

Idiosyncratic patterns of genetic differentiation in five bumblebee species of the Colombian Andes

(Hymenoptera, Apidae)

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Lotta-Arévalo, I., Vargas-Ramírez, M., Nates-Parra, G., Matta, N. E., Balke, M. & Ospina-Torres, R. 2025. Idiosyncratic patterns of genetic differentiation in five bumblebee species of the Colombian Andes (Hymenoptera, Apidae). *Spixiana* 48(1): 63–74.

Currently, pollinators and particularly bees are highly threatened by habitat loss and fragmentation or even the introduction of invasive species as well as intensive use of pesticides, among others. In Colombia, nine species of bumblebees have been reported. Yet, information about their genetic diversity and structure in Colombia remains scarce. Based on a comparative phylogeographic approach, we aim to assess the genetic variation and potential presence of biogeographical patterns for five montane species across the three Colombian mountain ranges, and discuss the possible drivers of such patterns in light of the evolutionary and biogeography history of each species. Partial sequences of the mitochondrial genes cytochrome *c* oxidase subunit I (*coxI*), and the 16S rRNA and of the nuclear gene phosphoenolpyruvate carboxykinase (PEPCK) were amplified from specimens of *Bombus rubicundus*, *B. robustus*, *B. pauloensis*, *B. funebris* and *B. hortulanus*. Our maximum parsimony haplotype network analyses showed that only *B. pauloensis* has a clear genetic structure for all molecular markers. For *B. funebris*, *B. robustus*, and *B. rubicundus* the structure was incipient, mostly driven by the *coxI* fragment. In *B. hortulanus*, no spatial pattern was detected. We hypothesize that mountain ranges, which act as barriers, along with climatic variations, most probably influenced the patterns observed for *B. pauloensis* 16S and PEPCK, as has been reported for many other Andean taxa. In contrast, the weak or absent genetic structure observed in *B. hortulanus*, *B. robustus*, *B. rubicundus* and *B. funebris* indicates the presence of gene flow, probably aided by the existence of passages within and across the mountain ranges which maintained páramo connectivity during glacial periods. Our results contribute to the estimation of the actual biodiversity and the distribution of the genetic variability of bumblebees in Colombia, much needed for the generation of policies oriented to the protection of pollinators.

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Introduction

Pollinators are globally threatened due to intensification of agriculture, pesticide use, climate change, habitat destruction, as well as the introduction of exotic species and pathogens (Potts et al. 2010). Given the functional diversity of bees, the functioning of a large number of ecosystems, both natural and agroecosystems, depend on these organisms, so their current decline can seriously affect the planet's food security (Potts et al. 2016, de la Peña Alonso et al. 2018).

At present, the charismatic bumblebees (genus *Bombus*) have experienced the most considerable loss of biodiversity in their evolutionary history (Condamine & Hines 2015). *Bombus* has about 240 recognized species, occupying a great diversity of habitats, from alpine grasslands to tropical forests (Cameron et al. 2007). In Colombia, there are nine species with an exclusively neotropical distribution, which have been divided morphologically into short-faced and long-faced species (Williams 1998). Except for *B. melaleucus* (Handlirsch, 1888), short-faced species are mainly distributed over 2100 meters above sea level, while long-faced species inhabit lowlands from sea level up to 3500 m (*B. excellens* (Smith, 1879) is distributed from 1740 m up to 2500 m) (Liévano et al. 1991). *Bombus* species are the most important pollinators of *Solanum quitoense*, *Physalis peruviana*, *Solanum lycopersicum* and other important crops (Almanza Fandiño 2007, Ramírez et al. 2018, Ramírez & Davenport 2021).

Neotropical bumblebees originate from lineages that migrated from the Holarctic. The date of both the arrival of ancestors and diversification remains an open question. According to a recent study, this could have occurred between the late Miocene (c. 20 million years ago (mya)) and the late Pliocene (c. 5 mya) (Santos Júnior et al. 2022). Migration occurred in independent events (so-called pulses), probably using emerging land portions that appeared intermittently as a consequence of volcanic processes, climate change, or fluctuations in the sea level (Hines 2008, Müller et al. 2008, Bacon et al. 2015). According to this hypothesis, the climate cooling in the middle Miocene favoured the migration towards an expanding niche (Condamine & Hines 2015), since similarities were established with the ecosystems of origin in the Nearctic (Hines 2008). At that time, the northern block of the Andes had

already begun its uplift process and the mountain ranges had about half their current height (Gregory-Wodzicki 2000), which created barriers that contributed in the diversification by vicariance (i.e. the split of *B. pullatus* (Franklin, 1913) and *B. pauloensis* (Friese, 1913)). In contrast, Williams et al. (2022) suggest that the bumblebee migration actually occurred about 3 mya when the Panama Isthmus presented suitable conditions. In this sense, the radiation of some of these groups occurred in Mesoamerica and later they migrate to South America.

