Journal of Insect Conservation (2005) 9:75–83
DOI 10.1007/s10841-004-4168-0
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Delineating species for conservation using mitochondrial sequence data: the taxonomic status of two problematic *Bombus* species (Hymenoptera: Apidae)

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Received 12 July 2004; accepted in revised form 3 October 2004

Key words: Bombus hortorum, *Bombus ruderatus*, bumblebees, conservation genetics, mtDNA sequence (cytochrome oxidase II, cytochrome *b*)

Abstract

Across Western Europe and North America, many bumblebee species are currently undergoing drastic declines in their abundance and ranges, primarily as a result of habitat fragmentation. In contrast, a smaller number of species are seemingly unaffected by this and remain common. The UK Biodiversity Action Plan-designated *Bombus ruderatus* belongs to the former group while *B. hortorum* belongs to the latter. These two species are sympatric and remarkably similar in morphology. There are no diagnostic characters for workers and male genitalia are illustrated with the same diagram in standard keys. Isolated records of putative *B. ruderatus* occur amongst a mass of records for *B. hortorum*. This raises two important issues: first, are *B. ruderatus* and *B. hortorum* 'good' species? Second, if they are, can the uncertainty over their identification be resolved? We present COII and cytochrome *b* mtDNA sequence data from these and other *Bombus* species. Molecular data and coat colour characters are in concordance and confirm that *B. ruderatus* and *B. hortorum* should be regarded as separate species (although coat colour alone is an unreliable diagnostic character for many individuals). Confirmation of the specific status of *B. ruderatus* allows the work on the conservation of this species to continue.

Introduction

Bumblebees are highly valued as important pollinators of crops and wildflowers (Corbet et al. 1991; Fussell and Corbet 1992, 1993). Currently many species in the UK, Europe, North and Central America and Japan are declining in range and abundance (Williams 1982, 1986; Rasmont 1995; Koisor 1995; Batra 1995). Of twenty-six species on the British list (including *Bombus hypnorum* (Linnaeus), a recently established species from continental Europe) three are now extinct. At least ten of the remaining twenty-three are in decline while six remain common and ubiquitous (Williams 1982). To understand the ecological processes underlying these trends, knowledge of current diversity and distributions of bumblebee fauna is important.

Bombus ruderatus (Fabricius) is one of Britain's rarest species: it is regarded as one of the southern local species by Williams (1982) and is designated on the UK Biodiversity Action Plan (UKBAP). *Bombus hortorum* (Linnaeus) is ubiquitous in distribution in the UK (Alford 1980) and is

apparently not undergoing declines in range and abundance (Williams 1982). Globally, *B. hortorum* is more widely distributed across the Palaearctic region than *B. ruderatus*, which is generally confined to Europe and North Africa (Williams 1998). Although these species have been regarded as separate by bumblebee taxonomists since 1900 (Williams and Hernandez 2000) they are remarkably similar in morphology. According to the standard key (Prys-Jones and Corbet 1991), in some cases it is not possible to distinguish whether an individual worker belongs to one or the other species; even male genitalia of both species are illustrated with the same diagram [male genitalia are perhaps the most reliable morphological character used in cladistic analysis in bumblebees (Williams 1994)]. Although some distinctive morphological characters based on the ratio of length of the malar space and head breadth have now been identified in queens (Williams and Hernandez 2000), these do not separate workers, and due to the scarcity of *B. ruderatus*, queens are rarely seen. Isolated records of *B. ruderatus* occur among many for *B. hortorum*; there is confusion over the naming of specimens and field studies of *B. ruderatus* by the bumblebee working group in the UK have been abandoned (Edwards 2002). This raises two issues: first, is the apparent lack of well defined populations of *B. ruderatus* solely because we cannot reliably distinguish this species from *B. hortorum*, or is the specific status of these species doubtful? Second, if they are separate species, is it possible to ascertain any degree of certainty over the identification of workers to enable autecological studies to continue? Here we attempt to clarify these issues with the aid of mitochondrial sequence data.

