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Niche differentiation of a cryptic bumblebee complex in the Western Isles of Scotland

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Abstract. 1. It has recently become apparent that there is cryptic diversity in bumblebees, notably in the *Bombus lucorum* complex which appears to contain three distinct taxa (*B. lucorum, B. magnus,* and *B. cryptarum*). We know almost nothing about how these species differ in their ecology or distribution.

2. Here, we use RFLP markers to identify workers of the *B. lucorum* complex from the west of Scotland, and we map the distributions, forage use and habitat associations of the three taxa.

3. In western Scotland, *B. cryptarum* was found to be the most abundant of the three related taxa, but all three occurred in almost all sample sites. In combination with similar work from Ireland, we are able to conclude that: *B. cryptarum* is a polylectic species associated with uplands and cool climates; *B. lucorum* appears to be a lowland bee particularly associated with urban areas and islands close to the mainland in Scotland, and feeding largely on *Erica cinerea* and Apiaceae; *B. magnus* appears to be a heathland bee strongly associated with feeding on *Calluna vulgaris*.

4. Our study demonstrates that a combination of molecular and ecological approaches can reveal aspects of the ecology of cryptic species.

Key words. Biogeography*, Bombus lucorum*, coexistence, forage use, RFLP.

Introduction

A feature of bumblebees (genus *Bombus*) is their tendency to converge on the same colour pattern in sympatry through Müllerian mimicry (Williams, 2007). Some species also exhibit considerable intra-specific variation in colour pattern, both within and between populations (Williams, 2007). Together these features can make bumblebee identification difficult or impossible in the field, and for some taxa, definitive identification remains problematic even when voucher specimens are available. This greatly hampers field studies in ecology or behaviour; for example workers of *B. terrestris* and *B. lucorum* have been grouped together in many published studies, obscuring differences between the two species (e.g. Goulson & Darvill, 2004; Goulson *et al*., 2005). Numerous studies of bumblebee ecology have been carried out in the UK, many of them including data on *B. lucorum*, and it now seems probable that much of this data actually refers to a complex of three cryptic species, *B. lucorum, B. magnus,* and *B. cryptarum* (Bertsch *et al*., 2005; Murray *et al*., 2008), one of which (*B. cryptarum*) had never been suspected as occurring in the UK before 2005. Our inability to reliably identify such species means that their ecological attributes are poorly known; the descriptions of their distribution, behaviour, forage use, nest site choice etc, to be found in standard texts are actually descriptions of pooled data for multiple species, and hence of limited value. We might expect niche-partitioning between closely-related species (e.g. Goulson *et al*., 2008a), but this is difficult to study when the bees cannot be separated.

The subgenus *Bombus sensu stricto* contains five species in Europe, *B. (Bombus) terrestris* (Linnaeus), *B. (B.) sporadicus* (Nylander), *B. (B.) lucorum* (Linnaeus), *B. (B.) magnus* (Vogt), and *B. (B.) cryptarum* (Fabricius). Some authorities regard the latter two as subspecies of *B. lucorum* and they are often referred to as the *B. lucorum* complex or simply synonymized to *B. lucorum* (Edwards & Jenner, 2005; Benton, 2006). In the field, morphological characters do not allow reliable identification of queens while workers and males are widely regarded as morphologically indistinguishable; even the male genitalia, which provide valuable distinguishing characters between most bumblebee species, do not differ (Alford, 1975; Rasmont *et al*., 1986). Morphological differences between queens have been reported

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(Fig. 1). However, Williams (2000) showed that there was no clear distinction between specimens that researchers had identified as *B. magnus* and *B. lucorum*. The extent of the yellow collar, the main morphological difference between *B. lucorum* and *B. magnus*, exhibits continuous variation. The aptly named *B. cryptarum* was not considered to be present in the British Isles until Bertsch *et al.* (2005) described it on the basis of both male pheromone composition and mitochondrial gene sequences, which suggest clear distinctions between *B. lucorum, B. cryptarum,* and *B. magnus*. Queens of *B. cryptarum* can apparently be identified by the presence of a curved black line crossing the collar (Fig. 1), but the reliability of this character is unknown.

