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Notas / *Notes*

DNA barcode confirms the distribution of *Bombus magnus* (Vogt, 1911) (Hymenoptera: Apidae) in the Iberian Peninsula

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ABSTRACT

Bombus magnus (Vogt, 1911) (Hymenoptera: Apidae) is one of the three cryptic species belonging to the *lucorum* complex besides *B. lucorum* (Linnaeus, 1761) and *B. cryptarum* (Fabricius, 1775). In the Iberian Peninsula, only *B. lucorum* and *B. magnus* are present but the presence of this last species south of the Pyrenees has not yet been confirmed. Given their morphological similarity, we used the DNA barcode region for the identification of 113 individuals of this species complex in an Iberian sampling. Results confirm the presence of *B. magnus* in the Pyrenees and extend its current distribution to the Northern Iberian Plateau. Given these results, we suggest that the distribution and conservation status of this species in the Iberian Peninsula should be revised.

Key words: Bumblebees; *Bombus magnus*; *B. lucorum* complex; Apidae; Hymenoptera; DNA barcode; *cox1*.

RESUMEN

El código de barras de ADN confirma la distribución de *Bombus magnus* (Vogt, 1911) (Hymenoptera: Apidae) en la península Ibérica

Bombus magnus (Vogt, 1911) (Hymenoptera: Apidae) es una de las tres especies crípticas pertenecientes al complejo *lucorum* junto con *B. lucorum* (Linnaeus, 1761) y *B. cryptarum* (Fabricius, 1775). En la península Ibérica solo se encuentran *B. lurocum* y *B. magnus* pero la presencia de esta última no ha sido confirmada al sur de los Pirineos. Dada su similitud morfológica, usamos la región del código de barras de ADN para identificar 113 individuos de este complejo de especies en un muestreo ibérico. Los resultados confirman la presencia de *B. magnus* en los Pirineos y amplían su distribución actual hacia la meseta Norte ibérica. Dados estos resultados, sugerimos que ha de revisarse su distribución y el estado de conservación de esta especie en la península Ibérica.

Palabras clave: Abejorros; *Bombus magnus*; complejo *B. lucorum*; Apidae; Hymenoptera; código de barras de ADN; *cox1*.

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Bumblebee species (*Bombus* Latreille, 1802) were initially identified by their colour patterns. However, different species can show convergent colour patterns and furthermore, one given species can display a high intraspecific variation in different geographical locations (Williams, 2007). This results in the presence of cryptic species very similar or identical in morphology which cannot be readily distinguished (Rasmont, 1984). To overcome this, a molecular approach based on the sequence variation of a region of the mitochondrial *cytochrome c oxidase subunit I* (*cox1*) gene known as DNA barcode, has proved useful for revealing species identical to those recognised by traditional methods (Schmidt *et al*., 2015). DNA barcodes are included together with other data in an integrative taxonomy approach to delimit, discover and identify meaningful, natural species and taxa at all levels (Will *et al*., 2005), and have been widely used in bumblebee identification (Williams *et al.*, 2012).

Within *Bombus*, the subgenus *Bombus s. str.* has presented more problems than other subgenera when it comes to species identification (Williams *et al*., 2012). In Western Europe, only two species were widely accepted until the mid-twentieth century: *B. terrestris* (Linnaeus, 1758) and *B. lucorum* (Linnaeus, 1761). However, a detailed review of the species *B. lucorum* based on morphological methods led to the confirmation of the species *B. magnus* (Vogt, 1911) by Krüger (1954) and *B. cryptarum* (Fabricius, 1775) by Rasmont (1984). Their status as species was supported by cross-breeding experiments (De Jonghe & Rasmont, 1983) and mating observations that indicated reproductive isolation between them (Bučánková *et al*., 2011). Later, molecular data and male labial gland secretions contributed enough evidence to accept all three as separate species (Bossert, 2015; McKendrick *et al*., 2017). This grouping is now known as the *B. lucorum* complex.

