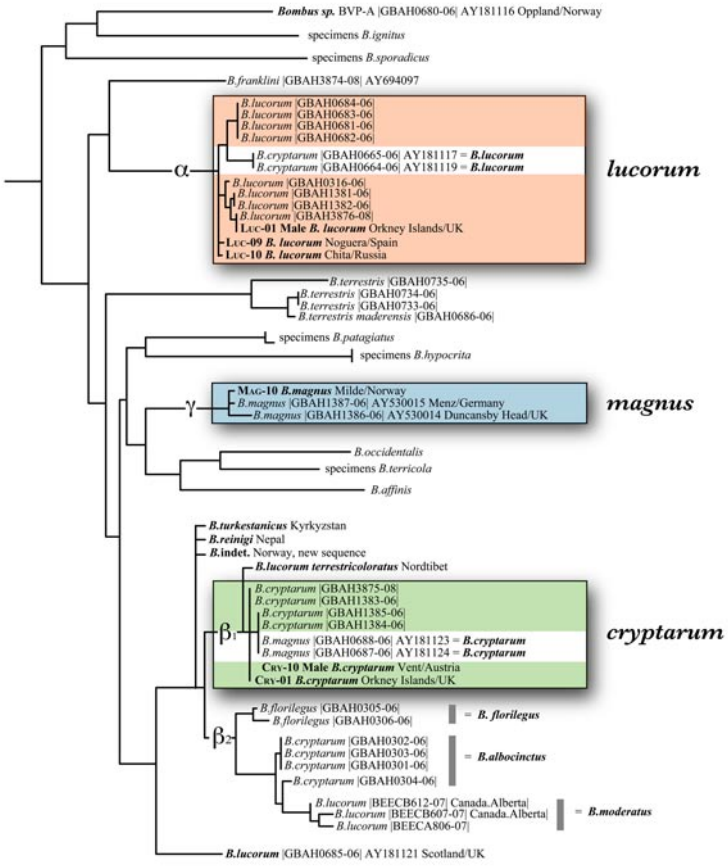

Fig. 4: Summary of Barcode engine identification requests for specimens with identification problems (Luc-01, Luc-09, Luc-10, Mag-10, Cry-01 and Cry-10) or degraded DNA (*B. lucorum terrestricoloratus*, *B. reinigi*, *B. magnus turkestanicus* and new sequence for *B.* sp. BVP-A, AY181116). For details of misidentifications and misnamings see text.

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and AY181124 from alpine habitats that were misidentified as *B. magnus* by Pedersen (2002). This explains the strange identification result of the Barcode identification engine in the case of Cry-01 and Cry-10. A second *cryptarum*-cluster β_2 contains specimens of *B. albocinctus* from the Russian Far East and *B. moderatus* from North America, a topological position that proves that both taxa are separate from *B. lucorum*.

So all five doubtful specimens were identified by the Barcode identification engine without any problem. This task requires much professional skill and experience if based on morphology, and requires a lot of time and facilities if carried out using artificial colonies and male labial gland secretions. It should be emphasized that the COI sequences in this investigation used only part of the barcoding region of 658 bp (overlap 435 bp), and the reference sequences were not from the *validated reference* barcode database but from the *species level records* barcode database.

Detecting new taxa?

A *new species* of the subgenus *Bombus* s. str. was detected by Pedersen (2002) and the sequence is available as "unclassified *Bombus* sp. BVP-A" from Norway (GenBank AY181116). No morphological details about this *new species* are available. I became interested in this unidentified *Bombus*

Tab. 5: Diagnostic Positions for *B. lucorum*, *B. magnus* and *B. cryptarum* and for misidentified specimens AY181124, AY181123 and AY181121. A new sequence for AY181116 (indet Norway), and sequences from museum specimens *B. reinigi*, *B. lucorum terrestricoloratus* Krüger, *B. magnus turkestanicus* Krüger. *T* interpreted as type II miscoding lesions (C → T) caused by degraded DNA.

