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Microsatellite analysis supports the existence of three cryptic species within the bumble bee *Bombus lucorum sensu lato*

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1 **Abstract**

2

3 Mitochondrial cytochrome oxidase I (COI) partial sequences are widely used in taxonomy for
4 species identification. Increasingly, these sequence identities are combined with modeling
5 approaches to delineate species. Yet the validity of species delineation based on such DNA
6 'barcodes' is rarely tested and may be called into question by phenomena such as ancestral
7 polymorphisms in DNA sequences, phylogeographic divergence, mitochondrial introgression
8 and hybridization, or distortion of mitochondrial inheritance through such factors as
9 *Wolbachia* infection. The common and widespread European bumble bee *Bombus lucorum s.*
10 *lato* contains three distinct mitochondrial DNA lineages that are assumed to represent three
11 cryptic species, namely *Bombus cryptarum*, *B. lucorum s. str.* and *Bombus magnus*. To test
12 whether nuclear gene pools of the three putative species were differentiated, we genotyped
13 304 sympatric members of the *lucorum* complex (54 *B. cryptarum* females, 168 *B. lucorum s.*
14 *str.* females and 82 *B. magnus* females, as defined using mtDNA COI haplotypes) from 11
15 localities spread across the island of Ireland at seven nuclear microsatellite loci. Multilocus
16 genotypes clustered into three discrete groups that largely corresponded to the three mtDNA
17 lineages: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*. The good fit of mitochondrial
18 haplotype to nuclear (microsatellite) genotypic data supports the view that these three bumble
19 bee taxa are reproductively isolated species, as well as providing a vindication of species
20 identity using so-called DNA barcodes.

21

22 **Keywords** DNA barcode; *cryptarum*; *magnus*; mitochondrial cytochrome oxidase I;
23 STRUCTURE software; PCoA; DAPC, sympatry

24

25 **Introduction**

26

27 Bumble bees (Hymenoptera: Apidae, genus *Bombus*) are of great ecological and economic
28 importance as major pollinators of both crops and wild flowers in the Northern Hemisphere,
29 yet they are in decline (e.g. Fitzpatrick et al. 2007; Goulson 2009; Cameron et al. 2011).
30 Though members of the subgenus *Bombus sensu stricto* (=*Terrestribombus* Vogt) are the
31 most abundant and widespread of all bumble bees, exhibiting a holarctic distribution (Hines
32 2008; Williams et al. 2008, 2012a), they can be difficult to identify in the field using the
33 minor morphological differences that separate species (Carolan et al. 2012; Bossert 2015);
34 the apparent abundance of members of the subgenus may mask the rarity of its
35 morphologically indistinguishable, or cryptic, species.

36

37 In Europe, five species of *Bombus s. str*. are recognised: *B. cryptarum* (Fabricius), *B. lucorum*
38 (L.), *B. magnus* Vogt, *B. terrestris* (L.) and *B. sporadicus* (Nylander). The taxonomic status
39 of *B. terrestris* and *B. sporadicus* is widely accepted (Williams 1998). Difficulties arise over
40 the other three species: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*, which are generally
41 grouped to form the *lucorum* complex (*B. lucorum sensu lato*). They are cryptic species that
42 appear very similar in colour and form, particularly as workers or males, and that are often
43 difficult to differentiate morphologically from *B. terrestris* even as queens (Figure 1;
44 Rasmont 1984; Rasmont et al. 1986).

45

46 Species classification based on morphological characters may not be suitable for cryptic
47 species and genetic methods may help support species identification. Correct identification is
48 of conservation importance because the taxonomic status of a species must be accurately
49 established in order to assign status and direct conservation efforts (Ryder, 1986; Crandall et

50 al. 2000). The presence of cryptic species, however, has potentially detrimental implications
51 since reproductively isolated groups should be managed independently of each other (Riddle
52 et al. 2000; Palsbøll et al. 2007). To facilitate identification of cryptic species, molecular
53 methods such as DNA barcoding can be used as a means of designating species on the basis
54 of sequence similarity (Hebert et al. 2003). Such approaches have confirmed the view that
55 cryptic species are particularly common in insects (e.g. Berkov 2002; Hebert et al. 2004).
56
57 In pre-DNA based studies, allozyme polymorphisms and variation in male cephalic odour
58 bouquet supported the view that the *lucorum* complex of bumble bees comprise two or three
59 species (reviewed in Bossert 2015). Bertsch et al. (2005) subsequently used mitochondrial
60 cytochrome oxidase I (COI) gene sequences of specimens morphologically well-
61 characterised as queens to show that the *lucorum* complex of bumble bees contained three
62 distinct mitochondrial DNA (mtDNA) lineages in Europe, albeit sampling of two putative
63 species was limited to two specimens apiece at each of two sites in Europe. Using a far larger
64 number of samples from across Europe, including >300 from the island of Ireland, Murray et
65 al. (2008) showed that the three mtDNA lineages exhibited considerable interspecific DNA
66 sequence divergence (≥ 2.3%) at COI compared to intra-taxon sequence variability (≤ 1.3%),
67 with overwhelming support for each lineage, supporting the idea that the three mtDNA
68 lineages represent species: *B. cryptarum*, *B. lucorum s. str*. and *B. magnus*. Williams et al.
69 (2012a) gave the distribution of these three taxa in Europe, Asia and, for *B. cryptarum*, even
70 into North America based on COI sequences, again demonstrating overwhelming statistical
71 support for each of the three COI lineages representing the *lucorum* complex. Murray et al.
72 (2008) also developed a relatively quick and economic restriction enzyme based mtDNA COI
73 marker system based on restriction fragment length polymorphisms (RFLPs) that
74 differentiated among lineages.

75

76 Notwithstanding the success of DNA barcoding in separating species and even entire regional
77 bee faunas (Sheffield et al. 2009; Magnacca and Brown 2010a, 2012; Schmidt et al. 2015),
78 mitochondrial lineages may not represent independent, reproductively isolated species.
79 Reasons include the retention of ancestral mitochondrial sequence polymorphisms,
80 mitochondrial introgression and biased inheritance of maternal genetic markers by such
81 factors as *Wolbachia* infection, known to be widespread in bees (Gerth et al. 2011, 2013,
82 2014; Gerth and Bleidorn 2013; but see Stahlhut et al. 2012), heteroplasmy (e.g., Magnacca
83 and Brown 2010a), and associated tissue segregation of haplotypes (Magnacca and Brown
84 2010b). Moreover, the use of DNA barcoding or any other mitochondrial DNA marker
85 system does not permit the detection of hybrids between taxa. This is all the more relevant for
86 the *lucorum* complex of bumble bees, in which Carolan et al. (2012) found apparent mis-
87 match between widely employed species-characteristic, discriminatory morphological traits
88 of queens and mtDNA lineage among Irish specimens.

89

90 A resolution to this problem is to incorporate multilocus sequence typing into DNA
91 barcoding studies (Gerth and Bleidorn 2013; Bossert 2015), an approach we report here for
92 the *lucorum* complex. We used microsatellites, biparentally inherited nuclear markers, to
93 determine the extent to which the nuclear gene pools of the *lucorum* complex taxa concur
94 with the three mtDNA lineages of Bertsch et al. (2005) and Murray et al. (2008), with the aim
95 of reducing taxonomic uncertainty in this group.