Nevertheless, there are not enough morphological characteristics to discriminate the species reliably. The first collar may be the only characteristic that allows discrimination of the three species (Williams, 2000; Bossert, 2015), but it is only useful for queens. Ecological data cannot distinguish the species as they live sympatrically, and there are no comparative studies about their habitat preferences over a wide geographic area (Bossert, 2015). Consequently, Rasmont *et al.* (2015) has considered *B. magnus* as a taxonomically problematic species.

In the Iberian Peninsula, only two species from this complex are known to be present: *B. lucorum* and *B. magnus*. *Bombus magnus* in particular has been located in the northern half of the Iberian Peninsula: between altitudes of 100 and 2000 m in the Cantabrian Range, the Pyrenees, Sierra de Guadarrama and provinces of Guadalajara and Teruel (Rasmont, 1984; Castro, 1996; Ornosa *et al.*, 2017). However, these reports have not been confirmed with molecular assays and have been the topic of further discussion (Williams *et al.*, 2012; Ornosa *et al.*, 2017). Only one report from a specimen collected in 1965 in Soria (also in the northern half of the Iberian Peninsula) has been confirmed with DNA barcoding (Williams *et al.*, 2012).

The lack of certainty in the *B. magnus* records from the Iberian Peninsula south of the Pyrenees (Bossert, 2015; Ornosa *et al.*, 2017) reveals the need for further sampling to clarify the distribution of this species in the Iberian Peninsula. In addition, specimens from the Iberian Peninsula are of particular importance since there are indications that queens of *B. lucorum* exhibit a yellow thoracic collar coloration similar to *B. magnus* queens in central Spain (Bertsch, 2009). *Bombus lucorum* queens in Spain can also be exceptionally large and this could lead to wrong identifications as *B. magnus* queens are supposedly larger, although specimens with medium and small size have been also observed (Bertsch, com. pers., 2017). In this study, we used the DNA barcode to molecularly identify individuals of this species complex. Although this species is not considered to be threatened in the IUCN Red List of European Bees (Rasmont *et al*., 2015), distribution models project a reduction of suitable areas by 2050, therefore an update of the distribution of *B. magnus* in the Iberian Peninsula is needed to propose conservation measures.

Bumblebees were sampled from different areas of the northern half of the Iberian Peninsula between the summers of 2013 and 2017 (Table 1). Individuals (N = 113) of different castes (Seven queens; 47 workers; 59 males) were preserved in absolute ethanol until analysed.

The posterior left leg was removed to extract the DNA with the Chelex method (Walsh *et al*., 1991). A fragment of the mitochondrial gene *cox1* of 614 bp was amplified with MyTaq™ Red Mix (Bioline) using two different methods. For the samplings between 2013 and 2016, we used the primers LCO/HCO (Folmer *et al*., 1994) and the following program: initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 48/50 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. For the sampling of 2017, we used the mini-barcode primers Barbee and MtD9 (Françoso & Arias, 2013) and the following program: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 46 °C for 1 min 20 s, 64 °C for 2 min, and a final extension step at 64 °C for 10 min.

PCR products were sequenced in Secugen (Madrid). Sequences were edited with Geneious 7.1 and species identification was performed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Both amplification methods amplified the same region of the gene *cox1*. DNA barcodes of two individuals showed an identity of 100% with the sequences GU705915 (*B. magnus* from Germany) and JN872621 (*B. magnus* from Denmark) from Genbank, therefore of 113 individuals sampled, 111 were found to be

Table 1.— Samples of the *B. lucorum*-complex collected between the summers of 2013 and 2017 in the Iberian Peninsula. The samples of *B. magnus* are shaded in grey (a.s.l.= above sea level; M= male, W= worker, Q= queen).

Tabla 1.— Muestras del complejo *B. lucorum* recogidas entre los veranos de 2013 y 2017 en la península Ibérica. Sombreado en gris se señalan las muestras de *B. magnus* (a.s.l.= sobre el nivel del mar; M= macho, W= obrera, Q= reina).