	312	328	330	334	381	396	408	414	441	453	540	546	588	603	645	657	666	687
lucorum			**C**		**C**	**A**		**C**	**C**		**C**				**C**		**T**	
magnus	**A**									**T**		**C**						**C**
cryptarum	T	**C**	**T**	**C**	T	T	**C**	A	T	A	T	T	**C**	**A**	T	**T**	A	T
AY181124	T	**C**	**T**	**C**	T	T	**C**	A	T	A	T	T	**C**	**A**	T	**T**	A	T
AY181123	T	**C**	**T**	**C**	T	T	**C**	A	T	A	T	T	**C**	**A**	T	**T**	A	T
indet Norway		**C**	**T**	**C**			*T*						*T*	**A**		**T**		
AY181121		**C**	**T**	*T*			**C**						*T*	T		**T**		
reinigi		**C**	**T**	**C**			**C**						Y	**A**		**T**		
lucorum		**C**	**T**	**C**			**C**						*T*	**A**		**T**		
turkestanicus		**C**	**T**	**C**			*T*						Y	**A**		**T**		
	708	747	751	763	822	846	864	964	1104	1116	1119	1131	1140	1146	1167	1204	1212	1222
lucorum				**G**			**C**		**G**				**T**		**T**			**C**
magnus					**A**			**C**						**G**		**T**	**C**	
cryptarum	**C**	**C**	**C**	A	T	**G**	T	T	A	**C**	**C**	**C**	A	A	A	A	T	T
AY181124	**C**	**C**	**C**	A	T	**G**	T	T	A	**C**	**C**	**C**	A	A	A	A	T	T
AY181123	**C**	**C**	**C**	A	T	**G**	T	T	A	**C**	**C**	**C**	A	A	A	A	T	T
indet Norway	*T*	**C**	**C**			A				**C**	*T*	*T*						
AY181121	*T*	**T**	**C**			T				**C**	**C**	**C**						
reinigi	*T*	**C**	**C**			**G**				**C**	*T*	-	-			-	-	-
lucorum	*T*	T	-	-		-	-	-	-	-	-	-	-			-	-	-
turkestanicus	*T*	**C**	Y			A				**C**	*T*	-	-			-	-	-

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species in summer 2006, when I obtained a strange sequence from a *B. magnus* specimen (from Leerstetten / Bavaria, Germany) that was sequenced in Belfast, and which had much similarity (simple Blast request 99.4 % similarity) with the sequence from *Bombus* sp. BVP-A from Norway. Because labial gland secretions from males of the artificial colony verified my morphological identification of the specimen from Leerstetten as *B. magnus* something must be wrong with both strange sequences. New sequences from both specimens resulted in quite different sequences and confirmed the identification of *B. magnus* from Germany (EF362728) and tentatively identified *Bombus* sp. BVP-A from Norway as *B. cryptarum*, which is clearly a specimen with degraded DNA, and thus cannot be identified by similarity but only by diagnostic positions (Table 5).

More specimens with degraded DNA

Pedersen (2002) also discussed in detail a specimen of *B. lucorum* from Scotland (GenBank AY181121), which shows an exceptionally large difference in base substitutions compared to all other specimen of *B. lucorum* by adding 38 polymorphic sites. As a consequence this specimen has a different topological position when identified by tree building (Fig. 5 in Pedersen 2002) and seems to be a near neighbour to *B. magnus*. But as the *B. magnus* in Pedersen is really *B. cryptarum*, it is obviously related to *B. cryptarum.* This specimen AY181121 corresponds with *B. cryptarum* at all diagnostic positions (Table 5) and the large difference in base substitutions is probably also due to degraded DNA. Sequences of *B. lucorum* from Scotland (Luc-01, AY694095) are more or less identical to sequences of *B. lucorum* from the continent.

To investigate how far the use of diagnostic positions allows the identification of museum specimens in which degraded DNA could be expected, three specimens of the subgenus *Bombus* s. str.