For many taxa in the Andes, diversification patterns have been analysed in the context of the glaciation periods of the late Pliocene and Pleistocene, and the refugia hypothesis, where populations experienced different processes of contractions and expansions, being fragmented and then only reconnected through temporary corridors that offered suitable life conditions (Ortiz & Miranda-Esquivel 2017, Sosa-Pivatto et al. 2020). Indeed, recent investigations indicate that there was a variable degree of connectivity between the different characteristic high altitude páramo ecosystems, along and across mountain ranges in the northern Andes during the last glacial period, that depended on the topography of the area, which may have favoured the gene flow between populations of resident flora and fauna (Flantua et al. 2019).

This study included five species, four short-faced *Bombus* species: *B. funebris* (Smith, 1854), *B. robustus* (Smith, 1854), *B. hortulanus* (Friese, 1904) and *B. rubicundus* (Smith, 1854), and one long-faced: *B. pauloensis* (Friese, 1913). *Bombus funebris* is distributed in high mountain ecosystems from Colombia to Chile. Although its highest abundance is in the montane floor (2800–3800 m), this species has been reported to inhabit over 4000 m (Liévano et al. 1991). It has a high preference for well-preserved natural habitats of the Andean Forest and páramo. Its tolerance to landscape transformation is low (Liévano et al. 1991, Abrahamovich et al. 2004, Nates-Parra 2005, Pinilla-Gallego et al. 2017). In regard to *B. robustus*, its distribution ranges from Venezuela to Ecuador (Abrahamovich et al. 2004). At an altitudinal level, it can be found from 2300 to 3800 m (Liévano et al. 1991, Pinilla-Gallego et al. 2017). This species is often confused with *B. hortulanus* (Williams 1998, Williams 2019), but it has less tolerance to the environmental intervention and reaches greater heights although in certain locations

they can be sympatric (Rubio Fernández 2012). *Bombus hortulanus* has the same geographic distribution as *B. robustus* (Abrahamovich et al. 2004), but altitudinally it can be found from 2100 to 3180 m. It can be found in places with mild to moderate levels of intervention, as well as pristine areas (Nates-Parra et al. 2006, Pinilla-Gallego et al. 2017). *Bombus rubicundus* has been reported from highlands of Venezuela to Bolivia (Abrahamovich et al. 2004), 2200 to 3700 m. Like *B. robustus*, its highest abundance is found in the montane floor (Liévano et al. 1991, Pinilla-Gallego et al. 2017).

The long-faced species *B. pauloensis* is widely distributed in South America. Although the documented altitudinal range for this species ranges between 150 to 2800 m (Ospina et al. 1987), in Colombia it only occurs in the lower montane zone (1800–2,800 m), capable of nesting in landscapes highly intervened (Liévano et al. 1991, Pinilla-Gallego et al. 2017).

The study of genetic diversity, phylogeographic patterns, the estimation of past and present distributions ranges as well as the characterization of the habitats of bumblebees has recently gained great relevance since this information allows for the identification and prioritization of species and suitable living areas for their conservation (Herrera et al. 2014, Françoso et al. 2016, Krechmer & Marchioro 2020). Nevertheless, for most bumblebee species inhabiting South America and particularly the Colombian Andes, genetic studies remain scarce (Santos Júnior et al. 2022, Williams et al. 2022). Thus, as another step forward, the present study aims to assess genetic differentiation patterns among populations of five widely distributed *Bombus* species of the northern Andes, and discuss the possible drivers of such patterns in light of the evolutionary and biogeographical history of each species.

Materials and methods

Material examined. We used museum specimens from the Bee Research Laboratory (LABUN) at the National University (Bogotá) collection as well as newly collected individuals (Electronic Supplement 1). Morphological determination of specimens was carried out following the characters proposed by Liévano et al. (1994). We also used the colour pattern and genitalia traits of species found in the taxonomic keys of the Natural History Museum of London (Williams 2019).

Collecting methods. Using entomological nets, bumblebees were collected in nine sampling sites located throughout each of the three Colombian mountain ranges (Fig. 1, Electronic Supplement 1). In the Eastern chain, six sites were sampled: 1. The Chingaza National Natural Park (NNP), 2. La Laja Natural Reserve (NR), 3. Matarredonda páramo at Choachí, 4. Salto Tequendama,

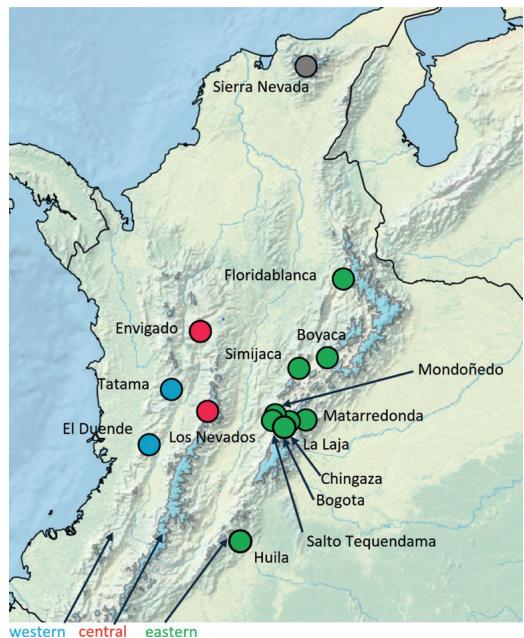


Fig. 1. Sampling sites on the tree cordilleras of the Colombian northern Andes. Localities in the Western Mountain range, are indicated with blue dots. Those in the Central chain of mountains are shown with red dots, and green dots depict places in the Eastern chain of mountains. In the north, the Sierra Nevada of Santa Marta is represented by a grey dot.