Sequencing of the relatively conserved mitochondrial gene cytochrome oxidase-1 (mtCO-1) has emerged as a reliable method of species identification within the *B. lucorum* complex. Several studies have used direct sequencing of a 700–1056 bp section of this gene to determine phlyogenetic relationships within the group and as a means of identifying specimens for subsequent analysis (Pedersen, 2002; Bertsch *et al*., 2005; Cameron *et al*., 2007; Murray *et al*., 2008). However, sequencing is expensive and slow for large numbers of samples. To provide a quicker and cheaper alternative, Murray *et al.* (2008) identified sites within the gene sequence that were unique to each of the three species and then matched these to restriction enzymes that were able to distinguish between them. By using PCR to amplify a 700 bp section of the mtCO-1 gene and then digesting the PCR product using the discriminatory restriction enzymes *Eco*NI and *Hinf*I, different patterns of DNA fragment length (restriction fragment length polymorphism, RFLP), could be used to reliably distinguish between species.

Ecological differences have been suggested between the three species. In Britain, *B. magnus* is considered a species of upland moors to the North and West (Alford, 1975; Prys- Jones & Corbet, 1991; Goulson *et al*., 2006). In Germany however it is considered a lowland species (von Hagen & Aichhorn, 2003). *B. lucorum* and *B. cryptarum* are thought to be widely distributed on mainland Europe with *B. cryptarum* considered to have an earlier phenology, with queens emerging in the spring before *B. lucorum* and *B. magnus* (Bertsch *et al*., 2005). The reliability of these ecological observations is debatable due to confusion about the taxonomic status and morphological differences between the species. The only clear insight we have into the ecology of these three species comes from Murray *et al.* (2008) who used RFLP to identify 391 *B. lucorum* complex bees from across Ireland. *B. lucorum* was the most abundant and widespread, comprising 56% of specimens. Contrary to the claims of earlier studies such as Alford (1975), *B. cryptarum* rather than *B. magnus* was associated with upland sites, while *B. magnus* was found to avoid urban areas. Clearly, there is much that has yet to be discovered about the ecology of these three widespread bumblebee species.

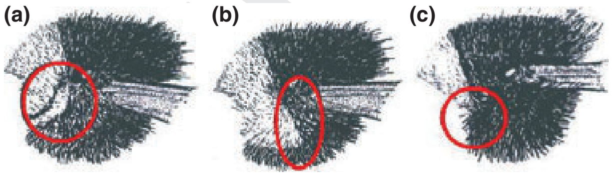

Fig. 1. Morphological characteristics for the separation of queens of *the B. lucorum* complex. The yellow collar of *B. cryptarum* (a) and *B. magnus* (b) extends to a point further below the wing than that of *B. lucorum* (c), while *B. cryptarum* is recognised by a thin 'S' shaped line of black hairs through the yellow collar (Alford, 1975; Macdonald, 1999).

Here, we collect data on the forage use and habitat associations of the *B. lucorum* complex from islands off the west coast of Scotland plus a small mainland sample. This region has been the focus of much recent research on bumblebees, in part because a number of rare species are present and also because this island system has become a useful model for examining patterns of gene flow and dispersal in bumblebees (Darvill *et al*., 2006; in press). We use Murray *et al*.'s (2008) RFLP method to identify our bee samples. Finally, we examine whether the extent of the anterior thoracic yellow collar (the only known variable morphological character for workers) is of value in distinguishing between these species.

Methods

All samples were collected during July and August in the summers of 2003, 2004, and 2005 by Darvill, Goulson or Waters. Worker bees only were sampled from 12 islands off the West coast of Scotland as well as from mainland Scotland (Table 1). Bees were caught from one or more specific locations on each island, depending on availability of bees to sample. Locations were separated by at least one kilometre. No more than 25 bees were sampled from any single location per year. This prevented the over-representation of bees from single locations and reduced the risk of large numbers of sisters within samples. If a bee was caught while foraging on a flower, the identity of the forage plant was recorded. The habitat within which the bee was caught was also noted, using the following crude classification:

Table 1. Sample sizes from 12 islands and one mainland sample, identified to species using RFLP markers.