96

97 **Materials and Methods**

98

99 Sample collection and DNA extraction

100

101 Females (queens and workers) of *B. lucorum s.l.* were collected in 2005 and 2006 from 11
102 localities spread across the island of Ireland from both rural and urban environments while
103 foraging on flowers (Table 1, Figure 2); they are a subset of the same Irish dataset originally
104 presented in Murray et al. (2008). Individuals were either frozen or stored in 99% ethanol at
105 4°C prior to DNA extraction from a single leg using 10% Chelex (Walsh et al. 1991) or from
106 half a thorax using a standard high salt protocol (Paxton et al. 1996).

107

108 Microsatellite genotyping and species identification by mitochondrial haplotyping

109

110 Individuals were genotyped at seven nuclear microsatellite loci (Supplementary Table S1)
111 described in Stolle et al. (2011) and developed for *B. terrestris*. Forward primers included a
112 19 bp M13 5' tail (CACGACGTTGTAAAACGAC) and reverse primers included a 7 bp 5'
113 tail (GTGTCTT). PCRs were carried out in a total volume of 10 µL containing 1-10 ng
114 genomic DNA, 1 µM of 6-FAM-, TET- or HEX-labelled M13 primer (see Supplementary
115 Table S1), 0.1 µM tailed forward primer, 1 µM reverse primer, 1x PCR reaction buffer
116 (Promega), 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.25 U GoTaq Flexi DNA polymerase
117 (Promega). PCRs were carried out on a MWG Primus thermal cycler using the following
118 parameters: initial denaturation at 96 °C for 3 min followed by 35 cycles of denaturation at
119 96 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 45 s, and a final extension
120 at 72 °C for 3 min. Genotyping was carried out on an AB3730xl capillary genotyping system
121 (Life Technologies; Carlsbad, California, USA). Allele sizes were scored using 500 LIZ size
122 standards and were checked by comparison with previously sized samples.

123

124 Microsatellite genotypes were obtained at 5-7 loci for 304 females (54 *B. cryptarum*, 168 *B.*
125 *lucorum s. str.* and 82 *B. magnus*; Table 1). All had already been classified to mt haplotype
126 by RFLP analysis of a mitochondrial partial COI gene sequence by Murray et al. (2008), the
127 results of which we use (and update by Sanger sequencing of the COI 'barcode' of eight
128 samples) here.
129
130 Data analysis
131
132 GENEPOP (version 3.4; Raymond and Rousset 1995) was used to test for linkage
133 disequilibrium between nuclear microsatellite loci and for deviation from Hardy-Weinberg
134 equilibrium (HWE) at these loci. We also tested for the presence of null alleles using MICRO-
135 CHECKER (Van Oosterhout et al. 2004). Genetic differentiation within each putative species at
136 microsatellite loci was calculated in MICROSATELLITE ANALYSER (MSA, version 4.05 for
137 OSX) (Dieringer and Schlötterer 1997) and isolation by distance tested using the online web
138 service IBDWS version 3.23 (Jensen et al. 2005).
139
140 We used three approaches to determine the fit between mtDNA lineage and multilocus
141 nuclear genotype. In the first approach, genetic clustering of individuals was assessed using a
142 Bayesian procedure implemented in the STRUCTURE software package (version 2.3.3;
143 Pritchard et al. 2000). The program was run without priors, and with or without the admixture
144 ancestry model. Twenty independent runs were carried out for each model and value of K, the
145 number of genetic clusters, from $K = 1$ to $K = 3$. Our rationale was to test the hypothesis of K
146 $= 3$ clusters (representing the three species: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*)
147 versus a null hypothesis of $K = 1$ or $K = 2$ clusters (species). Each Markov chain Monte Carlo
148 analysis used a burn-in of 50,000 followed by a further 500,000 iterations. STRUCTURE's Q

149 value, a probability of group membership, was calculated for each individual at $K = 3$ using
150 the admixture ancestry model.
151
152 Because our dataset suggested deviation from HWE (see results) whereas HWE is an
153 assumption of STRUCTURE, we employed two distance-based methods to test for the
154 association between genotypes and mitochondrial haplotypes, methods that do not make
155 assumptions about mating structure and that do not make *a priori* assumptions about group
156 membership. In the second approach, we visualised relationships among multilocus
157 microsatellite genotypes of the 304 females of the *lucorum* complex using principle
158 coordinate analysis (PCoA) in GENALEX version 6.5 (Peakall and Smouse 2006). In the third
159 approach, we used Discriminant Analysis of Principal Components (DAPC; Jombart et al.
160 2010) to cluster genotypes independently of *a priori* haplotype designation using the R
161 package *adegenet* version 1.4.2 (Jombart 2008) in R version 3.1.0 (R Core Team 2014). For
162 DAPC, we examined results after extracting 5, 10, 20 or 40 principal components from the
163 genotype data.
164
165 DNA sequencing to improve mt RFLP-based haplotyping
166
167 STRUCTURE Q values suggested that three individuals were in a different genotypic cluster to
168 that of the other individuals with the same mt RFLP haplotype (Supplementary Table S2).
169 Preliminary visualisation of the PCoA suggested that two of these three individuals and five
170 additional individuals were in a different genotypic cluster to those with the same mt RFLP
171 haplotype (Supplementary Table S2). All eight aberrant individuals were sequenced at the
172 COI ‘barcode’ (Hebert et al. 2003) and identified by a web-based BLASTn search against the
173 entire NCBI nucleotide database.

174

175 The original (in Murray et al. 2008) mitochondrial RFLP classification for four of these eight
176 individuals was incorrect; two individuals with *lucorum* RFLP patterns had *cryptarum* COI
177 DNA sequences and two individuals with *cryptarum* RFLP patterns had *magnus* COI DNA
178 sequences (Supplementary Table S2). Though error rates in defining an individual's mt
179 lineage using RFLPs were likely low, they nevertheless call into question the value of using
180 RFLPs to define unambiguously the mt haplotype, as has been proposed for the *lucorum*
181 complex of bumble bees (Murray et al. 2008; Versterlund et al. 2014). We suggest that DNA
182 sequencing of the COI barcode is a more reliable method of defining the mt lineage in the
183 *lucorum* complex of bumble bees in Europe and likely in other taxa, too. We recommend
184 Sanger sequencing rather than RFLP-based inference of haplotypes in future studies of the
185 *lucorum* complex.

186

187 The final, updated dataset is presented in Table 1 and in the Results section below.

188

189 **Results**

190

191 Approximately 10% of samples were duplicated per 96-well plate for PCR; duplicates gave
192 identical microsatellite genotypes, suggesting very low rates of error in amplifying and
193 calling genotypes. No consistent linkage disequilibrium (i.e. involving the same loci) was
194 detected between any of the seven nuclear microsatellites analysed across the three putative
195 species of the *lucorum* complex (Supplementary Table S3).