5. Mondoñedo, and 6. Simijaca. At the Central range, specimens were collected at the Nevados NNP. As for the Western range, samples from two sites, 1. The Tatamá NNP and, 2. the “El Duende” páramo were analysed. In addition, museum samples from 1. Floridablanca, 2. Huila, 3. Boyacá, 4. Bogotá, and 5. Envigado were processed.

DNA extraction, mitochondrial and nuclear molecular markers amplification. DNA extraction from legs or thoracic muscle was performed using the DNeasy (Qiagen) Kit. For museum samples, protocol modifications were made according to Lotta-Arevalo et al. (2020). We analyzed two mitochondrial markers that have been used in previous studies of bumble bees and which can comparably easily be amplified from museum specimen DNA. We used partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (*coxI*) (Folmer et al. 1994) and 16S (Cameron et al. 1992, Dowton & Austin 1994). For further confirmation, we targeted the nuclear phosphoenolpyruvate carboxykinase (PEPCK) (Cameron et al. 2007) for newly collected specimens. Meanwhile, for samples from the biological collection, five primer sets were used in eight combinations to obtain fragments from 152–404 base pairs (bp) (Lotta-Arevalo et al. 2020), that were assembled to get full length or near

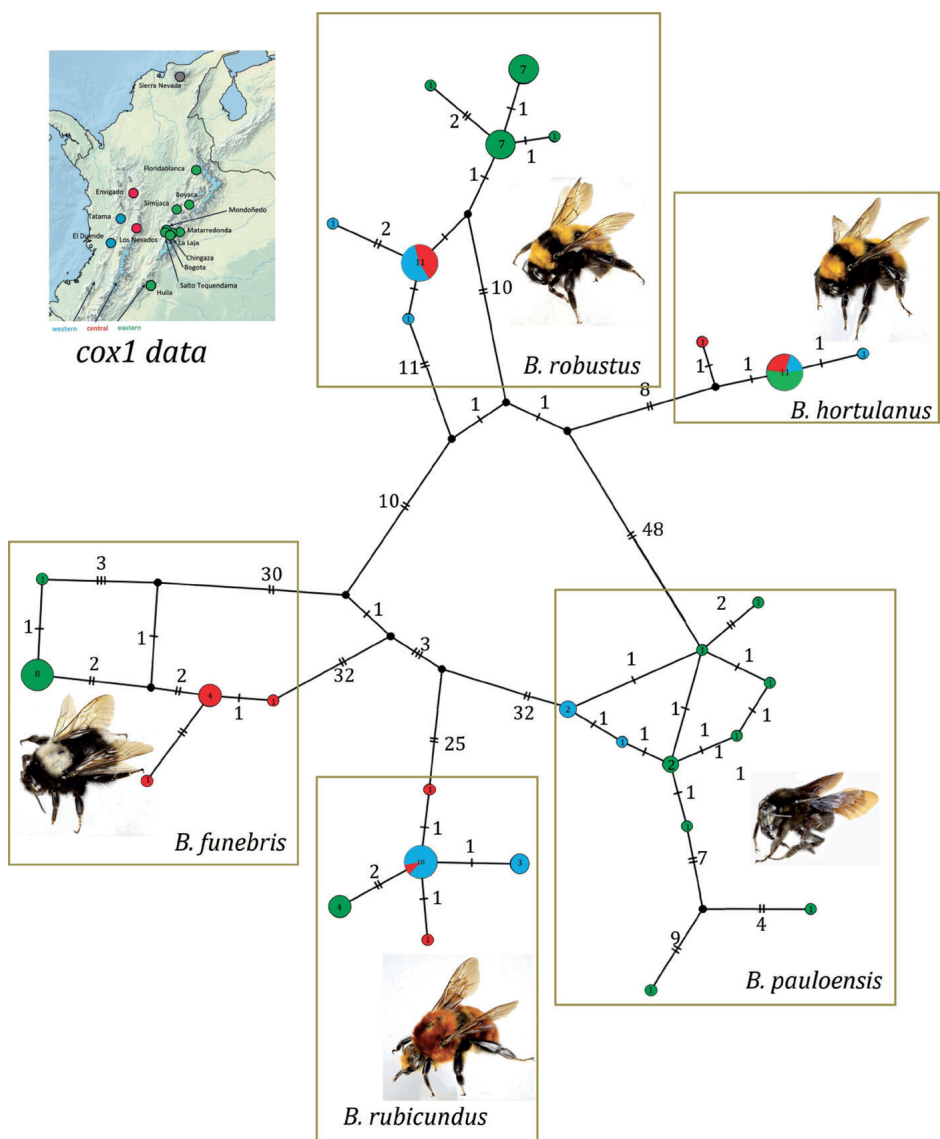


Fig. 2. Evolutionary relationships of haplotypes obtained with partial cytochrome *c* oxidase subunit I (*cox1*), gene fragment. Numbers close to the connector lines indicate the number of mutational steps. Colour code is as in Fig. 1.

full length barcode fragments. Amplification products were cleaned using the ammonium acetate purification protocol (Bensch et al. 2000), and sequenced in both directions.