	lucorum	*magnus*	*cryptarum*	Total
Arran	9	3	4	16
Benbecula	1	3	21	25
Canna	8	0	0	8
Coll	1	34	36	71
Colonsay	1	9	5	15
Gigha	14	9	13	36
Mainland	9	0	4	13
Muck	0	6	0	6
Mull	12	23	35	70
N. Uist	1	10	30	41
S. Uist	0	4	49	53
Skye	76	4	16	96
Tiree	1	66	16	83
Total	133	171	229	533

heathland; woodland; gardens; lowland meadows (including machair). Variation in the ventral extent of the yellow collar was noted amongst the 223 workers collected by Waters, and was used to nominally separate worker bees into *B. lucorum* and *B. magnus* (the extent of the yellow collar in *B. cryptarum* is unknown). A tarsal sample was then taken from each bee following Holehouse *et al.* (2003). A small number of individuals were retained whole as voucher specimens. Tarsi and whole bees were stored in absolute ethanol at ambient temperatures.

Restriction fragment length polymorphism (RFLP)

RFLP was used to identify all *Bombus lucorum* complex specimens using the protocol developed by Murray *et al.* (2008), who provide full details of primers and reaction conditions. Species identity was determined by comparing the pattern of digestion fragments for each individual with the characteristic patterns associated with *B. lucorum, B. cryptarum,* and *B. magnus* (see Fig. 3 in Murray *et al.*, 2008). When no clear banding pattern was evident, bees were discarded from further analysis.

Once bees had been identified, differences in the habitat use and forage use of the three bee species were examined using chi-squared tests of association upon data pooled for all sites, excluding habitats within which or plant species upon which fewer than 15 bees were recorded.

Analyses

To compare the diet breadth of the species recorded, a Simpson's index was calculated for the diversity of flowers visited by each caste (Simpson, 1949):

$$D_s = \sum_{i=1}^{s} \frac{(n_i(n_i - 1))}{(N_i(N - 1))}$$

where n_i is the number of flowers of the *i*th plant species that were visited, N is the total number of flowers visited, and *s* is the total number of flower species visited. As is usual, results are presented as 1/D, so that larger values indicate higher diversity. This index is insensitive to sample size (Magurran 1988) which is important because samples are inevitably larger for the more common species. Only species for which there were at least 10 records of either pollen or nectar collection per caste were included.

The use of Simpson's index for measuring diet breadth has been criticized; Williams (2005) argues that rarefaction is preferable to take into account varying sample sizes across bee species. Hence, we also calculated diet breadth for each bee species using rarefaction, randomly sub-sampling 10 visits from those recorded, without replacement, and repeating the procedure 100 times. This provides an estimate of the mean number of plant species each bee species would be expected to visit in a total of 10 flower visits.

The proportion of visits by bees of each species to each plant family was examined using principal components analysis in SPSS 11.0. Data on the forage use of the other bumblebee species present on these islands were collected concurrently with this study, and have been published elsewhere (Goulson *et al.*, 2005). To allow the forage use of *B. magnus*, *B. cryptarum,* and *B. lucorum* to be placed in a broader context, these data are included in the principal components analysis. We have previously applied this method to similar data sets for bumblebee communities elsewhere in the UK and Poland, allowing comparisons across studies (Goulson & Darvill, 2004; Goulson *et al.*, 2008a).

Results

Geographic distributions

Of the 533 bee samples identified using the RFLP method, 43.8% were identified as *B. cryptarum*, 32.1% were *B. magnus* and 30.0% were *B. lucorum* (Table 1). The three species exhibit marked differences in their distributions (Fig. 2). *B. lucorum* was found primarily on islands close to the mainland (Arran, Canna, Gigha, Mull, and Skye) and on the mainland itself (Table 1), and hence exhibits a strong easterly bias. *B. cryptarum* tended to be abundant everywhere but was most common in the west, comprising 84.0% of the bees caught on the Outer Hebridean island chain (North Uist, Benbecula, and South Uist). *B. magnus* was the least abundant of the three, but was the dominant bee species on the neighbouring islands of Coll and Tiree. With the exceptions of islands for which only small sample sizes were obtained, all three species were detected on all islands.