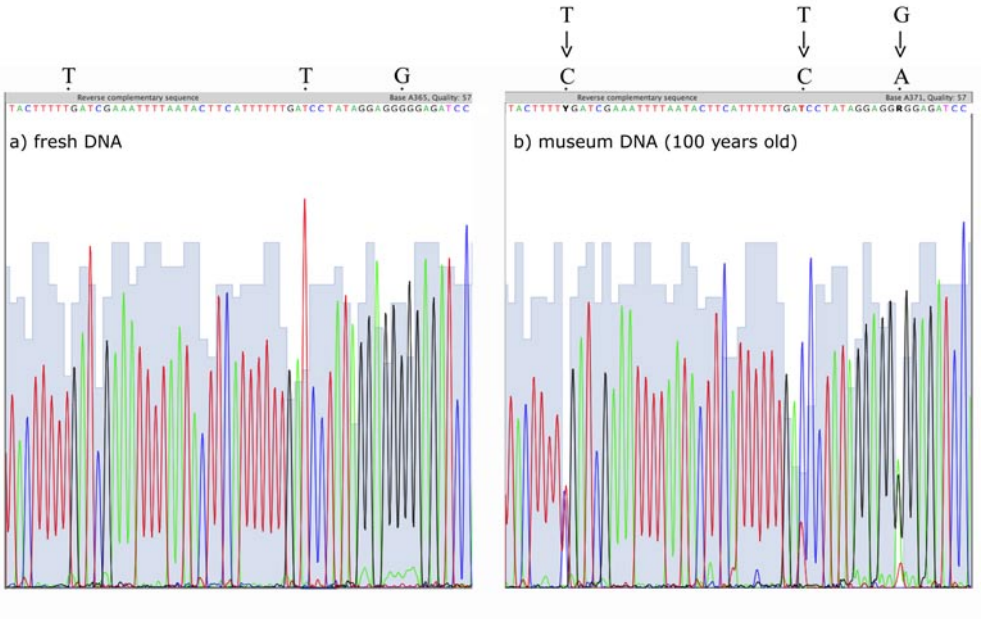

Fig. 5: Comparison of ABI colour traces of fresh (Cry-09) and 100 year old museum DNA (*B. magnus turkestanicus*), with two miscoding lesions of type T → C, one marked by Y (mixed bases IUPAC code for T/C) in the ABI output file, and one miscoding lesion of type G → A marked by R (mixed bases IUPAC code for G/A) in the ABI output file.

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of unknown relationships from Asia were sequenced: a paratype specimen of *B. reinigi* Tkalcu (1974a, p. 322, collected VI, 1973) from the Nepalese Himalayas, *B. lucorum terrestricoloratus* Krüger (1951, p. 195; Nr. 0277.2 Vogt collection, Amsterdam) from North Tibet and *B. magnus turkestanicus* Krüger (1954, p. 274; Nr. 0281.885 Vogt collection, Amsterdam, collected IV, 1909) from Central Asia. As expected the DNA of all three specimens was degraded with miscoding lesions, which are quite obvious when inspecting the ABI traces. Figure 5 compares partial sequences from fresh (deep frozen) DNA with hundred year old DNA from a museum specimen. The overall quality of the museum DNA is quite good (shaded area = quality about 60 %) but three sites of this partial sequence are ambiguous, with double peaks: A first miscoding lesion (T → C, marked Y by the ABI output file), a second miscoding lesion (T → C, not recognised by the ABI output file) and a third miscoding lesion (G → A, marked R). A barcoding request identified all three specimens as part of the *cryptarum*-cluster (Fig. 4) and analysis of the diagnostic positions also revealed a close relationship with *B. cryptarum* (Table 5). None of the diagnostic positions characteristic for *B. lucorum* or *B. magnus* was found in these specimens.

Discussion

Facing the facts: morphology versus molecules

So far accurate identification of specimens of *B. magnus* and *B. cryptarum* by morphological characters is only possible with females. Besides the characteristic colouration and shape at the lateral ends of the collare (Bertsch et al. 2004) the main morphological characters used are (Rasmont 1984):

- *forms* (e. g. form of the labrum),
- *sculptures* (e. g. surface of tergite 2),
- *numbers* (e. g. of "micropunctures" in the lateral corner of the ocellar field),
- *measures* (e. g. length of malar space, diameter of ocelles),
- and *morphometric indices* (e. g. labral-index, ocellar-index).