Materials and methods

Sampling and identification

Samples of *B. ruderatus* and *B. hortorum* were collected from various sites in New Zealand's South Island during January 2003 (four species of bumblebee (*B. hortorum*, *B. ruderatus*, *B. terrestris* and *B. subterraneus*) were introduced to New Zealand from the UK in 1895 and 1906 (Goulson 2003)) and from Cambridgeshire and Norfolk in the UK during July 2003. Further samples were kindly donated by C. Carvell (Cambridgeshire, summer 2002) and T. Benton (Essex, summer 2003). Individuals were killed either by immersion in 70% ethanol or by freezing on return from the field. A total of 74 bumblebees were collected. British specimens are currently lodged as vouchers in the private collection of M. Edwards.

Individuals were independently examined and assigned to either *B. ruderatus* or *B. hortorum* by seven people experienced in bumblebee identification (T. Benton, G. Else, M. Edwards, D. Goulson, B. Darvill, J. Ellis and P. Williams). As there are no reliable characters for workers of *B. ruderatus* and *B. hortorum*, individuals were separated according to the following criteria, all aspects of coat colour. Various combinations of these criteria have long been in use for separating *B. hortorum* and *B. ruderatus*, but it is not known which characters correspond best with the species boundary:

(1) The proportional width of the yellow band on the scutellum relative to the width of the yellow band on the pronotum. In *B. ruderatus* these bands are thought to remain relatively equal in width, regardless of the extent of black hairs on the centre of the thorax. In *B. hortorum* the band on the scutellum is said to be slightly narrower than the band on the pronotum.
(2) The extent of yellow hair present on the abdomen. In *B. ruderatus* the yellow hairs are generally present on the first tergite only. Often the extent of yellow hairs is greater at the sides of the tergite giving this yellow band a concave appearance. In *B. hortorum* the yellow hairs may extend onto the second tergite and the band is generally broader.
(3) Coat appearance: *B. ruderatus* is of a more neat appearance as opposed to *B. hortorum* which is often described as looking 'scruffy'. The yellow bands are supposedly of a slightly darker hue in the former species.

It must be emphasized that these differences are not obvious, and that many intermediates occur. There was disagreement over the identity of twenty-four of the seventy-four individuals collected from the UK, identified by the seven people named above. Old, depilated individuals and entirely black individuals occur and are particularly difficult to separate [Alford (1975) states that dark individuals are of both species].

DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from nine individuals of each proposed species either from wing muscle removed from the thorax or from an homogenized leg. The individuals that were chosen were those on which there was unanimous agreement as to their identity based on morphological characters, or were queens (bumblebees collected from New Zealand). Legs were homogenized in 600 μl SE buffer using a 1.5 ml polypropylene pellet pestle with microtube (Anachem, UK). DNA was extracted from legs or muscle tissue by a standard salt/chloroform extraction (Rico et al. 1992).

Partial regions of the COII and cytochrome *b* genes were sequenced. Although mitochondrial genes are encoded as a single linkage group and are hence not truly independent of one another (Moore 1995) it is useful to sequence more than one because different regions of mtDNA may have different mutation rates (e.g. Koulianos and Schmid-Hempel 2000) and patterns of substitution (e.g. transition/transversion ratios, distribution of non-synonymous distributions, Koulianos and Crozier 1999).

Individual PCRs were carried out in 30 μl volumes containing: 3 μl template DNA, 0.8 units of *Taq* (0.16 μl), 1.2 μl 25 mM $MgCl_2$, 3 μl 10x PCR buffer (including 15 mM $MgCl_2$), 0.6 μl dNTPS (10 mM each) 0.26 μl of each 25 mM primer and 215.2 μl H_2O. The PCR cycle was as follows: 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, with a final 10-min extension period at 72 °C. Primers are illustrated in Table 1. PCR products (total volume) were run out on a 3% agarose gel at 60 V for 120 min. The correct fragment (~500 bp for cytochrome *b* and ~900 bp for COII) was excised and purified using a QIAquick gel extraction kit (Qiagen, UK). Elution buffer was diluted 1:10 with HPLC grade water. The volume of the eluate was then reduced to 11 μl in a heat block at 70 °C in order to concentrate DNA for the sequencing reaction. For the sequencing reaction primers were diluted to 2.84 mM. Reaction volumes included 4 μl Big Dye (Big Dye® Terminator v1.1 cycle sequencing kit, Applied Biosystems), 5.5 μl purified DNA and 0.5 μl forward or reverse primer. The reaction was as follows: 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Products were sequenced on an ABI 377 sequencer.