Habitat and forage use

Marked differences are evident in the distribution of the three taxa among habitats (Fig. 3) ($\chi^2_4 = 200$, $P < 0.001$). Of the three species, *B. magnus* appears to have the most marked habitat specialisation, occurring almost exclusively on heathland. It should be noted that *B. magnus* was most abundant on the islands of Coll and Tiree, both of which contain extensive areas of flower-rich lowland meadows and some gardens, therefore this cannot simply be an artefact of the geographic distribution of the species. *B. cryptarum* was frequent in gardens and heathland. *B. lucorum* was the only species to be common in lowland meadows, although it also occurred frequently on heathland.

Forage use also differed markedly between the three species (Table 2) ($\chi^2_{10} = 232$, $P < 0.001$). In accordance with its association with heathland, *B. magnus* foraged almost exclusively on Ericaceae, predominantly on *Calluna vulgaris* and had the lowest diet breadth of the three species (Simpson's 1/D = 1.73). In contrast, *B. lucorum* was also commonly observed visiting Ericaceae but was largely recorded on the larger-flowered *Erica cinerea,* and had a broader diet breadth than *B. magnus* (Simpson's 1/D = 3.55). It was also the only species to make frequent use of *Angelica* spp., itself notable because Apiaceae are not generally considered to be favoured forage sources for bumblebees (see Goulson *et al.*, 2005). *B. cryptarum* appeared to be the most polylectic of the three species, visiting a broad range of foodplants including Ericaceae, Rosaceae, the non-native *Escallonia* spp. (a garden shrub) and *Solidago canadensis* (a garden plant

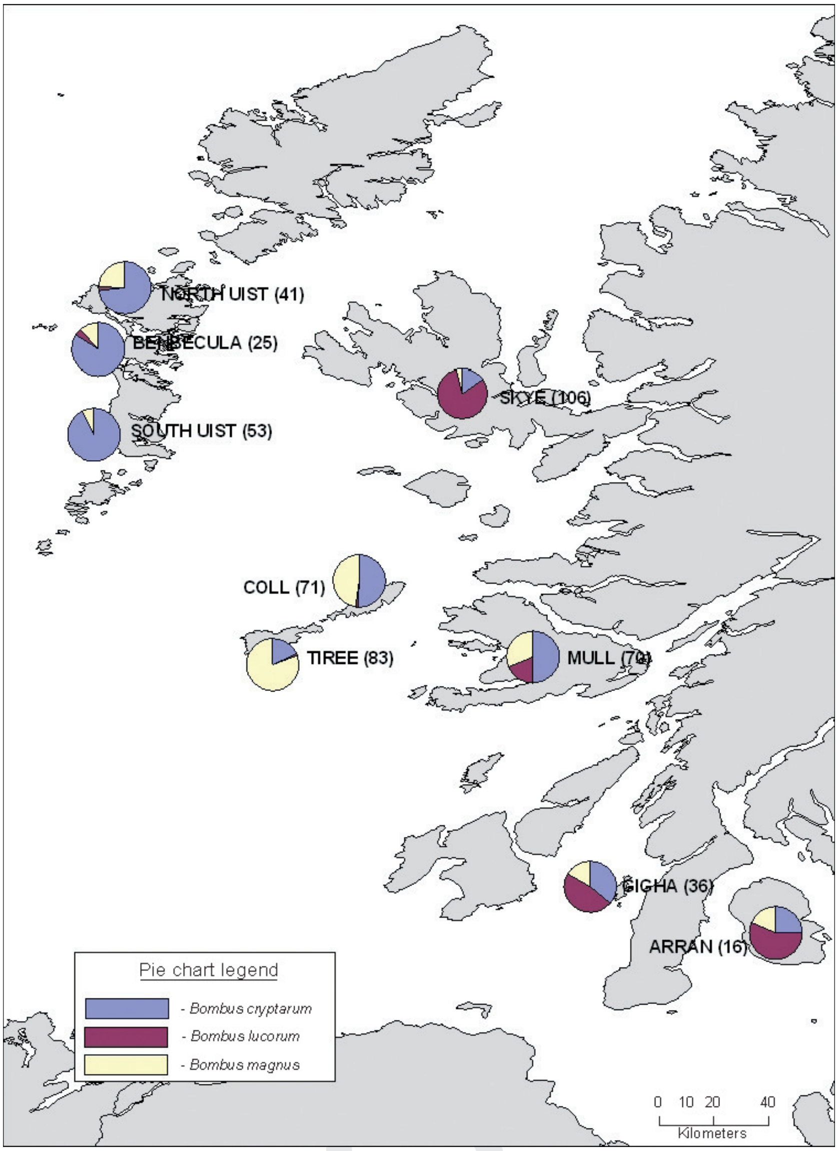

Fig. 2 The distribution of *Bombus lucorum* complex species in the Western Isles of Scotland. Species identification was by RFLP and only islands with > 15 identified specimens are included. Figure in brackets is the total number of individuals identified per island.