196

197 When individuals from all 11 populations were lumped together into their three putative
198 species, *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*, loci 255 and 278 exhibited deviation

199 from Hardy-Weinberg equilibrium in all three putative species and loci 198, 331 and 554
200 deviated in two putative species (Supplementary Table S4). In most of these cases, there was
201 evidence from MICRO-CHECKER for null alleles as the cause of the deviation (Supplementary
202 Table S4).
203
204 Lumping individuals from different populations into a single group could lead to deviation
205 from HWE and evidence for null alleles due to the Wahlund effect. To explore this
206 possibility, we tested for deviation from HWE using GENEPOP and for evidence of null
207 alleles using MICRO-CHECKER by testing each locus in each putative species at each sampling
208 location separately (Supplementary Tables S4.1-S4.11). In the majority of cases (147 of 172
209 locus by species by locality combinations), genotypes did not deviate from HWE and there
210 was no evidence of null alleles. These results suggest that all three putative species are
211 regular outbreeders and that deviation from HWE was a consequence of having lumped
212 individuals from different populations.
213
214 Interestingly, when we analysed deviation from HWE and sought evidence for null alleles by
215 lumping individuals from different putative species into a single taxon, *B. lucorum s. lato*,
216 either across all sampling localities (Supplementary Table S4) or for each sampling locality
217 separately (Supplementary Tables S4.1-S4.11), loci were often out of HWE and showed
218 evidence of null alleles (57 of 83 locus by location comparisons). These results provide a hint
219 that the three putative species are differentiated in sympatry.
220
221 We tested for population genetic differentiation for each putative species separately across
222 sampling localities for sampling site with $n \geq 5$ individuals. For each putative species,
223 differentiation across Ireland (Figure 2) was subtle, not significantly different from zero for

224 *B. cryptarum* (6 locations, global F_{ST} = 0.015, *P* = 0.109) but significant for *B. lucorum s. str.*

225 (9 locations, global F_{ST} = 0.008, *P* = 0.036) and *B. magnus* (8 locations, global F_{ST} = 0.018, *P*

226 = 0.023). For each putative species, Isolation by Distance was not significant (statistics and

227 population pairwise F_{ST}, Supplementary Table S5), probably due to low statistical power

228 (lack of sampling sites).

229

230 Differentiation between the three putative species was high (global F_{ST} = 0.268, *P* < 0.001);

231 all three putative species pairs were significantly differentiated (*cryptarum* versus *lucorum s.*

232 *str.*: F_{ST} > 0.205, *P* < 0.001; *cryptarum* versus *magnus*: F_{ST} > 0.219; *P* < 0.001; *cryptarum*

233 *lucorum s. str.* versus magnus: F_{ST} > 0.322, *P* < 0.001). There was no suggestion in the F_{ST}

234 values that *B. cryptarum* was closer to *B. magnus* than to *B. lucorum s. str.*, though *B.*

235 *lucorum s. str.* was most distant to *B. magnus.* When we lumped the three putative species

236 into one taxon, *B. lucorum s. lato*, differentiation across our 11 sampling sites in Ireland was

237 insignificant (F_{ST} = 0.046, n.s.). These results suggest that the three putative species are

238 genetically well differentiated.

239

240 Differences in allele frequencies of *B. lucorum s. str.* to the other two taxa were particularly

241 evident at locus 327 (Figure 3). Allele frequencies also differed markedly in *B. magnus*

242 compared to the other two putative species at locus 331 (Figure 3). Yet not one allele at any

243 of the loci was both private (restricted to a putative species) and at a sufficiently high

244 frequency within that species to allow it to be used to discriminate readily between species.

245

246 STRUCTURE analysis at *K* = 2 (mean likelihood Ln Pr (X|K) = -5668.6; admixture ancestry

247 model) revealed the mitochondrial lineage corresponding to *B. lucorum s. str.* to be well

248 differentiated from *B. cryptarum* and *B. magnus* (Figure 4), which may reflect the closer

249 phylogenetic affinity of the latter pair of species than either of them to *B. lucorum s. str.*
250 (Murray et al. 2008). The nuclear gene pools of the three putative species were clearly
251 separated at $K = 3$ (mean likelihood Ln Pr (X|K) = -5232.6**;** Figure 4), with greater model
252 support than for $K = 1$ (mean likelihood Ln Pr (X|K) = -6880.2) or $K = 2$. Results were
253 qualitatively the same using STRUCTURE's non-admixture model (Supplementary Figure S1).
254
255 Multilocus microsatellite genotypes of one out of the 304 individuals did not concur with the
256 COI mtDNA species delineation; an individual with a *magnus* mitochondrial haplotype was
257 assigned to the *cryptarum* nuclear gene pool cluster (individual Ff53, Supplementary Table
258 S6). Two additional individuals exhibited a major split in their nuclear gene pool assignments
259 between two putative species (individuals Ff19 and Ff27: Q value ≤ 0.5; Supplementary
260 Tables S6 and S7). STRUCTURE assignment of the other 301 individuals to their correct
261 mitochondrial lineage was generally with high posterior probability (Q value >0.93); only 21
262 of the other 301 individuals (~8%) analysed exhibited a major assignment (Q) value of < 0.9.
263 These results lend weight to the hypothesis that the *lucorum* complex comprises three
264 species, with good fit of multilocus nuclear genotypes to mitochondrial haplotypes and few
265 exceptions.
266
267 Because STRUCTURE makes the strong assumption that genotypic data are in HWE, we
268 repeated analyses by removing three loci that suggested marked deviation from HWE: loci
269 255, 331 and 198 (Supplementary Table S4). Results from STRUCTURE analyses with only
270 four loci gave largely similar results to those with the entire dataset (Supplementary Figure
271 S2).
272

273 Genotypes of the three mtDNA lineages each formed a separate cluster when mapped in
274 multivariate space by PCoA, with only slight overlap at the edges of clusters (Figure 5).
275 Seven of 304 individuals did not concur with COI mtDNA species delineation. These
276 included the three individuals (Ff19, Ff27, Ff53) whose STRUCTURE assignment suggested
277 their genotypes did not fit with other members of the same mitochondrial lineage
278 (Supplementary Table S7).

279

280 Clustering genotypes by DAPC also revealed three discrete clusters that largely concurred
281 with mitochondrial lineages (20 PCs extracted from the genotype data, Figure 6), providing
282 additional support for the hypothesis that *B. cryptarum*, *B. lucorum s. str.* and *B. magnus* are
283 discrete species. Five of 304 individuals were at the multivariate edge of mtDNA lineages.
284 These also included the same three aberrant individuals (Ff19, Ff27, Ff53) highlighted by
285 STRUCTURE and PCoA (Supplementary Table S7). The same five individuals were identified
286 as outliers when 5, 10 or 40 PCs were extracted from the genotype data for DAPC
287 (generating 7, 7 and 5 outlier individuals respectively).

288

289 Though STRUCTURE analysis suggested *B. cyrptarum* and *B. magnus* are genetically closer to
290 each other than either is to *B. lucorum s. str.* (Figure 4), PCoA and DAPC analyses did not
291 support this view. Using the multivariate distance-based approaches, all three putative taxa
292 were similarly differentiated (Figures 5 and 6).