However, the interspecific differences in all these characters are quite small, there is overlap and measuring length in three-dimensional space is not that simple. With much experience, it is possible to identify most females by a combination of these characters but as can be seen in most museum collections the number of misidentifications is substantial. I do not know of any attempt to extract all these morphological characters from a large number of unclassified specimens and to demonstrate that the result is not a continuum of characters, but character clusters separated by gaps. As it is quite simple to obtain large numbers of specimens from artificial colonies from a wide range of geographical provenances, it would be interesting to see which morphological character or combination of characters is best suited to identify specimens classified independently by male labial gland secretions or DNA sequences. Much work is waiting for the morphological taxonomists.

Empirical science relies on the ability to verify results independently in different laboratories. For identification of critical taxa and validation of morphological characters, this would imply that measurements (for instance of the *ocellar index = distance from right ocellus to preoccipital ridge / distance from ocellus to compound eye* measured by Løken 1973 to separate *B. lucorum* and *B. magnus*) can be repeated independently in the same specimens. However, Løken's measurements are available only in a complex diagram (Løken 1973, Fig. 53) and there is no reference to individual specimens. Whereas Løken (1973) came to the conclusion that *B. lucorum* and *B. magnus* can be separated by measurements of the *ocellar index*, a view confirmed by Tkalcu (1974), Pekkarinen (1979) came to the conclusion that the observed differences are caused by

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allometry and that species separation is not possible. This is a typical situation when dealing with morphological characters of specimens of the *lucorum*-complex, contradicting results and with no possibility of checking the original data. For these taxa Pierre Rasmont is most probably the only person who has the experience necessary to identify critical specimens.

Morphological characters can always be coded for cladistic investigations, trees can be constructed and homologies and possible character developments can be derived. With enough faculties of imagination or suitable mathematical models, any form can be changed into any other form and connected by intermediates. The question is whether such results are reliable. A good example for the problems involved in such approaches might be P. Williams' "reappraisal of morphology" (1985, 1994). A total of forty-four morphological characters (21 from the male genital capsule) of bumblebees were used and coded to construct a strict consensus tree. A minimally and a maximally resolved tree with all character state changes was given, and one of the results of this investigation was a close phylogenetic relationship between *B. (Rufipedobombus) rufipes*, *B. (Pressibombus) pressus, B. (Bombus) sporadicus* and *B. (Bombus) terrestris*. Figure 5 in Williams (1994) shows in detail and convincingly how the change in forms of male genitalia (penis valve) of these species may have occured. However, the results of recently published trees based on genetics (Cameron et al. 2007) contradict this reconstruction based on "morphological evidence," indicating that there is no close genetic relationship between the subgenus *Bombus* and *B. rufipes* (the *rufipes*-group is part of the subgenus *Melanobombus*) or *B. pressus* (which from genetic evidence is surprisingly part of the subgenus *Pyrobombus*). The nearest group to the species of the subgenus *Bombus* is the subgenus *Alpinobombus*. The theoretical considerations on why morphometric data and the concept of biological homologies are incompatible (Bookstein 1991, 1994) and why landmark data can be useful for delimitation and identification of taxa but are unsuitable to derive homologies and cladistically relevant trees should always be kept in mind.

Compared to morphological evidence the use of genetic evidence is relatively new, and what has been achieved in about 20 years is quite impressive. First, the sequence data are deposited in a public database, so the original data are available. Projects involving the long-term storage of well-documented DNA are underway (e. g. DNA Bank at the Zoologische Staatssammlungen München) and in the future it will be possible to extend and complement previous studies and to reinvestigate doubtful material. The need to make reference to specimens deposited in an accessible museum collection must be improved (Ruedas et al. 2000), and material preserved in alcohol can be restored such that it is useful for morphological inspection, including characters of colouration and hair (Milliron 1971, p. 29). Many reference sequences used by the Barcode identification engine are from GenBank sequences and difficulties with misidentifications within GenBank data are well known (e. g. Harris, 2003; Hebert et al., 2003; Seberg, 2004; Vilgalys, 2003), the possibilities to correct misidentified sequences should be improved. For the moment I prefer a simple BLAST (Basic Local Alignment Search Tool, Altschul et al. 1990) request because with GenBank data there is immediate access to all necessary information associated with the sequences (e. g. author, laboratory, publication, geographic provenance) whereas in the Barcode engine databank the original Genbank numbers have been changed and it is not straightforward to get this useful supplementary information. Thus the basis for every empirical science, that is, the ability to reproduce and check results independently, is guaranteed. Computer software for special purposes is increasing (e. g. *TaxonDNA*/*species_identifier* to identify taxa: Meier et al. 2006*; MOTU_define* to define molecular operational units: Blaxter et al. 2005; *bypassdegr* to check degraded DNA: Mateiu & Rannala 2008) and contrary to morphological taxonomy (somehow an endangered species) molecular taxonomy is exploding. In the future, it will be easier to check and correct morphological misidentifications by DNA methods than vice versa.