Analysis

Cytochrome *b* and COII sequences were edited and aligned using BioEdit version 5.0.9 and Clustal W 1.8 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Cytochrome *b* sequences of *B. ruderatus* and *B. hortorum* were aligned with published sequences for *Apis mellifera* (Crozier and Crozier 1993, GenBank accession number NC001566), *B. terrestris* (Linnaeus) (Koulianos et al. 1999, AF002721), *B. lucorum* (Linnaeus) (Koulianos et al. 1999, AF002722), *B. humilis* (Illiger) (Pirounakis et al. 1998, AF017517), *B. ruderarius* (Mueller) (Koulianos et al. 1999, AF002723) and *B. pascuorum* (Widmer and Schmid-Hempel 1999, AF081806). COII *B. ruderatus* and *B. hortorum* sequences were aligned with published sequences of *B. terrestris* (four haplotypes, accession numbers X90404-X90407 inclusive), *B. lucorum* (X90409) and *B. canariensis* (X90408) (all Estoup et al. 1996).

Table 1. Primers used to amplify the mitochondrial regions of interest (COII from Estoup et al. 1996)

Region	Primer sequence	Direction	T_m
COII	ATACCACGACGTTATTCAGA	Forward	47 °C
	GTTCATGAATGAATTACATCTG	Reverse	
	AATTCTGGATATTCATAACATC	Reverse	
CYTB	AGGATATGTACTACCATGAGGAC*	Forward	48 °C/ 50 °C
	CTAATCCGATTACACCTCCTC*	Reverse	
	TTCAGCAATTCCATATATTGGAC	Forward	
	ATTACACCTCCTCATTTATTAGG	Reverse	

The forward and reverse primers marked with an * only amplified individuals of *B. ruderatus*. The T_m for cytochrome *b* was 48 °C for *B. ruderatus* and 50 °C for *B.hortorum*.

MEGA version 2.1 (Kumar et al. 2001) and DnaSP version 4.00 (Rozas et al. 2003) were used for further analysis of a 247 bp alignment of COII and 264 bp alignment of cytochrome *b* for the species listed above (www.megasoftware.net and ww.ub.es/dnasp, respectively). Sequence divergence and transition/transervion ratios were calculated using the Tamura-Nei model (1993). This model accounts for inequality of nucleotide frequencies, difference between transition and transversion ratios, difference between purine and pyrimidine transitions and substitutional rate differences between nucleotides.

Results

Two groups of nine individual bumblebees each were separated from the sequence data. These matched exactly the groups of individuals separated into proposed *B. ruderatus* and *B. hortorum* from the morphological criteria outlined above.

Sequence data of COII and cytochrome b in B. ruderatus *and* B. hortorum

A 290-bp partial sequence of the COII region and a 273-bp partial cytochrome *b* sequence were obtained for *B. hortorum* and *B. ruderatus* with no gaps or missing values. Two cytochrome *b* haplotypes were found in *B. ruderatus* (differing by a single synonymous transition from T to C in the third base of the codon). Otherwise there was one haplotype found per species. Translation of the sequences obtained using the reading frame of *Apis mellifera* (Crozier and Crozier 1993) yielded no stop codons. Sequences can be obtained from GenBank under the accession numbers AY639371–AY639375 inclusive.

Comparison between all species was made using a 247-bp COII alignment and a 264-bp cytochrome *b* alignment. Base composition was strongly AT biased in both *B. ruderatus* and *B. hortorum* (81.8–86.7%, Table 2) as previously observed in mitochondrial sequences of *Apis mellifera* (Crozier and Crozier 1993) and other insects (Kambhampati and Charlton 1999; Linton et al. 2002). AT bias was particularly strong in the third codon (92.0–97.5%, Table 2). Transitions/transversion ratios were 2.016 (COII) and 1.467 (cytochrome *b*).