293

294 Discussion

295

296 Our multilocus nuclear (microsatellite) data of the *lucorum* complex of bumble bees collected
297 in Ireland were grouped into three discrete multivariate clusters that corresponded well to the

298 three mitochondrial lineages formerly assigned to the putative species *B. cryptarum*, *B.*
299 *lucorum s. str.* and *B. magnus*. Our data lend weight to the hypothesis, based on mtDNA COI
300 partial sequences, that the *lucorum* complex comprises three morphologically cryptic but
301 reproductively isolated species: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*.
302
303 Mallet (1995, 2007) has persuasively argued that, in the age of genetics and genomics, a
304 robust species definition for sexually reproducing organisms is that, when in sympatry, they
305 form discrete genotypic clusters. The multilocus genetic differentiation of the three *lucorum*
306 complex mitochondrial lineages that we found in our STRUCTURE analyses at $K = 3$ and using
307 PCoA and DAPC is consistent with reciprocal monophyly of three species in sympatry.
308
309 Carstens et al. (2013) have argued that robust species delimitation should be based upon
310 multiple, independent analyses. Here we employed three methods to differentiate nuclear
311 gene pools of the *lucorum* complex, all of which were consistent in their identification of
312 three discrete groups, with few outliers. STRUCTURE in particular makes the assumption of
313 HWE, which, in our case, was violated at several loci when individuals from multiple
314 locations were lumped together within a putative species. That STRUCTURE nevertheless
315 identified the same outliers as PCoA and DAPC suggests it may be robust to given degree of
316 violation of HWE in our dataset.
317
318 Though our nuclear microsatellite marker dataset suggests reproductive isolation between the
319 three taxa of the *lucorum* complex, eight of 304 individuals possessed a multilocus genotype
320 that did not concur with their mitochondrial haplotype. For three of these eight individuals,
321 all three methods (STRUCTURE, PCoA and DAPC) placed them in a cluster different to that of
322 other members of their mitochondrial lineage. One explanation for this incongruence is that

323 genotypic clusters of different species may overlap at their edges when based on a reduced
324 number of loci; use of additional microsatellite loci or genome-wide markers may resolve this
325 'lack of data' issue (Lozier and Zayed 2016). Secondly, a low degree of hybridization
326 between species may take place in the field. Individuals Ff19 and Ff27 could represent
327 hybrids because their probability of assignment (STRUCTURE *Q* value) was intermediate
328 between their mitochondrial lineage and that of another lineage. Artificial crosses between
329 members of the *lucorum* complex are needed to support this suggestion. Thirdly, aberrant
330 individuals might be a product of mitochondrial introgression; individual Ff53 could
331 represent such a case, with a *magnus* mitochondrial haplotype confirmed by Sanger
332 sequencing and a multilocus nuclear genotype assigned to *cryptarum*, *cryptarum/lucorum* and
333 *cryptarum* by STRUCTURE, PCoA and DAPC respectively. It may be of significance that all
334 three consistently aberrant individuals (Ff19, Ff27, Ff53) were collected at one site, Slieve
335 Gullion, as workers (Table 1). We note, though, that the three discrete multilocus genotypic
336 clusters we detected are unlikely to be maintained if hybridization or mtDNA introgression
337 were common and widespread. Thus our second and third explanations seem unlikely to
338 account for the mis-assigned individuals. Alternatively, if they do occur, they may not lead to
339 fertile sexual descendants (queens and males).
340
341 If, as seems likely, our first explanation is correct, it suggests that considerable effort will be
342 required for microsatellites to be used to separate among cryptic species or to detect
343 hybridization. Within European bees, there are many putative cryptic species pairs or cryptic
344 species complexes that share COI barcodes (Schmidt et al. 2015). Interspecific DNA
345 sequence divergence at COI of the *lucorum* complex in Ireland is >2.3% (Murray et al. 2008),
346 yet the 7 microsatellite loci we used to analyse 54-168 individuals per taxon were insufficient
347 to resolve unambiguously all 304 individuals. For comparison, in the cryptic Neotropical

348 orchid bee sister species *Euglossa dilemma* and *Euglossa viridissima*, species-typical alleles
349 at one locus have been found to differentiate between taxa (Eltz et al. 2011), though not
350 unambiguously. Indeed, as the number of analysed individuals per taxon increases, so too is
351 the likelihood of detecting greater allelic diversity, reducing the probability of finding private,
352 species-diagnostic alleles at highly variable loci.
353
354 Lack of resolution in separating between reproductively isolated nuclear gene pools using
355 microsatellites might be due to shared ancestral polymorphisms and homoplasy caused by
356 high mutation rates at microsatellite loci (e.g. Schlötterer 1998). Sequence divergence of
357 other cryptic species complexes of bee at COI is considerably less than 2.3%, suggesting a
358 more recent common ancestor than that of the *lucorum* complex; for example, three members
359 of the *Colletes succinctus* complex (species *hederae, halophilus* and *succinctus*) share the
360 same COI barcode (Kuhlmann et al. 2007). For these and other closely related species, a
361 larger sample size of sympatric individuals and, possibly more importantly, a larger number
362 of nuclear loci may be needed to separate unambiguously among reproductively isolated
363 nuclear gene pools (e.g. using deep sequencing via genome skimming, Cossiac et al. 2016),
364 calling into question the feasibility of using microsatellites for multilocus sequence typing so
365 as to differentiate readily among cryptic species.
366
367 Species-specific pheromones may play an important role in mate-recognition, presumably
368 decreasing the incidence of interspecific mating (Paterson 1985). *Bombus* male sex
369 pheromones contain over 50 different volatile compounds derived from the labial glands that
370 are used to scent-mark substrates and that are thought to attract unmated conspecific queens
371 (Bergström et al. 1981; Ayasse et al. 2001). It has been previously demonstrated that males of
372 *B. cryptarum*, *B. lucorum s. str.* and *B. magnus* differ in their cephalic secretions to the extent

373 that their multivariate chemical composition has been used to support specific classification
374 of the three taxa (Bertsch 1997a; Bertsch et al. 2004, 2005; Pamilo et al. 1997). It is plausible
375 that mate recognition based on male labial secretions plays a significant role as a prezygotic
376 isolating mechanism in maintaining species boundaries between members of the *lucorum*
377 complex.
378
379 The existence of three cryptic species within the *lucorum* complex of *Bombus* has several
380 implications for conservation and management. In terms of conservation *per se*, members of
381 the *lucorum* complex, like *B. terrestris*, are classified as of 'least concern' in Europe (Nieto et
382 al. 2014). Nevertheless, it is highly likely that these cryptic species have been previously
383 overlooked, and treatment of them as such should be borne in mind when addressing
384 conservation status, particularly in light of the on-going declines in bumble bees in Europe
385 (Goulson et al. 2005; Fitzpatrick et al. 2007; Rasmont et al. 2015). Indeed, in Ireland, *B.*
386 *lucorum s. str.* is classified as 'least concern' whereas *B. cryptarum* and *B. magnus* are more
387 cautiously and appropriately classified as 'data deficient' (Fitzpatrick et al. 2006). The three
388 *lucorum* complex species may exhibit ecological specialization. With regard to altitude, *B.*
389 *cryptarum* is has more of an upland distribution in Ireland (Murray et al. 2008) whereas *B.*
390 *magnus* is considered a lowland species in Germany (von Hagen 2003). More recent analysis
391 of their ecological associations has suggested that *B. cryptarum* and *B. magnus* prefer cooler
392 sites in comparison to *B. lucorum* (Walters et al. 2010; Scriven et al. 2015). Phenological
393 differences also exist; *B. cryptarum* is an early species that precedes *B. lucorum s. str.* and *B.*
394 *magnus* (Bertsch 1997b; Bertsch et al. 2004), which may also play a role in preventing
395 hybridization.
396