Species	Latitude/ Longitude	Collection date	Locality/ Province	Elevation (m a.s.l.)	Caste
B. lucorum	42.63683 0.00972	10/07/2013	Parque Nacional de Ordesa, Huesca	1720	1 W
B. lucorum	42.63483 -0.03838	11/07/2013	Parque Nacional de Ordesa, Huesca	1950	2 Q, 1 W
B. lucorum	42.61313 -0.20201	12/07/2013	Linas de Broto, Huesca	1420	1 M
B. lucorum	40.75388 -4.06388	20/08/2013	Cercedilla, Madrid	1323	3 M
B. lucorum	42.63896 -0.03253	08/08/2014	Torla, Huesca	1390	2 W
B. lucorum	42.63582 -0.02783	08/08/2014	Torla, Huesca	1407	1 M
B. lucorum	42.63527 -0.01416	08/08/2014	Torla, Huesca	1665	2 M, 1 W
B. lucorum	42.63519 -0.01220	08/08/2014	Torla, Huesca	1649	1 W
B. lucorum	42.63500 -0.01083	08/08/2014	Torla, Huesca	1648	1 M
B. lucorum	42.63767 -0.00165	08/08/2014	Torla, Huesca	1673	1 Q
B. lucorum	42.69796 -0.12189	09/08/2014	San Nicolás de Bujaruelo, Huesca	1477	1 W
B. lucorum	42.69847 -0.12945	09/08/2014	San Nicolás de Bujaruelo, Huesca	1591	2 M
B. magnus	42.69847 -0.12945	09/08/2014	San Nicolás de Bujaruelo, Huesca	1591	1 M
B. lucorum	42.70055 -0.11777	09/08/2014	San Nicolás de Bujaruelo, Huesca	1381	1 M
B. lucorum	42.63033 -0.31615	10/08/2014	Biescas, Huesca	914	1 M
B. lucorum	42.74025 -0.78453	11/08/2014	Ansó, Huesca	1053	2 M, 4 W
B. lucorum	42.86482 -0.81805	11/08/2014	Ansó, Huesca	1216	1 M
B. lucorum	42.58361 1.05916	07/07/2014	Parque Nacional Aigüestortes, Lérida	1548	1 W
B. lucorum	42.58750 1.00111	08/07/2014	Parque Nacional Aigüestortes, Lérida	2035	1 W
B. lucorum	42.58194 1.01138	08/07/2014	Parque Nacional Aigüestortes, Lérida	2035	1 W
B. lucorum	42.58916 0.98927	08/07/2014	Parque Nacional Aigüestortes, Lérida	2152	4 W
B. lucorum	42.58111 1.12583	10/07/2014	Jou, Lérida	1369	1 W
B. lucorum	42.69388 0.93611	10/07/2014	Ruda, Lérida	1440	1 W
B. lucorum	42.66666 0.91694	10/07/2014	Colomers, Lérida	1589	1 W
B. lucorum	40.79194 -4.05972	29/07/2014	Puerto de la Fuenfría, Madrid	1797	4 M, 1 W
B. lucorum	40.78611 -4.05305	29/07/2014	Puerto de la Fuenfría, Madrid	1750	3 M

Table 1.— (Continued)