Data analysis. Sequences were edited in Geneious software v. 11.0.4 (Kearse et al. 2012), and then, independent alignments for each marker and each species were generated. All sequences included are detailed in

Electronic Supplement 2. Haplotype networks were calculated for *cox1*, 16S and PEPCK fragments separately, and analysed by means of the TCS algorithm (Clement et al. 2000), implemented in PopART (Leigh & Bryant 2015).

Moreover, genetic distances were calculated using the Kimura 2 parameters (K2P) implemented in MEGA v.7 (Kumar et al. 2016).

Results

Haplotype networks

A total of 30 unique haplotypes of *coxI* were recovered from the five species analysed. Furthermore, for 16S and for PEPCK, 21 and 23 unique haplotypes were obtained respectively (Electronic Supplement 2).

Figure 2 shows the haplotype relationships of the *coxI* gene fragment of the five species analysed. *Bombus funebris* shows a genetic structure in which two haplogroups, one from the Central range (green dots) and one from the Eastern Mountain range (red dots), are separated by five to eight mutational steps

in a loop, and a genetic distance of 0.01 (Table 1). One to eight changes separate haplotypes within haplogroups. *Bombus pauloensis* haplotypes show a structured pattern, being separated according to the mountain chain to which they belong, most of the haplotypes were located in a loop, separated each from another by one to two changes.

Meanwhile, for *B. robustus* two well-differentiated haplogroups separated by three mutational steps were formed, clustering the haplotypes of the Central (blue dots) + Western chain of mountains in a mixed pattern, and the other grouping the haplotypes of the Eastern Cordillera. Furthermore, the Central chain haplogroup is separated from the Western chain group by a genetic distance of 0.0017. In contrast,

Table 1. Kimura-Two parameters genetic distances between species and groups within species in mountain ranges of the Genus *Bombus*. The colour code of the groups is as in Fig 1. Abbreviations: **ER**, eastern range; **CR**, central range; **WR**, western range; **SN**, Sierra Nevada.

Taxa 1 vs. Taxa 2			Genetic distance $\pm$ SD		
			cox1	16S	PEPCK
Species					
<i>Bombus funebris</i>	vs.	<i>Bombus hortulanus</i>	0.145 $\pm$ 0.0173	0.0790 $\pm$ 0.013	0.013 $\pm$ 0.0030
<i>Bombus funebris</i>	vs.	<i>Bombus robustus</i>	0.147 $\pm$ 0.0167	0.0790 $\pm$ 0.013	0.012 $\pm$ 0.0030
<i>Bombus funebris</i>	vs.	<i>Bombus rubicundus</i>	0.186 $\pm$ 0.0184	0.0870 $\pm$ 0.013	0.017 $\pm$ 0.0032
<i>Bombus funebris</i>	vs.	<i>Bombus pauloensis</i>	0.156 $\pm$ 0.0165	0.1200 $\pm$ 0.017	0.060 $\pm$ 0.0070
<i>Bombus hortulanus</i>	vs.	<i>Bombus robustus</i>	0.059 $\pm$ 0.0100	0.0120 $\pm$ 0.005	0.002 $\pm$ 0.0006
<i>Bombus hortulanus</i>	vs.	<i>Bombus rubicundus</i>	0.152 $\pm$ 0.0170	0.0742 $\pm$ 0.013	0.021 $\pm$ 0.0037
<i>Bombus hortulanus</i>	vs.	<i>Bombus pauloensis</i>	0.161 $\pm$ 0.0190	0.1130 $\pm$ 0.016	0.059 $\pm$ 0.0066
<i>Bombus robustus</i>	vs.	<i>Bombus rubicundus</i>	0.154 $\pm$ 0.0174	0.0800 $\pm$ 0.013	0.020 $\pm$ 0.0040
<i>Bombus robustus</i>	vs.	<i>Bombus pauloensis</i>	0.163 $\pm$ 0.0180	0.1100 $\pm$ 0.016	0.059 $\pm$ 0.0060
<i>Bombus pauloensis</i>	vs.	<i>Bombus rubicundus</i>	0.157 $\pm$ 0.0180	0.1170 $\pm$ 0.017	0.059 $\pm$ 0.0060
Groups within species					
<i>Bombus funebris</i>					
ER	vs.	CR	0.0100 $\pm$ 0.0040	0.01400 $\pm$ 0.0035	0.00020 $\pm$ 0.0001
<i>Bombus hortulanus</i>					
ER	vs.	CR	0.0010 $\pm$ 0.0090	–	0.00190 $\pm$ 0.0010
ER	vs.	WR	0.0400 $\pm$ 0.0040	–	0.00245 $\pm$ 0.0010
ER	vs.	SN	–	–	0.00120 $\pm$ 0.0006
CR	vs.	WR	0.0400 $\pm$ 0.0050	–	0.00210 $\pm$ 0.0009
CR	vs.	SN	–	–	0.00250 $\pm$ 0.0010
WR	vs.	SN	–	–	0.00120 $\pm$ 0.0007
<i>Bombus robustus</i>					
ER	vs.	CR	0.0057 $\pm$ 0.0020	0.00059 $\pm$ 0.0003	0.00200 $\pm$ 0.0006
ER	vs.	WR	0.0057 $\pm$ 0.0020	0.00012 $\pm$ 0.0001	0.00120 $\pm$ 0.0005
CR	vs.	WR	0.0017 $\pm$ 0.0007	0.00047 $\pm$ 0.0003	0.00150 $\pm$ 0.0005
<i>Bombus rubicundus</i>					
ER	vs.	CR	0.004 $\pm$ 0.0024	–	0.00040 $\pm$ 0.0003
ER	vs.	WR	0.004 $\pm$ 0.0023	–	0.00100 $\pm$ 0.0007
CR	vs.	WR	0.002 $\pm$ 0.0010	–	0.00040 $\pm$ 0.0003
<i>Bombus pauloensis</i>					
ER	vs.	WR	0.009 $\pm$ 0.0020	0.00380 $\pm$ 0.0025	0.01000 $\pm$ 0.0020