From a management perspective, it is necessary to be able to identify taxa correctly because bumble bees are important managed crop pollinators and colonies are increasingly being transplanted long distances to provide pollination services (Goulson 2003). This has become of particular conservation concern since bumble bee translocations have been implicated in colony declines, including through pathogen spillover, and in the replacement of native *Bombus* species (Inoue et al. 2008; Meeus et al. 2011; Schmid-Hempel et al. 2014). Indeed, in Asia, confusion remains over the identification of bumble bee species imported for crop pollination (Williams et al. 2012b). Importation of non-native bumble bee species not only brings risks associated with the introduction of a competitively superior congener and of a non-native's pathogens, there is also the risk of hybridization between native and introduced species. We note that inter-specific mating and hybridization can only be detected by using codominant nuclear markers such as microsatellites or SNPs; the former have been employed to demonstrate interspecific mating and hybrid inviability in crosses between native Japanese bumble bees and imported European *B. terrestris* (Kanbe et al. 2008; Tsuchida et al. 2010).

The utility of DNA barcoding versus morphological-based taxonomy in biodiversity inventorying has been hotly debated (Packer et al. 2009; Stahlhut et al. 2012; Gerth and Bleidorn 2013). Yet DNA barcodes continue to be used for species identification and even the characterization of new *Bombus* species (Williams et al. 2016). Our data vindicate their use for species identification within the *lucorum* complex.

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430

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632 **Figure legends**

633

634 **Fig. 1** Queens of the four Irish members of the *Bombus s. str.* group, namely *B. terrestris* and
635 the three members of the *lucorum* complex: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*
636 (photo credit: Andreas Bertsch 2004)

637

638 **Fig. 2** Sample sites (numbers correspond to those in Table 1) and numbers of individuals
639 genotyped at seven microsatellite loci of the *lucorum* complex of bumble bees. Species
640 designation was based on mtDNA COI RFLPs and updated by Sanger sequencing

641

642 **Fig. 3** Bubble plots of allele frequencies at seven microsatellite loci of 304 individuals of the
643 three putative species of the *lucorum* complex of bumble bees from 11 sites in Ireland;
644 species designation was based on mtDNA COI haplotypes and bubble diameters reflect allele
645 frequencies which, within a species (column), sum to one

646

647 **Fig. 4** Barplot of STRUCTURE (using the admixture ancestry model) output showing
648 percentage assignment of individuals of the three *lucorum* complex species of bumble bees
649 genotyped at seven microsatellite loci to a given putative species for $K = 2$ and $K = 3$ (species
650 designation based on mtDNA COI haplotypes)

651

652 **Fig. 5** PCoA of the multilocus microsatellite genotypes of 304 *lucorum* complex bumble bees
653 from Ireland; each of the three mtDNA lineages is coded by a different shading; the three
654 individuals (Ff19, Ff27, Ff53) with low multilocus group membership (low STRUCTURE Q
655 value) to others of the same mtDNA lineage are labelled and circled in grey

656

657 **Fig. 6** DAPC of the multilocus microsatellite genotypes of 304 *lucorum* complex bumble
658 bees from Ireland; each of the three mtDNA lineages is coded by a different shading; the
659 three individuals (Ff19, Ff27, Ff53) with low multilocus group membership (low STRUCTURE
660 *Q* value and DAPC assignment to a different lineage) to others of the same mtDNA lineage
661 are labelled and circled in grey
662

663 **Table 1** Sample sites in Ireland and numbers of each species (as defined by mitochondrial lineage) of 304 *Bombus lucorum sensu lato* (*B.*
664 *cryptarum*, *B. lucorum s. str.* and *B. magnus*) collected and genotyped at 5-7 microsatellite loci (see Table 1 of Murray et al. (2008) for dates
665 of specimen collection); specimens were queens except where given in parentheses as workers (w)

No	Location	Latitude (N)	Longitude (W)	*B. cryptarum*	*B. lucorum s. str.*	*B. magnus*
1	Dublin City, Co. Dublin	53°20'22"	06°13'38"	11	28	0
2	Clara, Co. Wicklow	52°58'07"	06°15'59"	2	13	17
3	Glenasmole, Co Dublin	53°13'54"	06°20'52"	3	12	8
4	Glencree, Co. Wicklow	53°11'41"	06°18'22"	9	4	7
5	Kippure, Co. Wicklow	53°10'47"	06°18'18"	6	3	11
6	Powerscourt, Co. Wicklow	53°09'59"	06°14'55"	5	6	8
7	Killarney, Co. Kerry	52°03'48"	09°29'55"	0	19	12
8	Belfast City, Co. Antrim	54°34'13"	05°55'09"	6 (w=5)	18 (w=12)	0
9	Cork City, Co. Cork	51°53'54"	08°25'29"	1	40	0
10	Slieve Gullion, Co. Down	54°06'47"	06°24'55"	7 (all w)	18 (all w)	14 (all w)
11	Benbulben, Co. Sligo	54°18'49"	08°23'13"	4 (all w)	7 (all w)	5 (all w)
			Total	**54**	**168**	**82**

666 **Supplementary files**

667

668 **Table S1** Microsatellite primers developed for *Bombus terrestris* by Stolle et al. (2011; see
669 Table S1 of Stolle et al. 2011) and employed in this study to genotype the *lucorum* complex
670 of three putative species: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*

671

672 **Table S2.** Details of the eight individuals of the *lucorum* complex whose RFLP-based mt
673 haplotype did not concur with nuclear (microsatellite) genotypes, as defined by STRUCTURE
674 *Q* value (Supplementary Table S6) or visually by PCoA (Figure 5). Code: the unique
675 identifier to the specimen; Location: site of collection; RFLP ID: haplotype designation by
676 RFLP (from Murray et al. 2008); PCoA ID: microsatellite genetic cluster membership (see
677 Figure 5); STRUCTURE *Q* ID: microsatellite genetic cluster membership by STRUCTURE *Q*
678 value (see Supplementary Table S6); COI-BLAST: COI barcode DNA sequence identity by
679 BLASTn search against entire NCBI nucleotide database; GenBank Accession Number of
680 sequence with 100% identity to Irish sample

681

682 **Table S3** Linkage disequilibrium for seven microsatellite loci in the three putative species of
683 the *lucorum* complex of bumble bees collected at 11 sites in Ireland; columns represent
684 respectively the pairwise combination of loci, probability of linkage disequilibrium (P) and
685 standard error of that P value (SE), calculated in GENPOP

686

687 **Table S4** Deviation from Hardy-Weinberg equilibrium (HWE) for seven microsatellite loci
688 in the three putative species of the *lucorum* complex of bumble bees collected from 11 sites
689 in Ireland, including the observed and expected heterozygosity (H_o and H_{exp} respectively), the
690 inbreeding coefficient, F_{IS}, the probability of deviation from HWE as calculated in GENEPOP

691 v. 4.2 (http://genepop.curtin.edu.au), and evidence for null alleles in MICRO-CHECKER; also

692 given are H_o, H_{exp}, F_{IS}, and the probability of deviation from HWE for all *B. lucorum s. l.*

693 collected from site 1 and combined into one group**. Tables S4.1-S4.11** give the same details

694 for sampling sites 1-11 respectively.

