

**Short scientific note**Submitted: November 26<sup>th</sup>, 2019 - Accepted: March 18<sup>th</sup>, 2020 - Published: April 15<sup>th</sup>, 2020**The spread of *Bombus haematurus* in Italy and its first DNA barcode reference sequence**Paolo BIELLA <sup>1,\*</sup> Andrea GALIMBERTI <sup>2</sup><sup>1</sup> University of Milano-Bicocca, Department of Biotechnology and Biosciences - Piazza della Scienza 2, 20126, Milano, Italy  
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**Abstract**

Climate change and human activities are impacting species distribution, and thus, tracking species movements is a key aspect for their conservation and for understanding their biology. Among the bumblebees that are changing distribution, one of the most striking cases of a fast and natural range expansion is the eastern Mediterranean *Bombus haematurus*. Here we report the first Italian records of this species, with observations from the N-E Italy at a distance of 332 Km from the edge of the historical distribution. These are the westernmost known occurrences of this species and they are not far from a large series of records in several Central European countries of recent colonization. Here, we also obtained and made publicly available the reference COI barcode sequence of *Bombus haematurus* and shown that is significantly different from other similar species at this genetic marker. Coupling morphology, field-data and genetic identity should greatly improve the efficiency of tracking species movements and therefore also their knowledge in both recently colonized and historical areas.

**Keywords:** Natural spread; range expansion; pollinator; fauna of Italy; molecular identification.**Introduction**

Tracking organisms' movements is a key aspect for their conservation and for understanding their biology, especially in view of the recent global change (Caplat et al. 2013; Lawler et al. 2013). This can be complicated by an inability of rapidly detect shifts in distribution, such as due to misidentifications and limited knowledge of species characters and life history traits (Crall et al. 2010; Verloove 2010). Invasive alien species are clear cases of how a slow identification process can impact the economy or the health of local ecosystems especially when they are introduced into new areas by human activities. Other cases of spreading species are those favoured by climate change, which causes natural shifts of their ranges or expansions into new areas. In both cases, the spread can be very fast (Chen et al. 2011; Crooks et al. 1999), and therefore distinguishing quickly and reliably newly arrived species from the local fauna is self-evidently advantageous. One of the best ways to identify species relies on the nucleotide sequence of a mitochondrial DNA region (COI region) which can be used as a DNA barcode to be matched to a genetic reference dataset (De Pasquale and Galimberti 2014; Mori et al. 2017). Using DNA identities more routinely for recognizing species can potentially contribute to

tracking species movements, their impact on the local ecosystems and even to address the conservation of species.

Several bumblebee species are declining, shifting or expanding their distribution due to human-related activities (e.g. see Cameron et al. 2011) and climate change (Kerr et al. 2015), among other factors. In the diversity of bumblebees (about 260 species worldwide), clear signs of distribution displacement have been shown either in entire bumblebee assemblages (Ornosa et al. 2017) or in single species (Biella et al. 2017). Perhaps, one of the most iconic cases is *Bombus alpinus helleri*, a taxon endemic to the Alps occurring at the highest altitudes, whose lower altitudinal limit has shifted uphill since 1980s without apparent changes to the upper boundary, resulting in a suspected shrinkage of available surface (Biella et al. 2017). Other species are instead expanding their distribution area, and have been spreading successfully into new regions, typically northwards or uphill: in Europe, the most evident cases are those of *Bombus argillaceus*, *Bombus semenoviellus*, *Bombus hypnorum* and *Bombus haematurus* (Rasmont et al. 2015). These latter cases are clear examples of successful natural spread into new areas, perhaps in connection with climate change.

In this study we communicate the recording of *Bombus haematurus* in Italy for the first time and produce its stand-

ard COI barcode DNA region, which was surprisingly not publicly available before. This species has been spreading very fast from Serbia into Central Europe in the last few decades (Jenič et al. 2010; Šima & Smetana 2009) and it has already been included in some recently published Central European identification tools for bumblebees (i.e. Amiet et al. 2017; Gokcezade et al. 2017). Yet, its DNA identity can help to confirm doubtful identifications to ensure that its fast spread is correctly tracked.

## Methods

The survey took place by walking free transects in urban gardens, flowerbeds, meadows and forest patches in the vicinities of the town of Cormons (Italy, Friuli Venezia Giulia) on the day 3<sup>rd</sup> of June 2017. The collection started at 5:00 AM. Later during the day, additional sites in that region were sampled (vicinity of Gorizia, of San Martino del Carso). An entomological net was used for capturing insects, binoculars (Pentax Papilio 8.5x21) were used for field identifications.

Most collected bumblebees were instantly killed with ethyl acetate but a few were kept alive to be later killed by deep freezing in the lab (these ones were used for the DNA analysis). The bumblebees were identified with standard keys (Amiet et al. 2017; Gokcezade et al. 2017; Pittiotti 1939) and reference entomological collections (Biologisches Zentrum Linz - Oberösterreichisches Landesmuseum).