Sequence divergence between Bombus ruderatus *and* Bombus hortorum

At both the mitochondrial regions sequenced, *B. hortorum* individuals from the UK and New Zealand were identical. This was also the case in *B. ruderatus*, with the exception of a single nucleotide substitution making up two haplotypes from the UK. These two haplotypes came from two individuals collected within the same kilometre square in Cambridgeshire.

Between *B. hortorum* and *B. ruderatus* there are several nucleotide substitutions in both COII and cytochrome *b* sequences. Observed sequence divergence was 6.2 and 9.2%, respectively (Tamura-Nei 1993, Table 3, Figures 1 and 2). Of the observed sequence differences between these two species, 42.9% of substitutions were non-synonymous in the COII region (cf. 25–23.1% of substitutions between the four haplotypes of *B. terrestris* and *B. lucorum*) and 32.2 and 33.3% in the cytochrome *b* region (between *B. hortorum and*

Table 2. AT bias (%) in *B. ruderatus* and *B. hortorum* at each codon position and in total for CO II and cytochrome *b*.

	A			T			A + T total
	1	2	3	1	2	3	
COII							
ruderatus	51.8	24.4	45.1	36.1	51.2	50.0	86.2
hortorum	50.6	23.2	46.3	38.6	50.0	51.2	86.7
CYTB							
rud hap1	42.0	27.3	42.0	34.1	51.1	52.3	82.9
rud hap2	42.0	27.3	42.0	34.1	51.1	51.1	82.6
hortorum	43.2	28.4	42.0	31.8	50.0	50.0	81.8

Table 3. Percentage sequence divergence (Tamura-Nei 1993) in a range of *Bombus* species pairs at COII and cytochrome *b*.

Species pair	Length (bp)	Tamura-Nei distance (%)
COII		
ruderatus and hortorum	247	6.2
terrestris hap1 and lucorum	247	5.2
terrestris hap2 and lucorum	247	4.7
terrestris hap3 and lucorum	247	5.6
terrestris hap4 and lucorum	247	5.6
canariensis and lucorum	247	5.6
CYTB		
ruderatus hap1 and hortorum	264	9.2
ruderatus hap2 and hortorum	264	8.7
terrestris and lucorum	264	5.2
pascuorum and humilis	264	6.2
pascuorum and ruderarius	264	7.1
humilis and ruderarius	264	2.4

B. ruderatus haplotype1 and 2 respectively, cf. 27.7–40.0% in the other *Bombus* species pairs examined at this region).

Discussion

Specific status of B. ruderatus *and* B. hortorum

Sequence divergence between *B. ruderatus* and *B. hortorum* was found to be 6.2% (COII) and 9.2% (cytochrome *b*). These values are both greater than those found between *B. terrestris* and *B. lucorum* (sub-genus *Bombus s.s.*) and between *B. pascuorum, B. humilis and B. muscorum* (sub-genus *Thoracobombus*) which are all well-recognized distinct species (Table 3). Differences were consistent within morphologically separated groups between a range of individuals from both the UK and New Zealand. We therefore confirm that *B. ruderatus* and *B. hortorum* are distinct species. Further morphological evidence based on the ratio of malar space length and head breadth of queens supports the specific status of these species (Williams and Hernandez 2000).

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
hortorum         AAAAATTTCT ACATGAAATA TATTAACATT TCAAGACTCA AATTCATTTT
ruderatus        AAAAATTTCT ACATGAAATA TATTAACATT TCAAGATTCA AATTCATTTT
Consensus        ********** ********** ********** ****** *** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
hortorum         ACTCTGATAA TTTAATTTCA TTTCATAATT TAACTATAAT AATAATAACA
ruderatus        ATTCTGATAA TTTAATTTCA TTTCACAACT TAACTATAAT AATAATAATA
Consensus        * ******** ********** ***** ** * ********** ******** *

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
hortorum         ATAATTATTA CTTTAACAAC ATTTTTTATT TTTGATTTTT ATTCAAATAA
ruderatus        ATAATTATTA CCTTAACAAC ATTTTTTATT TTAGATTTTT ATATAAATAA
Consensus        ********** * ******** ********** ** ******* **  ******