695

696 **Table S5** Semi-matrix of pairwise estimates of F_{ST} across sampling locations (a) of *B.*

697 *crypatrum* (n = 6 locations), (b) *B. lucorum s. str.* (n = 9 locations) and (c) *B. magnus* (n = 8

698 locations), for which the number of individuals genotyped per location ≥ 5 (location notation

699 follows that given in Figure 1); no pairwise values are significantly different from zero ($P <$

700 0.05) using MSA v. 4.05 (Dieringer and Schlötterer 2003)

701

702 **Table S6** Mitochondrial lineage and multilocus probability of group membership

703 (STRUCTURE *Q* value; admixture model) at seven microsatellite loci in the three putative

704 species of the *lucorum* complex of bumble bees collected at 11 sites in Ireland (see Table 1

705 for site names and location); the one individual with aberrant correspondence between

706 mtDNA lineage (mt sp.) and nuclear genotype (nuc cryp, nuc luc, nuc mag for *B. cryptarum*,

707 *B. lucorum s. str.* and *B. magnus*, respectively) is highlighted in blue; the two individuals

708 with borderline membership to their mtDNA lineage (STRUCTURE *Q* value ≤ 0.5) are

709 highlighted in green; the 21 individuals with non-aberrant but low probability (STRUCTURE

710 *Q* value > 0.5 and < 0.9) of membership to their mtDNA lineage are highlighted in yellow

711

712 **Table S7** Details of the eight individuals of the *lucorum* complex whose updated mt

713 haplotype did not concur with nuclear (microsatellite) genotypes, as defined by STRUCTURE

714 *Q* value (Supplementary Table S6) or visually by PCoA (Figure 5) or by DAPC assignment

715 (Figure 6). Code: the unique identifier to the specimen; Location: site of collection;

716 Haplotype ID: haplotype designation by RFLP (from Murray et al. 2008) or Sanger
717 sequencing; STRUCTURE *Q* ID: microsatellite genetic cluster membership by STRUCTURE *Q*
718 value (see Supplementary Table S6); PCoA ID: microsatellite genetic cluster membership
719 (see Figure 5); DAPC ID: microsatellite genetic cluster membership (see Figure 6);
720 comment: possible cause for lack of mt-nuclear concurrence
721
722 **Fig. S1** Barplot of STRUCTURE (using the non-admixture model) output showing percentage
723 assignment of individuals of the three *lucorum* complex species of bumble bees genotyped at
724 seven microsatellite loci to a given putative species for $K = 2$ and $K = 3$ (species designation
725 based on mtDNA COI haplotypes)
726
727 **Fig. S2** Barplot of STRUCTURE (using admixture model) output showing percentage
728 assignment of individuals of the three *lucorum* complex species of bumble bees genotyped at
729 only four microsatellite loci to a given putative species for $K = 3$ (species designation based
730 on mtDNA COI haplotypes). STRUCTURE criteria were the same as in the ms for all seven
731 loci (modelling without priors, and with the admixture ancestry model, burn-in of 50,000
732 followed by a further 500,000 iterations). The data fit a model with K=3 better (mean
733 likelihood Ln Pr (X|K) = -3206) than with K=2 (Ln Pr (X|K) = -3343) or K=1 (Ln Pr (X|K) =
734 -3997). However, STRUCTURE gave two optimal results with K=3 for the reduced dataset of
735 four loci, (a) a poorer fit (probably local) optimum in which 'cryptarum' and 'magnus'
736 formed one group whilst 'lucorum' was split into two groups (6 of 20 runs, Ln Pr (X|K) = -
737 3340) and (b) a better fit (probably global) optimum as in our original results presented in Fig
738 4 (14 of 20 runs, Ln Pr (X|K) = -3148). All 20 runs converged.
739