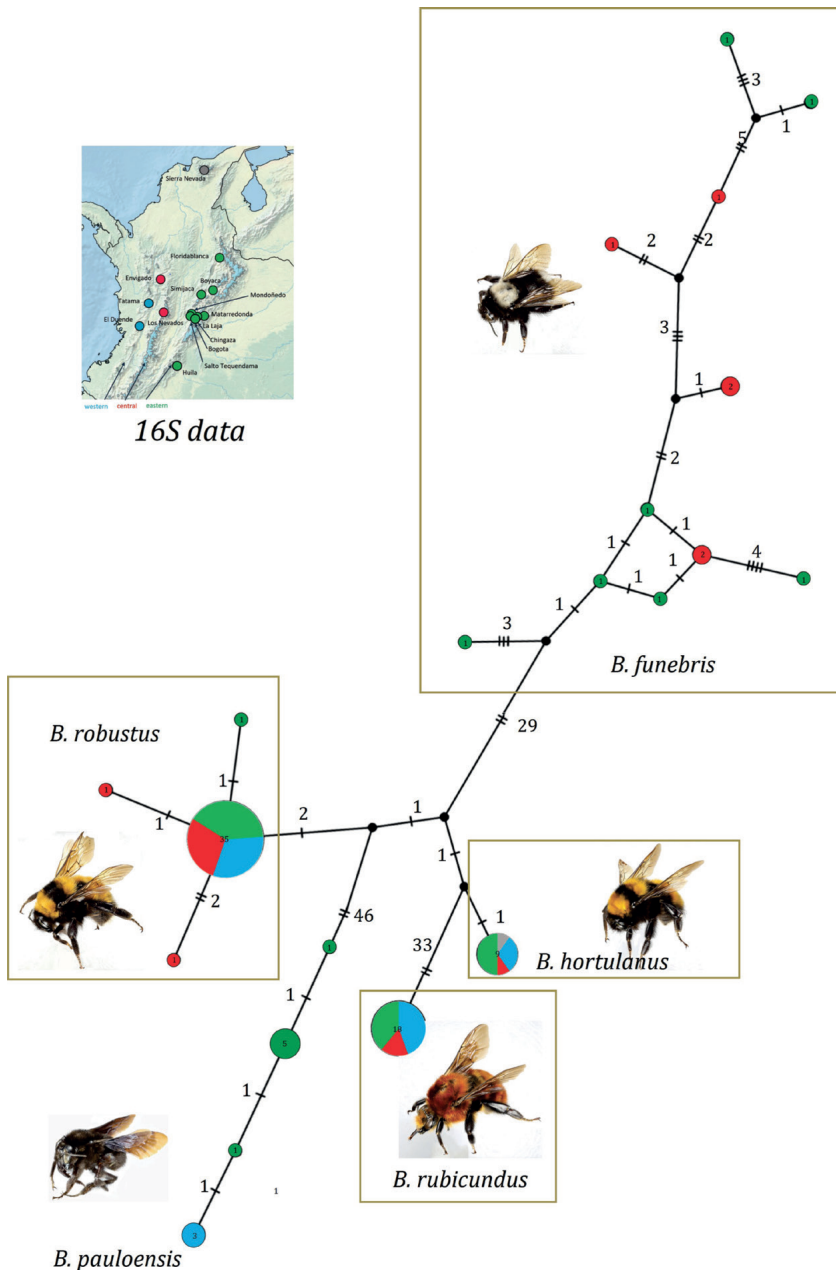


Fig. 3. Evolutionary relationships of haplotypes obtained with 16S gene fragment. Numbers close to the connector lines indicate the number of mutational steps. Colour code is as in Fig. 1.

both groups (Central + Western chain group) have a genetic distance of 0.0057 from the group formed in the Eastern Range (Table 1). The most common haplotype is distributed in the Central and Western Cordillera giving rise to two haplotypes that are

separated by one and two mutational steps. As for the Eastern group, the four haplotypes within it are separated by one to two mutational steps.

