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TECHNICAL NOTE

Cryptic species identification: a simple diagnostic tool for discriminating between two problematic bumblebee species

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Abstract

Distinguishing between cryptic species is a perennial problem for biologists. *Bombus ruderatus* and *Bombus hortorum* are two species of bumblebee, which can be indistinguishable from their morphology. The former species is in decline, whereas the latter is ubiquitous. In the UK, isolated records of *B. ruderatus* occur amongst many for *B. hortorum*. For ecological studies of *B. ruderatus* to be feasible, the two species need to be reliably distinguishable. We present a diagnostic tool for quick and reliable identification of problematic individuals based on a restriction enzyme digest of the cytochrome *b* region of mitochondrial DNA.

Keywords: *Bombus*, conservation, cryptic species, mtDNA, restriction enzyme

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Cryptic species often pose problems for biologists of many disciplines. Several examples of such species occur amongst bumblebees (Hymenoptera: Apidae) (e.g. Bertsch *et al*. 2005), an ecologically and economically important group of pollinators. *Bombus ruderatus* (Fabricius) and *Bombus hortorum* (Linnaeus) are one such case. The specific status of these species was recently questioned by individuals working on the conservation of *B. ruderatus* (Edwards 2002), currently seriously threatened in the UK and listed on the Biodiversity Action Plan (UK Biodiversity Group 1998). Doubts over specific status arose because workers of these two species were often found to be indistinguishable: a spectrum of morphology based on coat colour and pilosity exists with 'good' *B. ruderatus* and 'good' *B. hortorum* at either end, whereas individuals in the middle are difficult to assign to either species. Both species also produce melanic individuals for which there are no simple reliable taxonomic characters. The morphology of the male genitalia, usually a reliable taxonomic tool, if only for one sex, is illustrated with the same diagram in the standard key (Prys-Jones & Corbet 1991). Furthermore, isolated records of *B. ruderatus* occur among many records for *B. hortorum*, at least in the UK, and it is unclear how often these are cases of mistaken identity (Edwards 2002).

Concerns over the specific status of these two taxa have now been assuaged: individual bees unanimously assigned as either species by several people independently (individuals from the extremes of the morphological spectrum as described above) displayed significant genetic differences in the mitochondrial regions COII and cytochrome *b*, entirely consistent with separate specific status (Ellis *et al*. 2005).

To conduct much-needed studies on *B. ruderatus* and halt further declines, a reliable means of confidently assigning individuals to either species is urgently required. A sequencing approach as above is impractical on any large scale as it demands significant time and money to positively assign individuals to one or other species. Thus, the problem of positively assigning past, present and future samples of bees, particularly those in the mid-range of the morphological spectrum remains. Additionally, in order to identify traditional morphological characters that reliably distinguish workers (if any exist), it is necessary to first designate individuals to one or the other species with certainty. Here, a restriction enzyme-based molecular probe is described. This provides a relatively cheap, simple, quick and reliable diagnostic tool.

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Previously nine individuals each of *B. ruderatus* and *B. hortorum* had been sequenced at two mitochondrial regions (Ellis *et al*. 2005). These were used to identify potential restriction sites. The enzyme Tsp45I was selected as its restriction site GTSAC (where S is either G or C, in this case a G) is present in the cytochrome *b* sequence of *B. hortorum* but not of *B. ruderatus*. For this study, individuals of *B. ruderatus* and *B. hortorum* were collected from Cambridgeshire and Norfolk through the summers 2002–2004. Shortly after collection, legs were removed from these individuals and stored in absolute ethanol. DNA was extracted from a leg of an individual using a standard salt/chloroform protocol (Rico *et al*. 1992). Legs were cut into segments with a scalpel to aid successful extraction. Individuals were then amplified at the mitochondrial region cytochrome *b* by polymerase chain reaction (PCR) (using an Applied Biosystems GeneAmp PCR system 2700). Individual PCRs contained: template DNA (of variably quantity), 0.8 U of *Taq*, 30 pmol $MgCl_2$, 1× QIAGEN PCR buffer (containing an extra 45 pmol $MgCl_2$), 6 pmol dNTPs and 6.5 pmol primer (forward primer: TTCAGCAATTCCATATATTGGAC; reverse primer: ATTACACCTCCTCATTTATTAGG). The PCR cycle was as follows: 94 °C for 4 min followed by 35 cycles at 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. PCR products were then digested with the restriction enzyme Tsp45I (New England Biolabs). Individual reactions contained 15 μL PCR product, 1 U of enzyme, 0.02 μg bovine serum albumin (BSA) and 1× buffer N.E. number 1 [containing 10 mM Bis-Tris Propane HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.0 at 25 °C)]. Reaction volumes were made up to 20 μL with 2.55 μL H_2O. DNA was digested at 65 °C for 4 h. Following digestion, products were run out by electrophoresis on a 3% agarose gel for 180 min at 60 V. Fragment sizes were compared to a 100 bp ladder (Promega).

Three bands were present in digested cytochrome *b* sequences of known *B. hortorum*: a band at 426 bp in length representing undigested complete cytochrome *b* PCR product, and two other bands, one 306 bp in length (including overhang), the other 125 bp (including overhang), representing digested cytochrome *b* PCR product. In most cases, the 306 bp band is the most obvious, the other two bands may be dim or absent. In all subsequent amplifications (see below) there was no detectable band of undigested PCR product in *B. hortorum* under the same conditions. To avoid digests of large quantities of PCR product possibly producing a stronger band of undigested product in this species leading to potential misidentification, known controls must be run each time. It should be noted that 1 U of enzyme digests 1 μg λDNA in 1 h in a total reaction volume of 50 μL at 65 °C. For known individuals of *B. ruderatus*, only the 426 bp undigested complete cytochrome *b* PCR product is present. This banding pattern allows a straightforward and simple means of assigning an individual of unknown identity to either species. Again, it is important to note that individuals must be run with controls of known individuals of *B. ruderatus* and *B. hortorum* to avoid erroneous identification owing to inhibition of the enzyme or failure of complete digestion. All original 10 UK samples of certain species identity were positively identified using this restriction site. A further 70 individuals of uncertain identity were then tested. In total, 32 individuals were found to be *B. hortorum* and 56 *B. ruderatus*.

The success rate of the taxonomists in correctly identifying individuals of *B. ruderatus* and *B. hortorum* varied from 89 to 100%. This sample also included 5 melanic individuals, 4 of which were male and 1 was female. All of these melanics were assigned to *B. ruderatus*. As all individuals appear to be this species, it is possible that these melanics contain enzyme inhibitors and that they may actually be *B. hortorum*. Further work would be necessary to confirm this (e.g. sequencing of melanics). It was previously anticipated by taxonomists, however, that melanics are more commonly produced in *B. ruderatus* than in *B. hortorum* in the UK (P. Williams, personal communication) and thus far only five melanic individuals have been assigned using this technique. Although those experienced in bumblebee taxonomy can correctly assign 89–100% of individuals to either species, many workers of *B. ruderatus* and *B. hortorum* remain difficult to separate with confidence. Additionally, there are no reliable characters for melanic individuals. Avoiding laborious direct sequencing procedures, the restriction enzyme-based identification tool described here will therefore be of great use to those working in the conservation and taxonomy of the rare and declining *B. ruderatus*. Such tools are of increasing value in the face of declining taxonomic expertise, and the development of molecular methods of identification should go hand in hand with the creation of mtDNA-based taxonomic libraries of DNA barcodes that are currently being advocated (Hebert *et al*. 2003). Similar methods could be applied to other problematic taxa, both within and without the Apidae, in the future.

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