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Figure 1

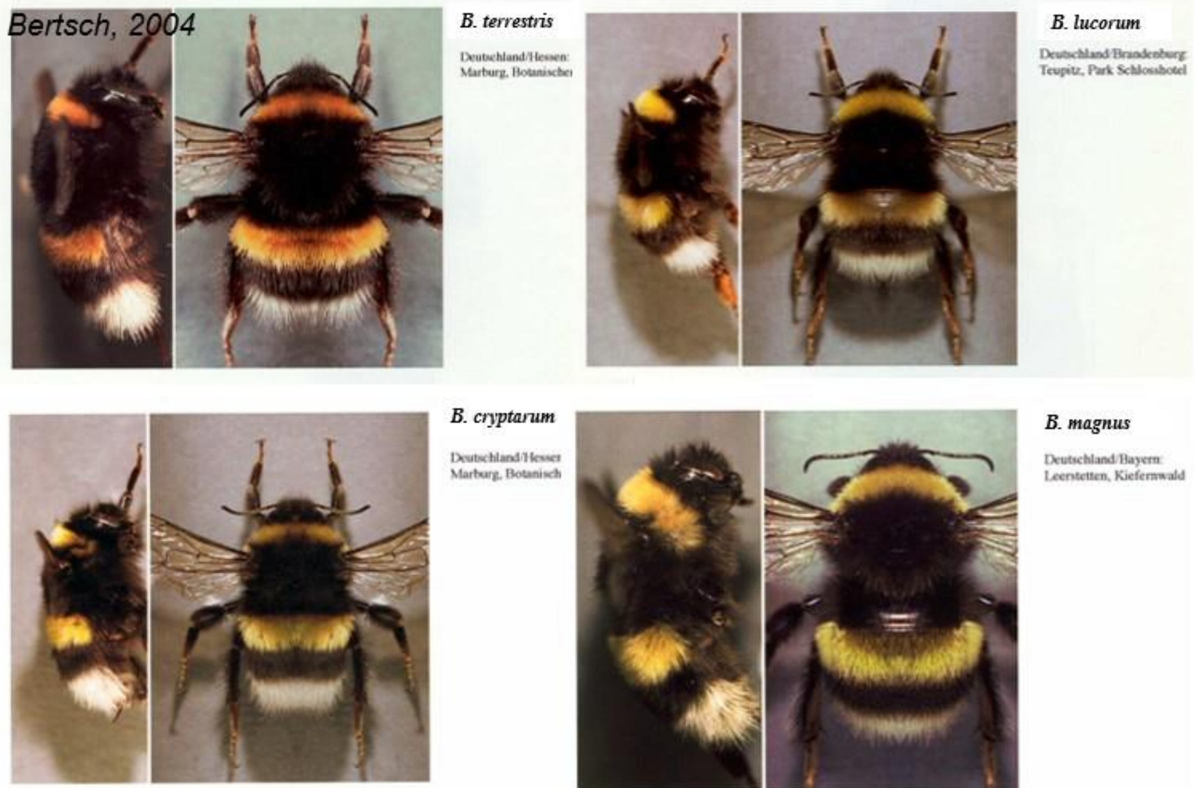

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Figure 2

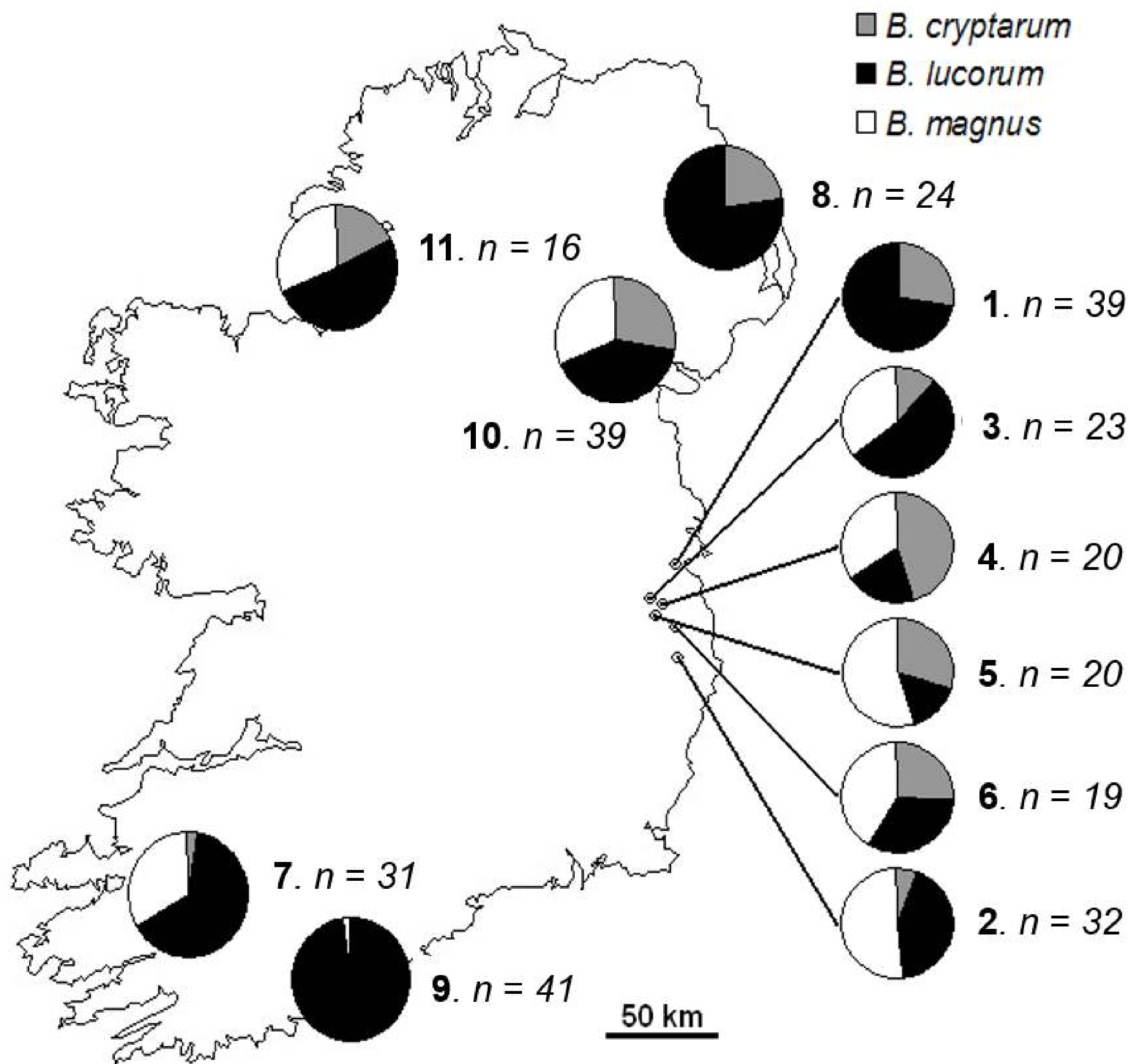

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Figure 3