Bombus rubicundus showed a less differentiated pattern where the most common haplotype was

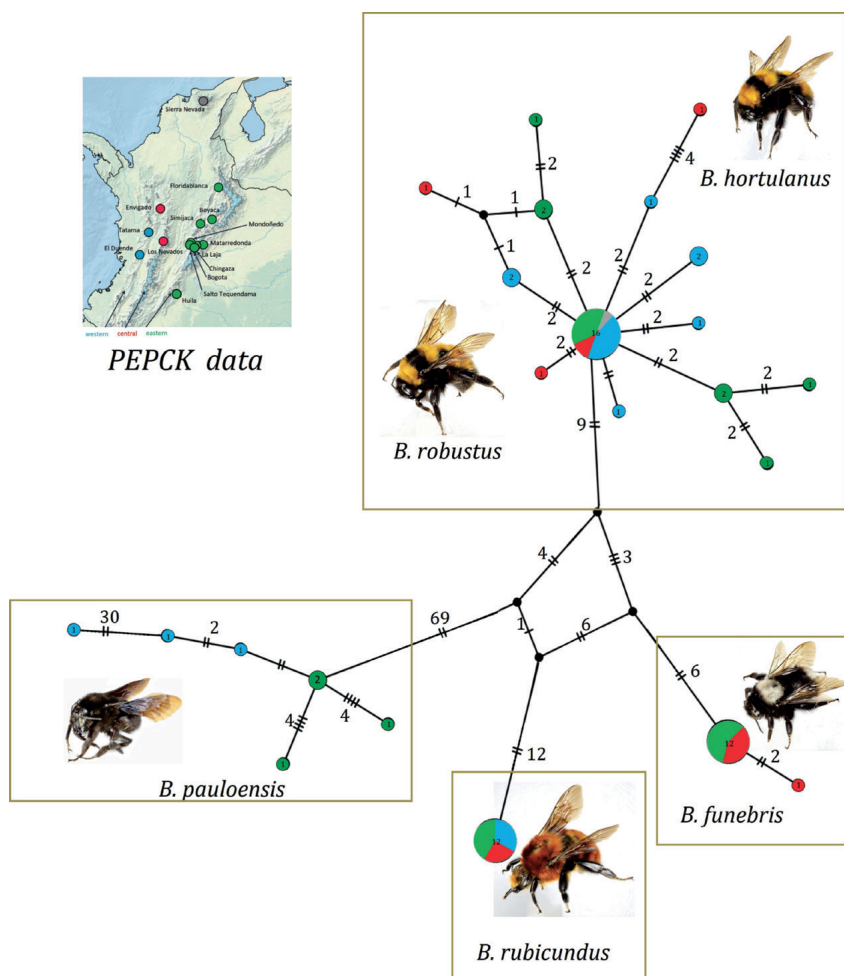


Fig. 4. Evolutionary relationships of haplotypes obtained with partial sequences of PEPCK gene. Numbers close to the connector lines indicate the number of mutational steps. Colour code is as in Fig. 1.

found in the Western and Central Mountain ranges. From this, other haplotypes emerge from the Eastern, Central, and Western Mountain ranges separated by one or two mutational steps.

B. hortulanus showed no genetic structure, the most common haplotype was found at the tree chain being separated from two other haplotypes by four mutational steps.

Of the five species analysed, only *B. pauloensis* showed a genetic structure for both, the 16S and PEPCK (Figs 3–4). Four 16S haplotypes were recovered, one from the Western and three from Eastern Mountain ranges. These haplotypes are separated by one to three mutational steps. In addition, six PEPCK haplotypes were organized according to their place of origin into two groups, with only one

mutational step separating the haplotypes from the Eastern range from those from the Western range. Interestingly, the inner genetic distance of the *B. pauloensis* haplogroup in the Western Mountain Range exceeds that between the species *B. funebris* and *B. hortulanus* (Table 2).

The eleven haplotypes of *B. funebris* obtained with 16S were connected one to another indistinctly of the chain of mountains where they belong. Meanwhile, the two PEPCK haplotypes were separated by a single mutational step and, one of them was shared between mountain ranges.

The *B. robustus* most common haplotype of 16S is shared between the three mountain chains giving rise to two haplotypes from the Central and one haplotype from the Eastern Cordilleras. *Bombus*

hortulanus showed one 16S haplotype. However, PEPCK gene showed a mixed pattern of *B. robustus* + *B. hortulanus* haplotypes separated by one to 3 steps between them. *Bombus rubicundus* showed just one haplotype of both 16S and PEPCK partial sequences.

Discussion

Over the past decade, an alarming decline in the abundance and distribution of bumblebees has been observed globally. Notably, these estimations may be subjected to the taxonomic scale of the studies, where the lack of information on genetic diversity could hide a greater loss of biodiversity in these bees (Cameron & Sadd 2020).

Whereas mitochondrial molecular markers offer valuable insights, their variability can be influenced by several confounding factors. These include the presence of nuclear copies of mitochondrial DNA (numts), hybridization between species followed by backcrossing (introgression), the coexistence of multiple mitochondrial DNA variants within an individual (heteroplasmy), and the persistence of

ancestral mitochondrial lineages within populations (incomplete lineage sorting) (Galtier et al. 2009, Meiklejohn et al. 2021). Such phenomena can lead to misinterpretations of population- or species-level relationships.