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         160        170        180        190        200
hortorum         ATTTTTAAAT TTAACTTTTT TAAAAAATCA TACAATTGAA ATTATTTGAA
ruderatus        TTATTTAAAT TTAACTTTTT TAAAAAACCA TACAATTGAA ATTATTTGAA
Consensus         * ******* ********** ******* ** ********** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
hortorum         CTTTAATTCC AATAATTATT TTAATAATTA TTTGTTTTCC ATCATTAAAA
ruderatus        CTTTAATTCC TATAATTATT TTAATAATTA TTTGTTTTCC ATCACTAAAA
Consensus        **********  ********* ********** ********** **** *****

                 ....|....| ....|....| ....|....| ....|....|
                         260        270        280        290
hortorum         ATTCTATATT ATATTGATGA AATTATAAAT CCTTATTTCT
ruderatus        ATTTTATATT ACATTGATGA AATTATAAAT CCTTATTTTT
Consensus        *** ****** * ******** ********** ******** *
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Figure 1. Clustal W 1.8 alignment of *B. hortorum* and *B. ruderatus* nucleotide sequences for a partial region of COII, with the consensus sequence.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
hortorum        ATACATTAAA TCGATTTTAT TCATTTCATT TTTTATTACC ATTTATTATT
ruderatus1      ATACATTAAA TCGATTCTAT TCATTTCATT TTTTATTACC ATTTATTATC
ruderatus2      ATACATTAAA TCGATTCTAT TCATTTCATT TTTTATTACC ATTTATTATC
Consensus       ********** ****** *** ********** ********** *********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
hortorum        TTATTAATAG TATATATACA TTTAATAATT TTACATATTA CTGGATCATC
ruderatus1      ATATTTATAG TATTTATACA TTTAATAATT TTACATATTA CCGGATCATC
ruderatus2      ATATTTATAG TATTTATACA TTTAATAATT TTACATATTA CCGGATCATC
Consensus        **** **** *** ****** ********** ********** * ********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
hortorum        AAATCCAATA CATTCAAAAA TTAATATTTA CAAAATTAAT TTTCATCCAT
ruderatus1      TAATCCAATT CATTCAAAAA TTAATATTTA TAAAATTAAT TTTCACCCAT
ruderatus2      TAATCCAATT CATTCAAAAA TTAATATTTA TAAAATTAAT TTTCACCCAT
Consensus        ********  ********** **********  ********* ***** ****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
hortorum        ATTTCACTAT TAAAGACCTA ATCACAATGA TTATTACATT TTTTATTTTT
ruderatus1      ATTTTACAAT TAAAGATTTA ATCACATTAA TTTTAACATT TTTCATTTTT
ruderatus2      ATTTTACAAT TAAAGATTTA ATTACATTAA TTTTAACATT TTTCATTTTT
Consensus       **** ** ** ******  ** ** *** * * ** * ***** *** ******

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
hortorum        ATAATTATTA ATCTTCAATT TCCATATATA TTAGGTGACC CTGATAACTT
ruderatus1      ATAATTATTA ATCTTCAATT TCCATATATA TTAGGTGATC CTGATAATTT
ruderatus2      ATAATTATTA ATCTTCAATT TCCATATATA TTAGGTGATC CTGATAATTT
Consensus       ********** ********** ********** ******** * ******* **

                ....|....| ....|....| ...
                       260        270
hortorum        TAAAATAGCA AATCCAATAA TTA
ruderatus1      TAAAATAGCA AATCCAATAA TTA
ruderatus2      TAAAATAGCA AATCCAATAA TTA
Consensus       ********** ********** ***
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Figure 2. Clustal W 1.8 alignment of *B. hortorum* and *B. ruderatus* nucleotide sequences for a partial region of cytochrome *b*, with the consensus sequence.

Confirmation of the specific status of *Bombus ruderatus* and *Bombus hortorum* allows ecological research on these species to continue. Comparative studies of closely related species such as these are important to aid our understanding of the processes behind the current pattern of declines in range and abundance of many *Bombus* species, but not others. Presently this trend is not well understood (Goulson et al. 2005).