From a sample morphologically identified as *Bombus haematurus*, total DNA was extracted from one posterior leg, by using the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) following manufacturer's instructions. Purified DNA concentration of the sample was estimated fluorometrically with a NanoDrop™1000 Spectrophotometer (Thermo Scientific, USA). DNA barcoding characterization of sample was conducted by comparing nucleotide sequence differences at the standard barcode region for metazoans (i.e., 658 bp at the 5' end of the mitochondrial COI, Hebert et al. 2003) following the same procedures described in Bellati et al. (2014). The presence of stop codons was verified for the obtained sequences by using the on-line tool EMBOSS Transeq ([http://www.ebi.ac.uk/Tools/st/emboss\\_transeq/](http://www.ebi.ac.uk/Tools/st/emboss_transeq/)). Sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI). The taxonomic status of the sampled bee was also tested by comparing the COI sequence with reference DNA barcodes stored in the Barcode of Life Database (BOLD System) using the Identification Engine tool (IDS) ([http://www.boldsystems.org/index.php/IDS\\_IdentificationRequest;SpeciesLevelBarcodeRecordsdatabase](http://www.boldsystems.org/index.php/IDS_IdentificationRequest;SpeciesLevelBarcodeRecordsdatabase)), which returns unique species assignments based on 99% sequence similarity of the barcode sequence.

## Results

**Findings: Italy:** Friuli Venezia Giulia, Cormons (Gorizia), 45.9603 N, 13.4713E, 106 m, 3 JUL 2017, on *Tilia* sp., leg & det. Paolo Biella, 2 ♂♂ and 9 ♀♀; Friuli Venezia Giulia, Cormons loc. S. Maria (Gorizia), 45.9652N, 13.4671E, 106 m, on *Lavandula* sp., leg & det. Paolo Biella, 1 ♀; Friuli Venezia Giulia, Brazzano (Gorizia), 45.9755, 13.4579, 117 m, on *Tilia* sp., leg & det. Paolo Biella, 1 ♀; the specimens collected are stored in Paolo Biella's collection.

As reported above, the current Italian range of *B. haematurus* includes only the north-easternmost region of the Italian peninsula (Friuli Venezia Giulia). These locations are currently the westernmost records of the global distribution area of *B. haematurus* and are as far as 331.9 Km in straight distance from the upper boundary of the historical area (north Serbia, Fruska Gora).

DNA extracted from the sample was of high quality (ratios of absorbance, A260/280 and A260/230 ~ 1.80 and >1.90, respectively) and provided good yield (> 30 ng/μl). Amplification with the selected primer pair was successful and resulting DNA concentration of purified amplicons was >50 ng/μl. High quality sequences showing a strong chromatogram signal along the entire read were obtained. The DNA barcode sequence contained no stop codons and was not biased by NUMT (*sensu* Bensasson et al. 2001) interference.

The DNA barcoding identification, performed on the *B. haematurus* sample (GenBank accession number LR738830), unequivocally confirmed that our sequence was the first complete DNA barcode record (658 bp) ever deposited in a public database. Interestingly, when it was used as query in BOLD, the algorithm returned reliable matches (i.e., similarity % > 99%) with two sequences of unidentified "*Apoidea* sp." from Plavi and from Bochorma in the Caucasus area of Georgia, respectively (Accession KY121930.1 and KY121844.1). When searching "*Bombus haematurus* AND COI" in the Nucleotide database of GenBank (NCBI), a COI sequence of *Bombus haematurus* was returned (418 bp, accession MH319126.1) but this only marginally overlap with the 5' 'Folmer' region of the gene that is usually referred to as 'standard DNA barcode'. The complete COI region of *B. haematurus* is well differentiated from a panel of putatively similar or co-subgeneric species occurring in the regions where *B. haematurus* occurs (Table 1).

## Discussion

In this study we report the first records for Italy of a bumblebee, *Bombus haematurus*. This species has undergone a remarkable natural spread since the late 1980s by colonizing several Central European countries in a North and North-Western direction from the historical distribution, whose northern limit was in northern Serbia. This species

**Table 1** – Dissimilarity at the COI DNA barcoding region between *Bombus (Pyrobombus) haematurus* and a panel of putatively similar species in morphology or in nesting ecology which occur in the same regions. Codes mean: H=High, M= Medium, L= Low, VL= Very low. The dissimilarity is calculated as 100 - similarity percentage at the COI regions from the output of the BLAST algorithm and their range is reported.