LOW RESOLUTION COLOUR FIG

and invasive weed) (Simpson's 1/D = 4.92). The broad pattern of differences in polylecty between the three species remain following rarefaction to take into account any influence of sample size on diet breadth (Table 2). It must be noted that forage use is likely to be limited by floral availability on the islands and in the habitats where a bee species occurs, so forage use as described here may not accurately reflect forage preferences.

Comparisons with forage use by other bumblebee species

The principal components analysis allows us to compare differences in forage use more broadly across bumblebee species present in this island group (Fig. 4). In this context, the differences between *B. magnus, B. cryptarum,* and *B. lucorum* appear small compared to the large differences in forage use found between the short-tongued bees (which includes these three species and also *B. jonellus*), and the medium and long-tongued bees (*B. hortorum, B. ruderarius, B. lapidarius, B. pascuorum*). The short-tongued bees are all broadly associated with Ericaceae, while the longer-tongued species are strongly associated with Fabaceae. In terms of forage use, *B. muscorum* is intermediate between the two groups (see also Goulson *et al*., 2005).

The value of the extent of the anterior thoracic collar in identifying worker bees

Of the 223 bees for which the extent of the yellow collar was examined, 150 (67.3%) were scored as *B. magnus* and 73 (32.7%) as *B. lucorum* (note that there is no known way to

Table 2. Forage use and measures of diet breadth for *B. lucorum* complex workers (identified by RFLP analysis), pooled across sample sites. Diet breadth is measured as Simpson's 1/D and via rarefaction to estimate the number of plant species each bee species would be expected to visit in a total of 10 flower visits.

Flower species	*cryptarum*	*lucorum*	*magnus*	Total
Ajuga repens	1			1
Angelica spp.	3	43	2	48
Anthriscus sylvestris		1	1	2
Calluna vulgaris	79	18	114	211
Centaurea nigra		2		2
Circium vulgare			1	1
Dipsacus spp.			1	1
Erica cinerea	26	49	13	88
Erica tetralix	2	3	5	10
Escallonia spp.	27	3	6	36
Filipendula			1	1
Fuchsia spp.	5		2	7
Knautia arvensis	4			4
Lotus corniculatus	1			1
Odontites vernus			1	1
Rosa spp.	30		2	32
Rubus spp.	2	3		5
Solidago canadensis	41			41
Trifolium pratense	1			1
Trifolium repens	1	5	1	7
Veronica spp.			1	1
Total	223	127	151	501
Simpsons 1/D	4.92	3.55	1.73	
Rarefaction (±SD)	4.95 ± 1.00	4.00 ± 0.96	3.08 ± 1.09	

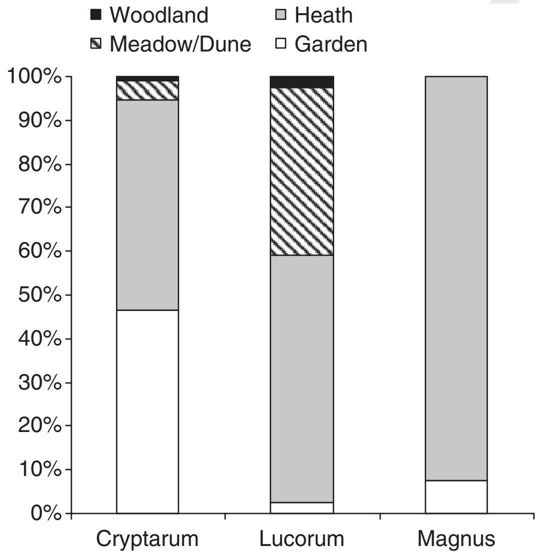

Fig. 3. Habitat use by workers of *B. cryptarum, B. lucorum* and *B. magnus* indicated by the percentage of bees caught in each habitat type, pooled for all sample sites.