Species concepts

Some authors strongly criticize studies of biodiversity that separate species on lineage-based species concepts, but do not present hypothetical criteria against which the species concepts/diagnosis can be empirically tested (Sites and Crandall 1997). We argue that the consistent genetic differences found in coding regions of the genome between sympatric populations of these bumblebees provide strong support for the separate specific status of *B. ruderatus* and *B. hortorum*. Such differences support species status under both the phylogenetic species concept (Cracraft 1983) and the biological species concept (Mayr 1963).

Using mitochondrial DNA for taxonomic delimitation

There are several potential pitfalls in delimiting species and inferring phylogeny from mtDNA data. Mitochondrial DNA is haploid and maternally

inherited thus the effective population size (Ne) is four times smaller than for diploid nuclear genes (Ballard and Whitlock 2004; see also Avise et al. 1987; Moritz et al. 1987). This results in mitochondrial haplotypes becoming exclusive more rapidly than nuclear genes (Moore 1995; Wiens and Penkrot 2002; Ballard and Whitlock 2004). While this has an advantage in that mtDNA resolves shallow-level phylogenies that nuclear genes often cannot (Wiens and Penkrot 2002; Ballard and Whitlock 2004), it can also be disadvantageous because temporarily isolated populations can be mistakenly accorded specific status (Sites and Crandall 1997). We suggest that the extent of the observed sequence differences found here along with the sympatric distribution of the collected individuals renders this an unlikely explanation of our results.

Introgressive hybridization events can create mtDNA haplotype distributions that confuse species boundaries (see Ballard and Whitlock 2004; Seehausen 2004). Such events can bring about scenarios with a bias in favour of species delimitation or a bias against it (e.g Bernatchez et al. 1995; Thelwell et al. 2000; Sota 2002; Testa et al. 2002; Pesson et al. 2004). In the latter case populations would share mtDNA haplotypes, but nuclear alleles would prove diagnostic. This is clearly not the case here since haplotypes were not shared. Alternatively, introgression occurring between one allopatric sub-population and a second species can lead to patterns of mtDNA haplotype diversity that erroneously imply species status (e.g. Bernatchez et al. 1995). In such a case analysis of mtDNA haplotypes alone would lead to the false conclusion that the allopatric sub-population was a sub-population of the donor species. While complete replacement of mtDNA is rare (Bernatchez et al. 1995) mitochondrial introgressions are not uncommon (again for example see Bernatchez et al. 1995; Thelwell et al. 2000; Sota 2002; Testa et al. 2002; Pesson et al. 2004). We do not believe this to be the case between *B. ruderatus* and *B. hortorum* . Current distributions are sympatric and if these were truly just reunited allopatric populations then we would not expect any extant structure in mtDNA haplotypes. In this study we found direct agreement between morphology and mtDNA data (see Sites and Crandall 1997; Puorto et al. 2001; Wiens and Penkrot 2002). Current male-mediated gene flow between *B. ruderatus* and *B. hortorum* would also remain undetected from analysis of mtDNA data alone (Avise 1994; Wiens and Penkrot 2002). Again, if this were occurring here we would not expect to see consistency between mtDNA haplotypes and morphology.

In summary, the sequence divergence observed in this study at two regions of the mitochondrial genome is in concordance with morphological variation and favours the recognition of *B. ruderatus* and *B. hortorum* as separate species. Studies of the natural history of *B. ruderatus*, one of Britain's rarest bumblebee species, can resume.

Acknowledgements

For contribution and collection of specimens we wish to thank Claire Carvell, Ted Benton, Bryan Pinchen, Bryan Banks and Mike Edwards; for identification of *B. ruderatus* and *B. hortorum*, Mike Edwards, George Else, Ted Benton, Paul Williams and Ben Darvill. Additionally, the wardens of the RSPB reserves at Whittlesey and Manea for allowing access to their reserves. We extend particular thanks to Mike Edwards, head of the Bumblebee Working Group. Thanks also to Prof Norman Maclean and Dr M. Dixon for use of their laboratories. Thanks to Paul Williams for comments on the manuscript. This work was funded by NERC as part of a PhD study of population genetics of rare bumblebees based at the University of Southampton.

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