Species	Latitude/ Longitude	Collection date	Locality/ Province	Elevation (m a.s.l.)	Caste
B. lucorum	40.75388 -4.06388	02/08/2014	Cercedilla, Madrid	1323	1 M
B. lucorum	40.82305 -3.96166	08/08/2014	Parque Natural de Peñalara, Madrid	1830	1 M
B. lucorum	40.77666 -4.05527	21/08/2014	Puerto de la Fuenfría, Madrid	1696	2 M
B. lucorum	40.79194 -4.05972	26/08/2014	Puerto de la Fuenfría, Madrid	1797	3 M
B. lucorum	40.78611 -4.05305	26/08/2014	Puerto de la Fuenfría, Madrid	1750	1 M
B. lucorum	42.58900 0.98700	02/09/2015	Clots de Rialba, Lérida	2174	2 W
B. lucorum	42.45116 1.07388	04/09/2015	Llessuí, Lérida	1405	1 Q
B. lucorum	42.42666 2.26444	08/07/2015	Valle de Camprodón, Gerona	2175	1 W
B. lucorum	40.75388 -4.06388	01/05/20155	Cercedilla, Madrid	1260	1 Q
B. lucorum	42.38472 1.94361	09/07/2015	Estación La Molina, Gerona	1730	1 Q, 4 W
B. lucorum	42.32027 1.96777	09/07/2015	Estación La Molina, Gerona	1800	5 W
B. lucorum	40.79194 -4.05972	20/07/2015	Puerto de la Fuenfría, Madrid	1797	6 M, 3 W
B. lucorum	40.78611 -4.05305	04/08/2015	Fuente de Antón Velasco, Madrid	1750	16 M, 1 W
B. lucorum	40.79194 -4.05972	20/07/2015	Puerto de la Fuenfría, Madrid	1797	2 W
B. lucorum	40.79194 -4.05972	26/08/2016	Puerto de la Fuenfría, Madrid	1797	1 W
B. lucorum	40.75388 -4.06388	18/06/2017	Cercedilla, Madrid	1230	1 M
B. magnus	42.44222 -4.26388	20/06/2017	Naveros de Pisuerga, Palencia	800	1 W
B. lucorum	43.04138 -4.45861	21/06/2017	Piedrasluengas, Palencia	1355	1 W
B. lucorum	40.75388 -4.06388	25/06/2017	Cercedilla, Madrid	1230	1 W
B. lucorum	40.79194 -4.05972	26/07/2017	Puerto de la Fuenfría, Madrid	1797	1Q, 4M, 3W
B. lucorum	40.79194 -4.05972	01/09/2017	Puerto de la Fuenfría, Madrid	1797	1 M

B. lucorum and two individuals firstly identified as *B. lucorum* by morphometrical data were found to be *B. magnus*. One of the individuals was a male caught in San Nicolás de Bujaruelo (Huesca) in the Pyrenees, at 1591 m above sea level, 9 VIII 2014 (P. De la Rúa leg.). The second individual was a worker sampled in Naveros de Pisuerga (Palencia), at 800 m above sea level, 20 VI 2017 (C. Ornosa leg.), foraging on *Rubus ulmifolius* Schott (Fig. 1).

These new reports of *B. magnus* confirm the current distribution of the species in the Iberian Northern Plateau. The fact that only two individuals were sampled in four years strongly suggests that the species is rare and much less abundant than *B. lucorum*. Although both species are more widely distributed in northern Europe, previous works have already reported that *B. lucorum* is more frequent than *B. magnus* in mainland Europe (Bossert, 2015).

These reports agree with previous studies, which have pointed to a patchy distribution for *B. magnus*, with an association to heathlands with low diversity of plant species in Europe (Bossert, 2015) and other Mediterranean