Nevertheless, the advantages of mitochondrial markers – such as their high mutation rate, abundance of copies per cell, uniparental (typically maternal) inheritance, and the vast amount of available comparative data – often outweigh these limitations, making them valuable tools in evolutionary and population genetic studies (Freeland et al. 2011). Furthermore, many of the issues caused by these confounding factors in phylogenetic analysis can be mitigated by methodological precautions, such as those employed in this study. These include translating nucleotide sequences into amino acids to check for internal stop codons, using multiple PCR replicates to confirm sequence consistency, employing different primer sets to minimize amplification bias, and integrating nuclear molecular markers to provide a complementary genetic perspective (Song et al. 2008, Fisher-Reid & Wiens 2011). Further approaches to mitigate such issues include the use

Table 2. Genetic distances within species of the genus *Bombus* calculated with Kimura-Two parameters. Along with genetic distances the standard deviations are provided. The colour code of the groups is as in Fig 1. Abbreviations: AS, all sequences; ER, eastern range; CR, central range; WR, western range.

Species	Genetic distance $\pm$ SD		
	cox1	16S	PEPCK
<i>Bombus funebris</i>			
AS	0.0073 $\pm$ 0.0021	0.0138 $\pm$ 0.0030	0.0002 $\pm$ 0.0001
ER	0.0007 $\pm$ 0.0005	0.0160 $\pm$ 0.0040	–
CR	0.0080 $\pm$ 0.0020	0.0100 $\pm$ 0.0030	0.0004 $\pm$ 0.0003
<i>Bombus hortulanus</i>			
AS	0.0019 $\pm$ 0.0007	–	0.0019 $\pm$ 0.0007
ER	0.0003 $\pm$ 0.0003	–	0.0012 $\pm$ 0.0006
CR	0.0020 $\pm$ 0.0020	–	–
WR	0.0800 $\pm$ 0.0080	–	0.0017 $\pm$ 0.0008
<i>Bombus robustus</i>			
AS	0.0040 $\pm$ 0.0014	0.0004 $\pm$ 0.0002	0.0014 $\pm$ 0.0004
ER	0.0030 $\pm$ 0.0010	0.0002 $\pm$ 0.0002	0.0012 $\pm$ 0.0005
CR	0.0018 $\pm$ 0.0010	0.0009 $\pm$ 0.0005	0.0020 $\pm$ 0.0007
WR	0.0015 $\pm$ 0.0007	–	0.0007 $\pm$ 0.0003
<i>Bombus rubicundus</i>			
AS	0.0030 $\pm$ 0.0010	–	0.0005 $\pm$ 0.0004
ER	0.0005 $\pm$ 0.0006	–	0.0005 $\pm$ 0.0003
CR	0.0020 $\pm$ 0.0015	–	0.0004 $\pm$ 0.0005
WR	0.0017 $\pm$ 0.0009	–	–
<i>Bombus pauloensis</i>			
AS	0.0110 $\pm$ 0.0020	0.00230 $\pm$ 0.0014	0.0086 $\pm$ 0.0010
ER	0.0140 $\pm$ 0.0030	0.00108 $\pm$ 0.0010	0.0020 $\pm$ 0.0007
WR	0.0010 $\pm$ 0.0010	–	0.0140 $\pm$ 0.0020

of next-generation sequencing (ngs) to generate high-coverage sequences of longer mitochondrial fragments, allowing for more accurate discrimination between true mitochondrial variants and nuclear or contaminant sequences (Meiklejohn et al. 2021). Additionally, the application of statistical and phylogenomic methods – such as coalescent-based species tree estimation, admixture analyses, and tests for gene flow – can help distinguish introgression from incomplete lineage sorting and assess the evolutionary relationships with greater confidence (Joly et al. 2009).

Using partial sequences of three molecular markers, *coxI*, 16S, and PEPCK, we characterized individuals of five bumblebee species distributed in highland ecosystems of the three mountain ranges of Colombia. Although the sample size was small for most species, high genetic variation within species regarding the barcode fragments was found.

Bombus pauloensis was found to have structured populations in all molecular markers. The disposition of haplotypes in two or more different clades or haplogroups, strongly suggest the presence of at least two different Evolutionary Significant Units (ESU) (Moritz 1994), each one corresponding to either Central or Eastern mountain range. The existence of different ESU in *B. pauloensis* populations, probably generated by the separation due to mountain ranges, is highly relevant for the species conservation, since this is a species widely used for managed pollination; and then subjected to translocation in different areas.

According to Hines (2008), the ancestor of the long-faced species *B. pauloensis*, *B. pullatus*, *B. brasiliensis*, and *B. transversalis* was one of the last to enter South America, about 2 mya, while the northern Andes reached the present altitudinal levels. During the following glacial period, *B. pauloensis* diverged from its sister taxa, *B. transversalis*, both are typical lowland species. It is been hypothesized that parapatric speciation must be the diversification mechanism for this species in which the highlands act as corridors to maintain a modest genetic flow between populations (Françoso et al. 2016). Furthermore, palaeoecological studies indicate that during the glacial periods, most of the extension of superpáramo ecosystems was covered by glaciers and páramo ecosystems were linked forming continuous belts (van der Hammen 1974). Such a scenario could avoid the crossing of individuals of a species whose current life zone is the middle mountain between 1800 and 2800 meters above sea level with sporadic higher registers (Liévano et al. 1991), to the Western flank of the Cordillera to further meet other populations from the Central or Western range, making high mountains, along with climatic conditions, isolating barriers (Lozier et al. 2011).