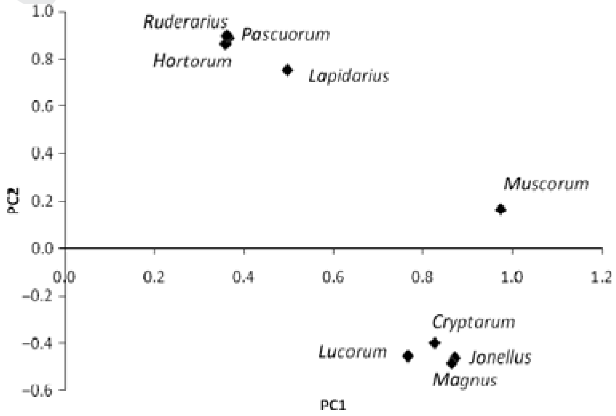

Fig. 4. The proportion of visits to different plant families by each bumblebee species was subjected to principal components analysis, and the first two components are plotted here. The first and second components account for 48.4% and 41.5% of variation in forage use, respectively. Component 1 is strongly positively correlated with visits to Ericaceae, while component two is most strongly (positively) correlated with visits to Fabaceae and negatively correlated with visits to Ericaceae and Apiaceae.

distinguish workers of *B. cryptarum* using this character). Following identification using RFLP, it is clear that the extent of the yellow collar is crudely related to species (Fig. 5). Of those specimens identified as *B. magnus* using morphology, 58.0% were correctly identified, while *B. magnus* comprised just 15.0% of bees ascribed to *B. lucorum* using morphology alone. A chi-squared test of association reveals that the proportions of the three species, as identified by RFLP, differs significantly between bees identified as *B. magnus* and those identified as *B. lucorum* on the basis of morphology ($\chi^2_2 = 68.2$, $P < 0.001$). Were it

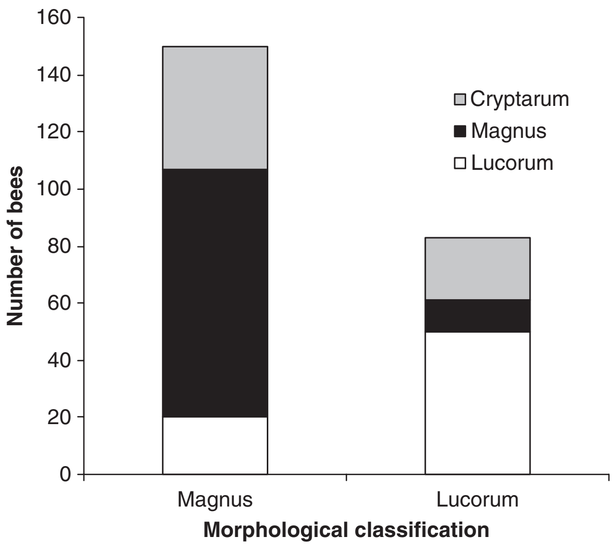

Fig. 5. The accuracy of the extent of the yellow thoracic collar in distinguishing between workers of the *B. lucorum* complex. Morphological classification is compared to that based on RFLP.

not for the presence of *B. cryptarum* at high frequency in both groups, this morphological classification would be moderately accurate.

Discussion

It seems probable that *B. lucorum*, *B. cryptarum,* and *B. magnus* should be considered as distinct species since they can be reliably distinguished on the basis of mitochondrial gene sequences and male pheromones (Bertsch *et al*., 2005). Our study, in conjunction with Murray *et al*. (2008) suggests that they have differing geographic patterns of abundance but demonstrate considerable overlap in range. We now provide evidence that these species differ quite markedly in habitat use and forage use. Hence, for the purposes of this discussion we make the assumption that they are good species, although clearly there is scope for further examination of this issue (for example by investigating genetic differences in other parts of the genome).