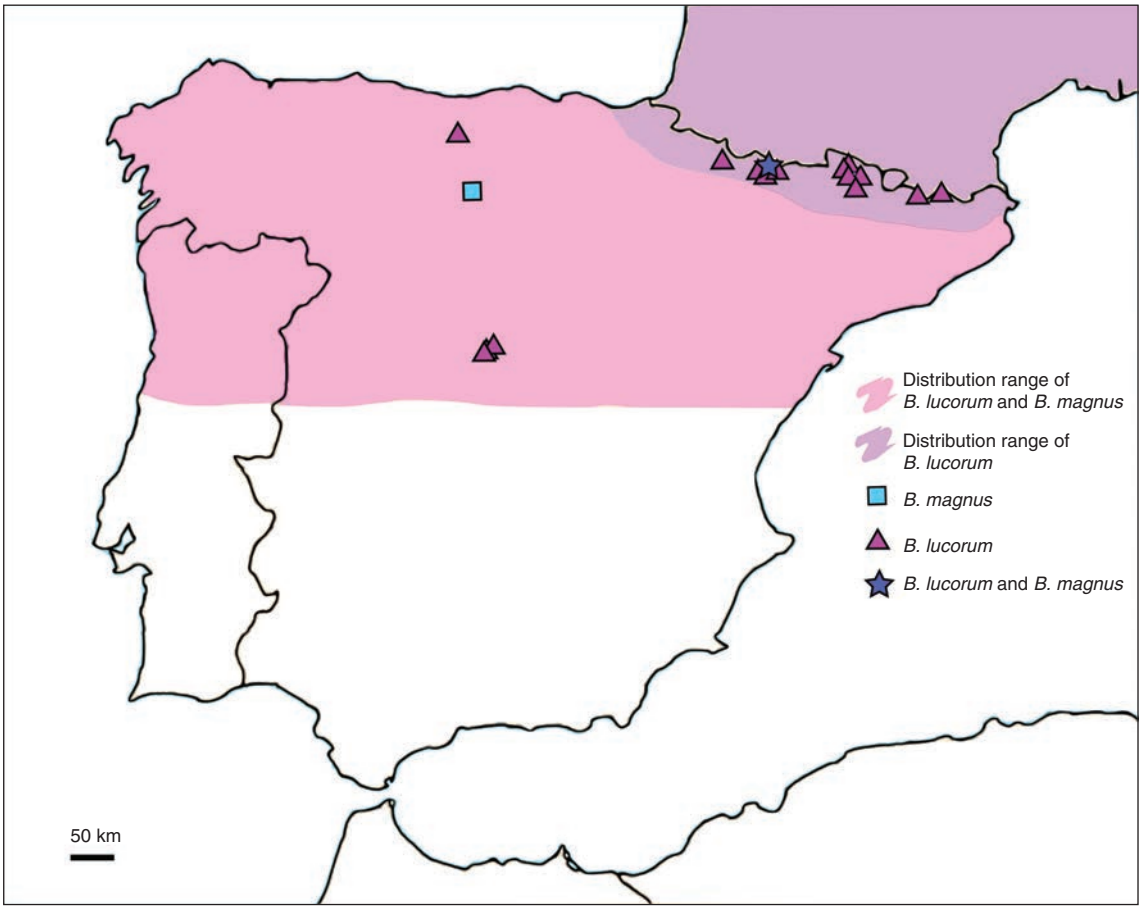

Fig. 1.— Known distribution range of *B. magnus* (Vogt, 1911) and *B. lucorum* (Linnaeus, 1761) according to Bossert (2015) and Ornosa & Ortiz-Sánchez (2004), respectively, and sampling sites of the present study. A blue square indicates a site where only *B. magnus* was found, a pink triangle indicates sites where only *B. lucorum* was found and a purple star indicates a site where both species were found.

Fig. 1.— Rango de distribución conocido de *B. magnus* (Vogt, 1911) y *B. lucorum* (Linnaeus, 1761) según Bossert (2015) y Ornosa y Ortiz-Sánchez (2004) respectivamente, y localidades de muestreo de este estudio. El cuadrado azul indica la localidad donde se encontró sólo *B. magnus*, un triángulo rosa indica las localidades donde sólo se encontró *B. lucorum* y una estrella morada indica la localidad donde se encontraron ambas especies.

landscapes in the Iberian Peninsula, where it was previously reported based on morphological data. These results highlight the need for sampling more individuals in the Iberian Peninsula, and the utility of the DNA barcode region for identifying them. Obtaining more samples could help pinpoint both the period of the year when *B. magnus* nests are active and their habitat, facilitating the discrimination of the species from *B. lucorum*. It could also help to assess its conservation status in Spain which is unknown at present.

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