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There is a certain uneasiness in relying on data from only one gene, but even in a very conserved gene like COI a sequence of 1000 bp delivers enough genetic variability: In the European taxa of the subgenus *Bombus* s. str. each taxon delivers up to 12 unambiguous diagnostic positions, enough to guarantee accurate identification. And as COI is a coding gene without indels the alignment lacks gaps and inconsistencies, so all base substitutions at diagnostic positions can be used as homologies to reconstruct phylogenetic relationships. Instead of the ongoing discussion about molecules versus morphology, close cooperation using both methods could bring rapid progress in difficult and controversial cases.

Molecular taxon identification: tree-based versus character-based

Most recently published approaches using DNA data have utilized *distance measures* to make the inference regarding species designation. Distances are generally measured in two ways. The first is a simple Blast-based approach where a raw similarity score will determine the nearest neighbour to the query sequence. The second approach utilizes distances in tree building (Hebert et al. 2003). A major shortcoming of using distances in DNA data is that all classical studies and taxonomic schemes are *character*-based, making the union of classical and DNA data a difficult process. Character-based methods have the logical advantage that when diagnostic character data are lacking, they will fail, allowing at least some hypothesis testing, whereas similarity scores will always give a nearest neighbour. However, this nearest neighbour is sometimes not the nearest relative (Koski & Golding 2001). There is also a lack of an objective set of criteria to delineate taxa when using distances. A universal similarity cutoff to determine species status will simply not exist.

Like distance methods, each of the multitudes of available variations of phylogeny estimation via maximum likelihood relies on an explicit underlying model of character transformation. Because methods that rely on explicit, a priori models of evolution are acknowledged to be poor estimators of hierarchical patterns when the assumptions of the models are violated (Yang et al. 1994; Felsenstein 2004) a model has to be taken on empirical grounds. Different models frequently produce the same best-supported tree for the same data: the maximum-likelihood approach seems robust to violation of some assumptions. However, caution is needed because false or overly-simple models can be misleading about the reliability of the estimated tree, tending to suggest that the tree is significantly supported when, in fact, it is not.

A practical alternative is the exploration of character diagnostics in the DNA sequences themselves, without reference to trees. Thus morphological and molecular "characters" can easily be integrated and the procedures follow the two-step procedure of traditional taxonomic studies in which relationships among species are assessed only after the minimal biological units appropriately employed as terminal units are first identified by *diagnostic characters*. This approach, its relevance to diagnosing *entities in nature* and its relevance to species delimitation has been discussed at length both from the technical and theoretical standpoints (Davis & Nixon, 1992; Wheeler 2004; Will & Rubinoff 2004; Ebach & Holredge 2005; DeSalle et al. 2005).

Conclusions

Morphological, physiological, and molecular *operational taxonomical units* (OTUs) clearly separate the specimens of the *Bombus lucorum* complex into three clusters that correspond with the taxa defined as *B. lucorum*, *B. cryptarum* and *B. magnus*. The differences in morphological characters, the composition of the species recognition signals (male labial gland secretions) and genetic distance are consistent with other taxa of *Bombus* where the species status is not in debate. All three taxa are thus good morphological, biological, and phylogenetic species.

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It seems appropriate to first define terminal biological units as *entities in nature* and to use and discuss the *logical class* species in a second step. One hundred and fifty years ago, Ch. Darwin (1859) found a good formulation for such a two-step procedure: *The endless disputes whether or not some fifty species of British brambles are true species will cease. Systematists will have only to decide (not that this will be easy) whether any form be sufficiently constant and distinct from other forms, to be capable of definition; and if definable, whether the differences be sufficiently important to deserve a specific name*.

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