| Taxon                                   | Dissimilarity % at COI | Habitus silarity |      | Male genitalia silarity | Nesting silarity |
|---|------------------------|------------------|------|-------------------------|------------------|
|   |                        | Female           | Male |                         |                  |
| <i>Bombus (Pyrobombus) pratorum</i>     | 6.70 - 7.43            | M                | M    | M                       | VL               |
| <i>Bombus (Kallobombus) soroeensis</i>  | 15.17 - 15.72          | M                | M    | VL                      | VL               |
| <i>Bombus (Bombus) terrestris</i>       | 10.49 - 10.97          | L                | L    | VL                      | VL               |
| <i>Bombus (Bombus) lucorum</i>          | 11.40 - 12.15          | L                | L    | VL                      | VL               |
| <i>Bombus (Bombus) cryptarum</i>        | 11.09 - 12.44          | L                | L    | VL                      | VL               |
| <i>Bombus (Bombus) magnus</i>           | 12.16 - 12.42          | L                | L    | VL                      | VL               |
| <i>Bombus (Pyrobombus) jonellus</i>     | 7.31 - 8.4             | VL               | VL   | M                       | VL               |
| <i>Bombus (Pyrobombus) pyrenaeus</i>    | 7.00 - 7.15            | VL               | VL   | M                       | VL               |
| <i>Bombus (Pyrobombus) brodmannicus</i> | 6.71 - 6.74            | VL               | VL   | M                       | VL               |
| <i>Bombus (Pyrobombus) monticola</i>    | 8.52 - 8.83            | VL               | VL   | M                       | VL               |
| <i>Bombus (Pyrobombus) hypnorum</i>     | 8.68 - 8.83            | VL               | VL   | M                       | H                |

has been historically distributed in scattered localities from Serbia to the Black Sea, and eastwards to E-Iran through the shores of the Black Sea, the North Anatolian region, the Caucasus area and the south coasts of the Caspian sea (Reinig 1974). The natural expansion of its distribution has been first documented in several European countries, with records from Croatia in 1987 (A. Četković Pers. Coll.), from Hungary in 1987 and from Austria in 1995 (Józan 1995), from Slovenia in 2007 (Jenič et al. 2010), from Slovakia in 2003 (Šima & Smetana 2009) and from the Czech Republic in 2013 (Straka et al. 2015). The newly discovered Italian records are not surprising because they are in continuity with a number of recent records from W-Slovenia (A. Gogala pers. comm.). However, the numerousness of the specimens recorded in Italy and the presence there of both workers and queens suggest that *B. haematurus* is successfully established in Italy.

Although the morphological identifications of the recorded specimens of *B. haematurus* were certain due to the distinguishing characters of the species, we produced the first COI reference DNA barcode sequence in order to make it publicly available in international databases. When querying the NCBI and the BOLD genetic databases for sequences that would be very similar to the one produced by us, we have found it matching with two sequences of unidentified bees from Georgia (as “*Apoidea* sp.” in Kirkitadze et al. 2017). Although these two specimens were not accessible to us for a morphological identification, the very high similarity in DNA barcode with the sequence from the Italian sample hints that they could belong to *B. haematurus* specimens. This could be considered as an identification *a-posteriori*, made possible only after the creation of the DNA reference entry from the Ital-

ian sample. This posterior identification is also supported because the collection localities of the two samples of Kirkitadze et al. (2017) fit the historic distribution of *B. haematurus* and namely the Caucasian region where this species occurs (Kirkitadze & Japoshvili 2015). It is very unfortunate that the two sequences of Kirkitadze et al. (2017) belong to un-identified bees and that the accession MH319126.1 only marginally overlaps with the standard COI DNA barcode, and so they cannot be considered as reference DNA barcodes; thus the importance of the COI DNA sequences produced in our study is hampered by the fact that complete and standard DNA barcoding sequences (658 bp) were not previously available in international databases. The high levels of dissimilarity to the COI region of other species (either putatively similar in habitus, or in the shape of male genitalia, or in nesting ecology; Table 1) is a clear evidence of the potential benefit of using the genetic identity for both the passive and the active monitoring in the historical and in the newly colonized areas of occurrence.

In conclusion, the first Italian records of *B. haematurus*, an eastern European species naturally spreading into Central Europe since the 1980s, were reported from N-E Italy in this study. Although the presence of this bee in Italy was expected when considering the current distribution in the newly colonized area, this species is surprisingly not included in a recently published identification tool for the Italian peninsula and adjacent territories (Cappellari et al. 2018). Therefore, it is urgent to update the national identification literature. Future studies should also be dedicated to investigating the rate of spread west- and north-wards and the resource utilization in the recently colonized areas; This is because *B. haematurus* is one of the fast-spreading

pollinator species that have been naturally colonizing new areas, and this case can help investigating general topics such as niche utilization and the effect of climate change on it (Polidori et al. 2018). Progress towards these goals could clearly be favoured by the integration of morphological features, field-data and DNA-based identification techniques.

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