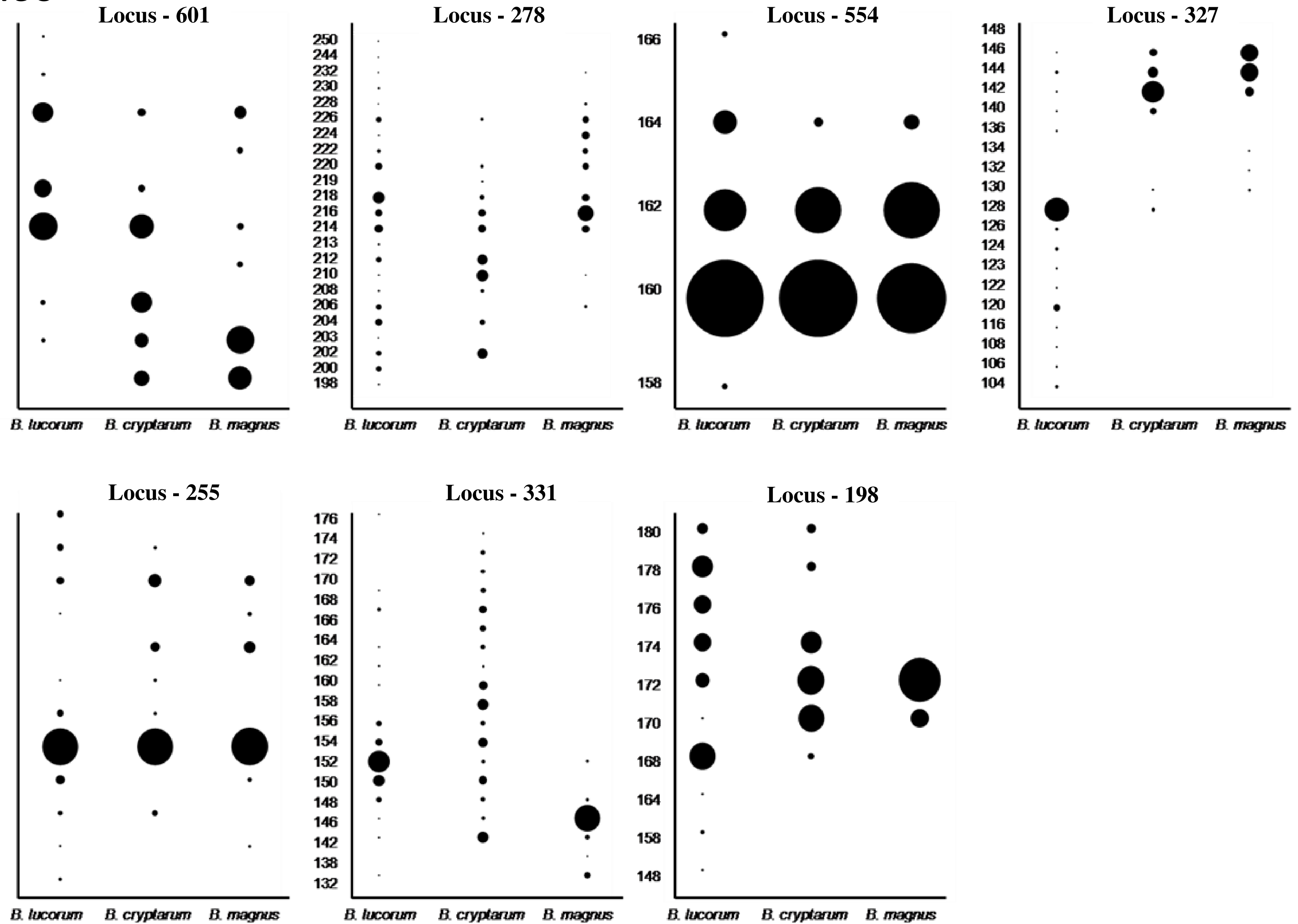

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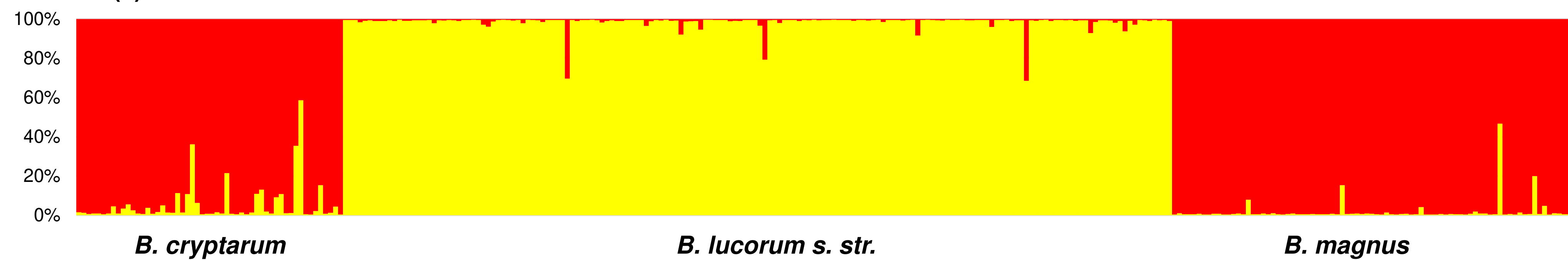
Figure 4



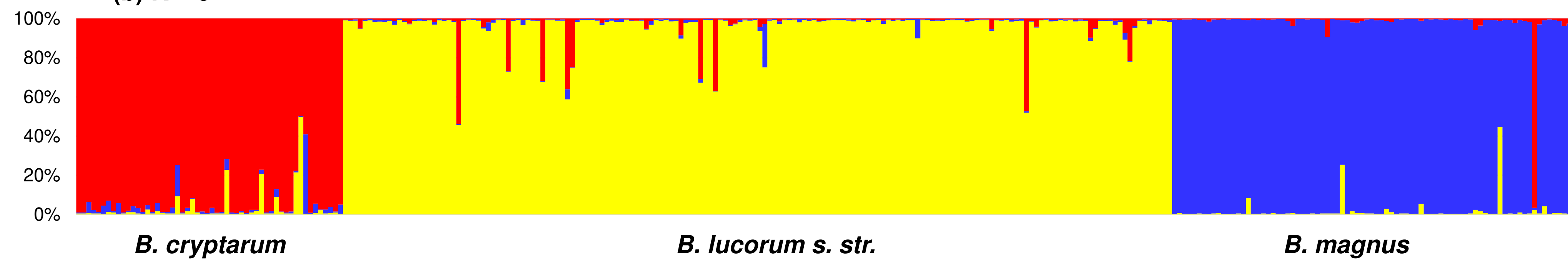
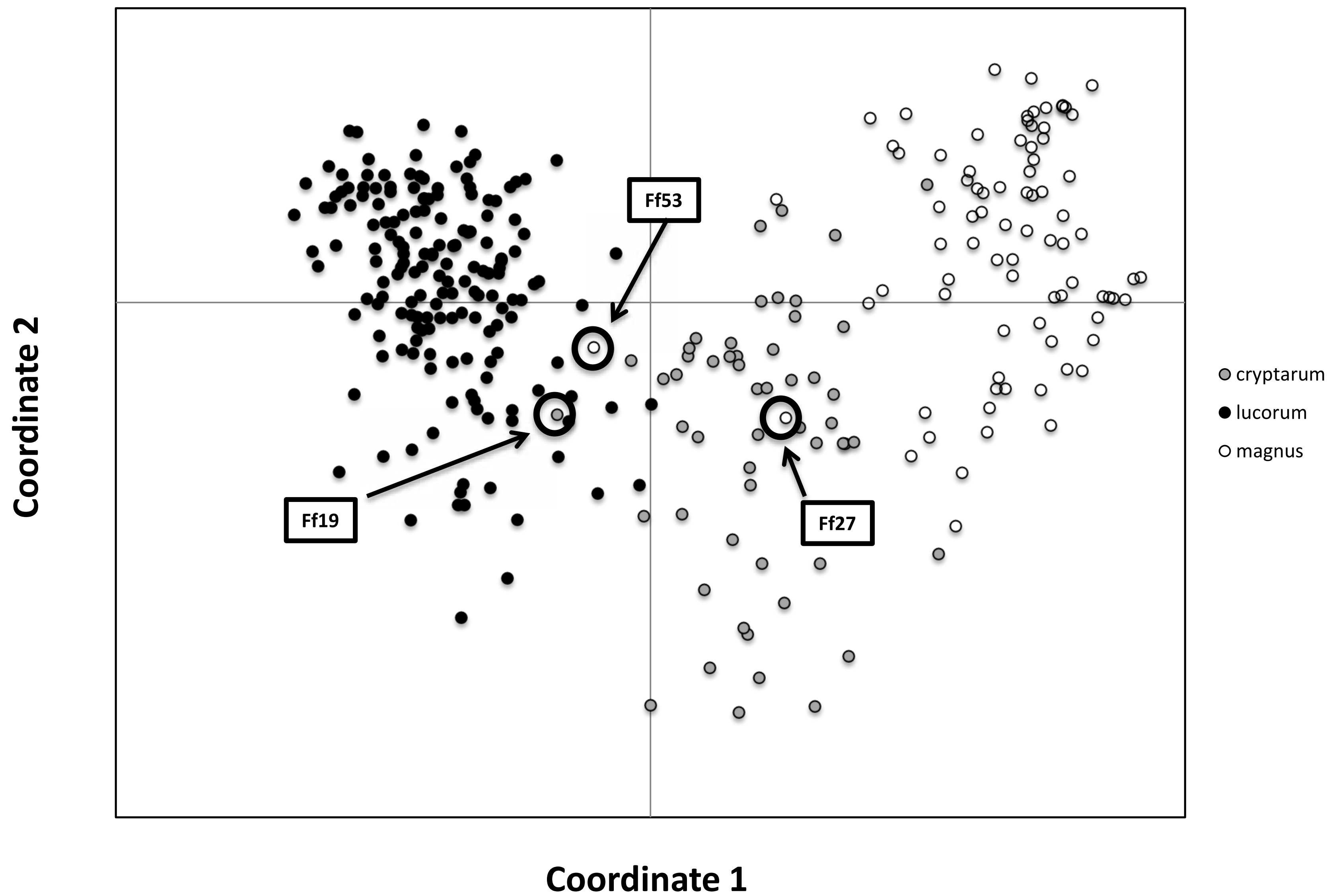


Figure 5



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