In contrast, the short-faced species *B. funebris*, *B. robustus*, and *B. rubicundus* showed a more perceptible structured pattern, where *coxI* haplotypes from the Eastern range of the Andes formed a separated group from the other mountain ranges. PEPCK and 16S, slow-evolving markers, have not shown structured patterns in these species suggesting a recent divergence.

Bombus rubicundus diverged about 7 mya; however, its ancestor was present in South America as early as 11 mya. Meanwhile, *B. funebris* diverged from the clade of Central and North American species *B. mcgregori* + *B. morrisoni* circa 7 mya. The ancestor of the *Robustobombus* clade came into South America about 6.5 mya. *B. robustus* and *B. hortulanus* nearest common ancestor is dated c. 2.5 mya, however, they diverged from their respectively sister taxa about 1 mya (*B. robustus*) and 0.5 mya (*B. hortulanus*) (Hines 2008).

Thus, all these currently cold-adapted species or their closest ancestors have experienced and accompanied part of the uprising of mountain ranges and the last glacial period, which, for some of the Andean species, act as barriers that favour their diversification (Chaves et al. 2011). Interestingly, the most common haplotypes of *coxI* in the structured species were found in the Central or Western mountain ranges which began their orogeny process in the late Cretaceous, before the Eastern mountain range uplift in the Oligocene (Mora et al. 2020); probably indicating that haplotypes in the Eastern Andes range are derivate from those in Central (Neigel & Avise 1993).

Besides, the relatively weak genetic structure observed in *B. funebris*, *B. rubicundus*, and *B. robustus*, as well as the absence of it in *B. hortulanus* indicates the existence of gene flow between populations of different mountain ranges. The small variations in *coxI* reported in the above-mentioned species may be more recent even than the glacial maximum.

The palynological evidence suggests that during the glacial period in the Pleistocene, the super páramo, páramo, sub páramo and forest ecosystems descended and expanded about 1000 to 1500 meters with respect to the current altitudinal limits (van der Hammen 1974, Thouret et al. 1996). Furthermore, recent investigations indicate that, during this period, connectivity between paramo areas was intermittent and highly dynamic, where Central and Eastern Cordilleras had a similar pattern in duration and frequency while, for Western Cordillera, different levels of connectivity were found. Thus, apart from the two passages reported as bridges between páramos connecting the Eastern Cordillera and the Colombian massif (from where the three branches of the mountain range emerge), and the passage

to the Magdalena River, a far more complex net of corridors, between and along Cordilleras, seemed to have existed. Such corridors would have been able to maintain the gene flow between populations (Flantua et al. 2019), particularly for *B. rubicundus*, *B. robustus*, and *B. hortulanus*, which seem to be the most tolerant of variations in their life zones (Pinilla-Gallego et al. 2017). These results are similar to those reported for euglossine bees, for which a cross-Andean dispersion pattern has been reported (Dick et al. 2004).

On the other hand, it is noteworthy that for *B. funebris*, *B. rubicundus*, and *B. robustus*, haplogroups have diversified along the sampled stripe in the Eastern Cordillera. This is probably related to the glacial-interglacial cycles of formation, posterior fragmentation, and further isolation of high mountain ecosystems at the top of mountain ranges. The formation of glacial environments reduces the genetic flow between populations in both flanks (Funk et al. 2005). Further studies, including samples from the Western flank of the Cordillera, should shed light on the actual influence that such climate changes in the glacial period had on the diversification of the analysed bumblebee species.

The global decline of pollinators has promoted research to mitigate the disappearance of these organisms. Despite this, in Colombia, the knowledge of the genetic diversity of bumblebees is scarce and fragmentary. In this sense, this study provides an understanding of patterns of genetic structure that can be used in further studies focused on determining the actual genetic diversity, the population size, and many other traits important in the design of policies for pollinators preservation.

Acknowledgements

The authors would like to thank the members of LABUN group, especially Natalia Flórez, Juan Diego Maldonado and Jesus Gomez Llano. We also want to thank the Administrative Special Unit of National Natural Parks for the sampling permissions and their kind collaboration. This study was funded by Minciencias contract N° FP44842-071-2016. We finally acknowledge extremely valuable support from the Alexander von Humboldt Foundation Research Group Linkage Programme: Evolution of the high Andean insect fauna project, which enabled us to meet in person in both Colombia and Germany on a regular basis.

Collection permits: The specimens deposited in biological collections were accessed in accordance with the provisions of Decree 1076 of 2015, and the newly collected samples were collected under permit 0255 of March 12, 2014, and permission N° 016 of 2016, granted by the National Environmental Licensing Authority (ANLA).

The authors declare no conflict of interest.

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