It is clear from our study and that of Murray *et al*. (2008) that these three species are common, widely distributed and sympatric throughout Ireland and Western Scotland. They are probably all widely distributed elsewhere, for the *B. lucorum* complex occurs from western Europe across the Palearctic to Japan, China and the western Nearctic (Williams, 1998). Until further studies are carried out, we have no way of knowing what the global distributions of the three component taxa are. Previous studies of the ecology and behaviour of *B. lucorum* should be re-evaluated in this light. For example, Goulson *et al*. (2005, 2006) describe *B. lucorum* as a generalist forager, feeding on a broad variety of plant species when collecting both nectar and pollen. However, it now seems certain that this is misrepresentation of the facts; the three component taxa differ in their feeding behaviour, and pooling them for this analysis inevitably led to the conclusion that the composite *species* had a broad foraging range.

Bombus magnus

The view espoused by books such as Alford (1975) and Benton (2006) that *B. magnus* is a species associated with upland, northerly, and westerly areas in the UK (i.e. cool and wet areas) is not supported by the data gathered so far (although studies from elsewhere in the UK are needed). Within western Scotland and Ireland, *B. magnus* appears to be widely distributed across both lowland and upland sites, but absent from urban areas (Murray *et al*., 2008). In our study this species was most abundant on Coll and Tiree, both low-lying islands. However, it is apparently strongly associated with heathland and particularly with *Calluna vulgaris* as a food plant. As a result, its forage use very strongly overlaps with that of *B. jonellus*, another short-tongued bumblebee species which specialises in visiting *C. vulgaris* (Fig. 4).

Bombus lucorum

B. lucorum was the most common of the three species in Ireland (comprising 56% of individuals), but the least common in our study (30%). In Ireland this species is most common at low altitudes and in urban areas; in Scotland it is most common on islands near the mainland, and is hence more common in the east. It occurs in both meadows and heaths, feeding primarily upon Ericaceae and Apiaceae.

Bombus cryptarum

B. cryptarum was the most common of the three species in our Scottish samples (43.8%), and the least common in Ireland (18.4%). In Ireland it appears to be more common in upland areas, and in Scotland it was the dominant member of this species group in the Outer Hebrides (the most westerly islands sampled). It would appear that, of the three species, *B. cryptarum* is the one most associated with cool upland, northern, and western areas, attributes previously ascribed to *B. magnus* (Alford, 1975). Our analyses of the value of the extent of the thoracic yellow collar in relation to species identity suggests that *B. cryptarum* may be intermediate between *B. magnus* and *B. lucorum*, and it seems likely that the purported association of *B. magnus* with upland areas may be the result of the misidentification of *B. cryptarum* and *B. magnus*. Of the three species, *B. cryptarum* was the most polylectic, visiting a broad range of foodplants from many families, including non-native garden plants.

There is a clear need to further improve our understanding of the ecology of these three taxa; we know nothing about how they may differ in nest site choice, susceptibility to parasites, or any number of other potentially important ecological factors. If one of these taxa were in sharp decline, we would have no way of knowing. Unfortunately it is hard to address these knowledge gaps. We have no reliable morphological means of distinguishing between the three species; for workers, the extent of the yellow thoracic collar is crudely indicative of a distinction between *B. lucorum* and *B. magnus*, but of no use for separating *B. cryptarum*. There are no known characters which can be used to differentiate between males of these three species. Although field identification is currently not possible, our study demonstrates that it is relatively easy to combine collection of ecological data with RFLP identification to flesh out our understanding of differences in their ecology, and this approach can readily be applied to other areas such as parasite load or nest site use. Regular sampling over time would enable detection in changes in the relative proportions of the three species.

Overall, it is clear that taxonomy of bumblebees based on morphological characters has led us to overlook the presence of cryptic species. Given ongoing concern over declines of bumblebee species (e.g. Goulson *et al*., 2008b), it is particularly alarming that, even in a well-studied region such as western Europe, there appear to be species about which at present we know almost nothing. Further work integrating ecological and genetic approaches is urgently needed to elucidate the biology of these organisms.

Acknowledgements

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Article: 101

Dear Author,
During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

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USING E-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

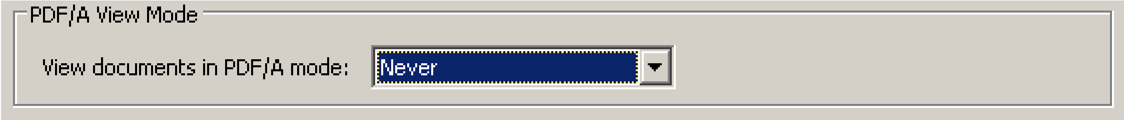
Required Software

Adobe Acrobat Professional or Acrobat Reader (version 7.0 or above) is required to e-annotate PDFs. Acrobat 8 Reader is a free download: http://www.adobe.com/products/acrobat/readstep2.html

Once you have Acrobat Reader 8 on your PC and open the proof, you will see the Commenting Toolbar (if it does not appear automatically go to Tools>Commenting>Commenting Toolbar). The Commenting Toolbar looks like this:

If you experience problems annotating files in Adobe Acrobat Reader 9 then you may need to change a preference setting in order to edit.

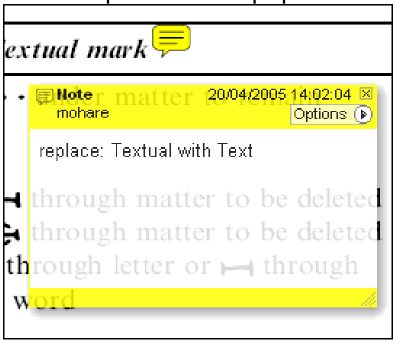
In the “Documents” category under “Edit – Preferences”, please select the category ‘Documents’ and change the setting “PDF/A mode:” to “Never”.



View documents in PDF/A mode: Never

Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.



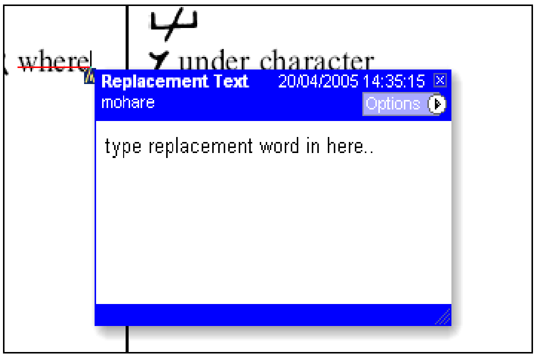
replace: Textual with Text

How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.



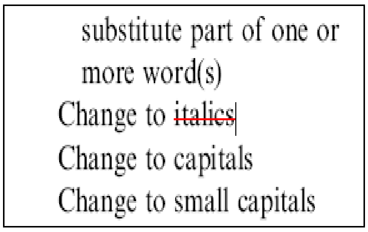
type replacement word in here..

How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



substitute part of one or more word(s)
Change to ~~italics~~
Change to capitals
Change to small capitals

How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

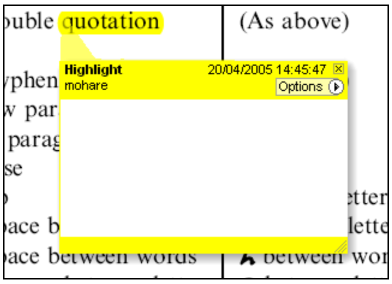
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the ‘standard business’ selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

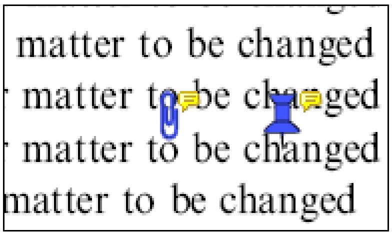


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

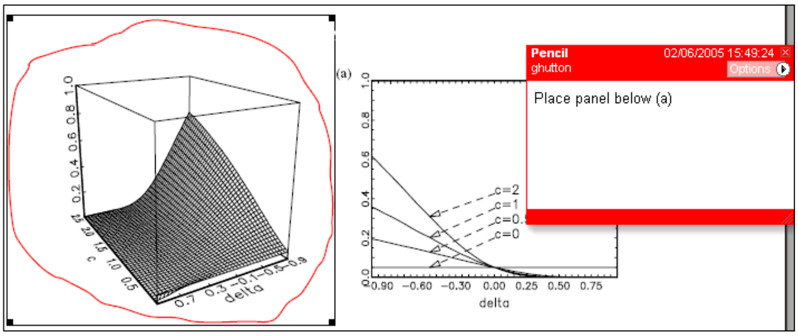


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



Place panel below (a)

How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: