RESEARCH ARTICLE

Safeguarding the genetic integrity of native pollinators requires stronger regulations on commercial lines

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[Correction added on July 7, 2020, after first online publication: Peer review history statement has been added.]

Abstract

1. Every year more than 1 million commercial bumblebee colonies are deployed in greenhouses worldwide for their pollination services. While commercial pollinators have been an enormous benefit for crop production, their use is emerging as an important threat. Commercial pollinators have been linked to pathogen spillover, and their introduction outside their native area has had devastating effects on native pollinators. A more pervasive but underappreciated threat is their potential impact on the genetic integrity of native pollinators.

2. We set up a sampling and genotyping-plus-phenotyping protocol to evaluate the presence and extent of hybridization between commercial and native individuals of *Bombus terrestris* in south-western Spain, a region experiencing a huge propagule pressure of non-native genotypes due to the massive use of commercial colonies for crop pollination.

3. Our genomic data show clear evidence of generalized hybridization between native and introduced commercial bumblebee lineages in southern Spain. Only 19% of analysed individuals were assigned with high confidence to the pure native genetic cluster and >45% of sampled specimens were first-generation hybrids or backcrosses between native and commercial genotypes, indicating that genetic introgression is pervasive in southern Spain.

4. Although the frequency of commercial genotypes sharply declined with the distance to greenhouses, non-native alleles have introgressed into native populations inhabiting protected natural parks >60 km away from commercial bumblebee release areas.

5. As pollination services demand will increase in the coming years, only a more restrictive regulation of commercial lines could mitigate their negative impacts on the genetic integrity of native pollinators, avoid processes of genetic homogenization, and prevent the potential disruption of local adaptations.

KEYWORDS
Bombus terrestris, bumblebees, hybridization, managed pollinators, subspecies

1 | INTRODUCTION

In 1987, commercial rearing of bumblebees started in the Netherlands for the pollination of tomato crops. Nowadays, more than 30 commercial producers worldwide supply pollination services in more than 60 countries (Velthuis & van Doorn, 2006). While five species of bumblebees are reared commercially, most of the market is dominated by two species: *Bombus terrestris* and *Bombus impatiens.* *Bombus terrestris* colonies have been used for commercial pollination not only in its Eurasian native range but also in East Asia (Japan, South Korea, and China), South America (Chile), and New Zealand, and the eastern North American *B. impatiens* has been used in western North America and Mexico (Dafni, Kevan, Gross, & Goka, 2010; Velthuis & van Doorn, 2006). As each bumblebee colony can produce over 200 queens, it is not surprising that commercial species have escaped into the wild and established naturalized populations in the introduced areas (Seabra et al., 2019; Trillo et al., 2019). The consequences for native pollinators, including direct competition and the spread of pathogens (Colla, Otterstatter, Gegear, & Thomson, 2006), have been in some cases devastating (e.g. the decline of *Bombus dahlbomii* in Chile; Morales, Arbetman, Cameron, & Aizen, 2013; but see Revainera et al., 2020), and most countries, but not all, regulate nowadays the import of exotic species (Aizen et al., 2019).

The movement of a bumblebee species even within its natural area of distribution contains a more silent threat. *Bombus terrestris* is a widespread species divided into nine well-defined subspecies with contrasting coloration patterns and local geographical adaptations (Rasmont, Coppee, Michez, & De Meulemeester, 2008). For example while northern European subspecies hibernate, awakening from diapause in spring, southern subspecies aestivate and start their cycle in autumn. However, commercial colonies of some of the subspecies of *B. terrestris* have been widely used outside their natural distribution area. Several subspecies of *B. terrestris* were initially used in the early years of commercial rearing, but from the commercial point of view, *B. terrestris dalmatinus* proved to have superior characteristics and is the most commonly sold subspecies nowadays (Velthuis & van Doorn, 2006). Commercial producers often argue that queen production of commercial colonies, escape from greenhouse conditions, and survival in the wild are unlikely. This view has resulted in no measures taken in most countries to regulate subspecies trade within Europe. In contrast, evidence is piling up that both male and queen production of commercial colonies are high, the produced queens can survive in the wild (Owen, Bale, & Hayward, 2016), and mating is not only happening among subspecies (Ings, Raine, & Chittka, 2005), but also among related species (Kondo et al., 2009). Some recent studies have also demonstrated that different subspecies can interbreed and produce viable hybrids in the field (Kraus et al., 2011; Seabra et al., 2019). The genetic risks associated with releases of commercial species are largely neglected in conservation plans (Laikre, Schwartz, Waples, Ryman, & Ge, 2010); however, genetic pollution can lead to the breakdown of co-adapted gene complexes, erode local adaptation processes, and reduce

the ability of populations to deal with different components of global change (Prentis, White, Radford, Lowe, & Clarke, 2007; Rhymer & Simberloff, 1996; Todesco et al., 2016). Unfortunately, economic interest usually dominates decision-making, and in the absence of solid evidence of genetic pollution from commercial pollinators, only a few countries (see below) have regulated the genetic lines that can be used commercially.

Spain is one of the main vegetable producers in Europe and several crops, mainly tomato and different species of berries, use commercial bumblebees to supplement pollination. Commercial bumblebees have been used in Spain since 1992 (Ortiz-Sánchez, 1992; Ornosa, 1996), and albeit actual commercial species are not necessarily pure lines, most sold bumblebees probably belong to the subspecies *B. t. dalmatinus* or *B. t. terrestris*. However, in the Iberian Peninsula the native subspecies is *B. t. lusitanicus*, a taxon characterized by its distinctive legs with reddish setae (Rasmont et al., 2008). Recent studies show that the commercial subspecies actively forage in natural areas, can produce viable queens (Trillo et al., 2019), and hybridize with local subspecies (Seabra et al., 2019). However, current evidence for hybridization between commercial and native subspecies is either anecdotal (Seabra et al., 2019) or based on molecular markers that resulted unreliable (microsatellite markers) or are not able (mtDNA) to quantify levels of genetic introgression (Cejas, Ornosa, Muñoz, & De la Rúa, 2018; Cejas, López-López, Muñoz, Ornosa, & De la Rúa, 2020). This poses the question of whether hybridization and introgression from commercial subspecies represents an important threat to the genetic integrity of native populations or if, rather, this is a sporadic phenomenon with limited impact and ephemeral ecological and evolutionary consequences (Rhymer & Simberloff, 1996; Todesco et al., 2016). Here, we set up a sampling and genotyping-plus-phenotyping protocol to evaluate the presence and extent of hybridization between commercial individuals and the native subspecies in south-west Spain, an area with a huge propagule pressure of non-native genotypes. Our genomic data demonstrate for the first time generalized hybridization between native and introduced bumblebee subspecies, which urges for more restrictive regulations on commercial lines.

2 | MATERIALS AND METHODS

2.1 | Sampling

During 2017 and 2018, we collected via sweep-netting a total of 66 free-foraging individuals of *B. terrestris* from 28 sampling sites located at different distances from main greenhouse areas in southwest Andalusia, Spain (Figure 1; Table S1). Additionally, we sampled four individuals from purchased commercial colonies of the two main companies operating in the region (Agrobío S.L. and Koppert España S.L.; Table S1). We placed all sampled specimens in vials with 4 ml of ethanol 96% and stored them at −20°C until needed for phenotypic and genomic analyses.
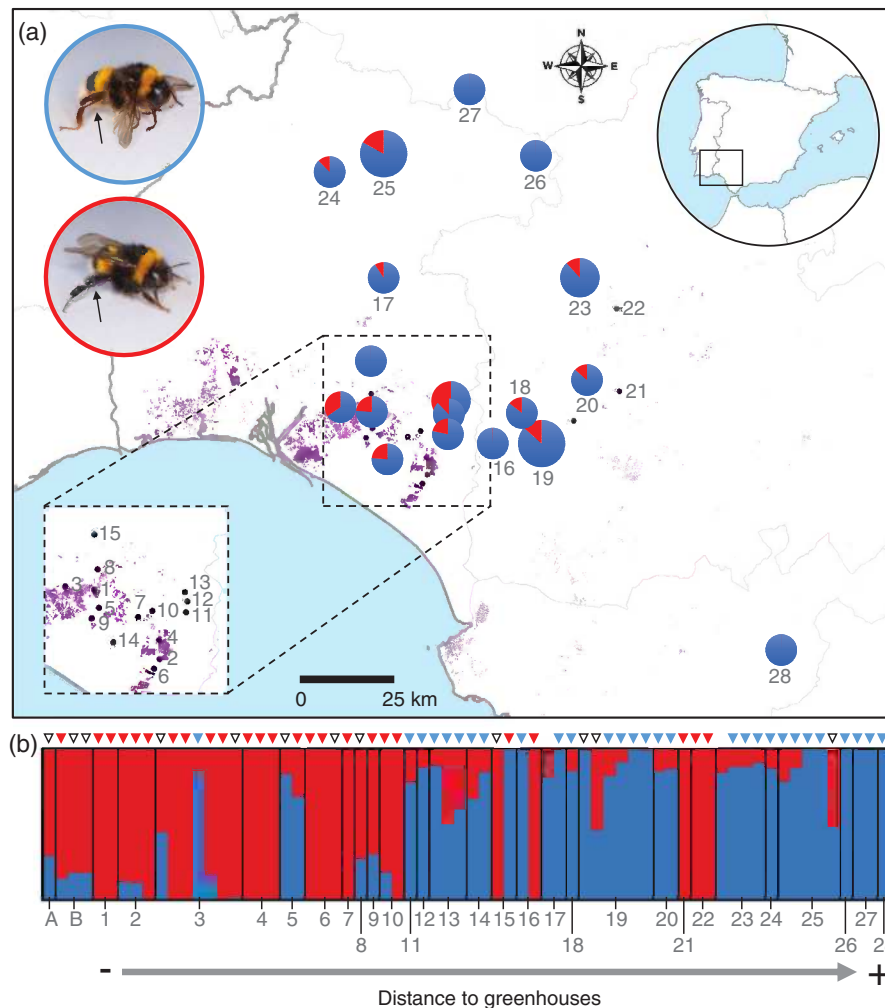
FIGURE 1 Hybridization between native and commercial bumblebees. (a) Map showing greenhouse areas (in purple) and sampling localities (black dots and pie charts) in south-western Spain. Pie charts (size proportional to number of genotyped individuals) show the posterior probabilities of assignment (q) to the native (blue) and non-native (red) genetic cluster inferred by the STRUCTURE program for each sampling locality. Pie charts are only shown for localities with individuals assigned with a probability >30% to the native genetic cluster (i.e. conservatively excluding potential commercial individuals with high admixed ancestry). (b) Bar plot shows the genetic assignment for all genotyped individuals, including four specimens sampled from commercial colonies (localities A and B) and 64 wild-caught individuals collected at 28 localities (sorted by distance to main greenhouse area). Inset pictures on panel (a) show a native *Bombus terrestris lusitanicus* (top, blue border) and a typical commercial individual (bottom, red border), with arrows indicating their respective reddish and black setae used to tentatively assign genotyped specimens to native, non-native, and hybrid (intermediate) phenotypes (blue, red, and white, respectively, triangles on top of STRUCTURE bar plot from panel b). Locality codes as in Table S1

2.2 | Phenotypic data

The main phenotypic trait characterizing the Iberian native subspecies *B. t. lusitanicus* is its distinctive legs with reddish setae, in contrast with the black or dark brown setae present in the subspecies *B. t. dalmatinus* and *B. t. terrestris*, which are most commonly used in commercial lines (Rasmont et al., 2008) (see Figure 1). Intermediate phenotypes (i.e. putative hybrids) show red cuticle and black setae (Figure S1). We used this trait to tentatively assign sampled specimens to native, non-native, and hybrid (intermediate) phenotypes. The same person (F.P.M.) phenotyped all specimens without a priori information about their respective genotype.

2.3 | Genomic library preparation and genomic data processing

We processed genomic DNA into one genomic library using the double-digestion restriction site–associated DNA sequencing procedure (ddRAD-seq) described by Peterson, Weber, Kay, Fisher, and Hoekstra (2012) with minor modifications detailed in Methods S1. We sequenced the library in a single-read 150-bp lane on an Illumina HiSeq2500 platform at The Centre for Applied Genomics (SickKids, Toronto, ON, Canada) and used the different programs distributed as part of the STACKS v.1.35 pipeline (*process_radtags, ustacks, cstacks, sstacks,* and *populations*) to assemble our sequences into de novo loci

and call genotypes (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). Methods S2 provide all details on sequence assembling and data filtering.

2.4 | Genetic assignment and hybrid identification

We identified hybrid and purebred individuals in our dataset using the Bayesian Markov chain Monte Carlo (MCMC) clustering method implemented in the program STRUCTURE v.2.3.3 (Pritchard, Stephens, & Donnelly, 2000). We conducted 15 independent runs for each value of $K = 1$–10 using 200,000 MCMC cycles after a burn-in step of 100,000 iterations, assuming correlated allele frequencies and admixture, and without using prior population information (Hubisz, Falush, Stephens, & Pritchard, 2009). We retained the 10 runs having the highest likelihood for each value of K and identified the number of genetic clusters best fitting the data using the ΔK method (Evanno, Regnaut, & Goudet, 2005). In STRUCTURE, the posterior probability (q) describes the proportion of an individual genotype originating from each of the K genetic clusters. We considered a q-value of .95 to classify individuals as purebreds or hybrids, an adequate threshold according to validation analyses based on genotypes simulated for different hybrid classes (F1, F2, and first-generation backcrosses) using HYBRIDLAB (Nielsen, Bach, & Kotlicki, 2006), and run with STRUCTURE considering the same settings than for our empirical dataset (Vähä & Primmer, 2006) (see Methods S3, Table S2, and Figure S2). Complementary to Bayesian clustering analyses and in order to visualize the major axes of genomic variation, we performed an individual-based principal components analysis (PCA) using the R v.3.3.3 (R Core Team, 2019) package *adegenet* (Jombart, 2008).

3 | RESULTS

3.1 | Genomic dataset

We obtained 132,125,053 reads (mean ± SD = 1,887,500 ± 610,189 reads/individual) across all genotyped individuals, of which 92% were retained after the different quality filtering steps in STACKS (Figure S3). After removing one of two individuals identified as full-siblings and another individual with a very low sequencing depth, the final dataset contained 68 non-sibling individuals (see Methods S2). The final exported dataset obtained with STACKS after removing loci that did not meet the population filtering requirements retained 9,063 single-single nucleotide polymorphism (SNP) loci.

3.2 | Genetic assignment and hybrid identification

Log probabilities [$Pr(X|K)$] of STRUCTURE analyses for the empirical dataset sharply increased from $K = 1$ to $K = 2$ and steadily from $K = 2$ to $K = 10$ (Figure S4). The ΔK method (Evanno et al., 2005) indicated that the best-supported number of clusters was $K = 2$ (Figure S4). Visual
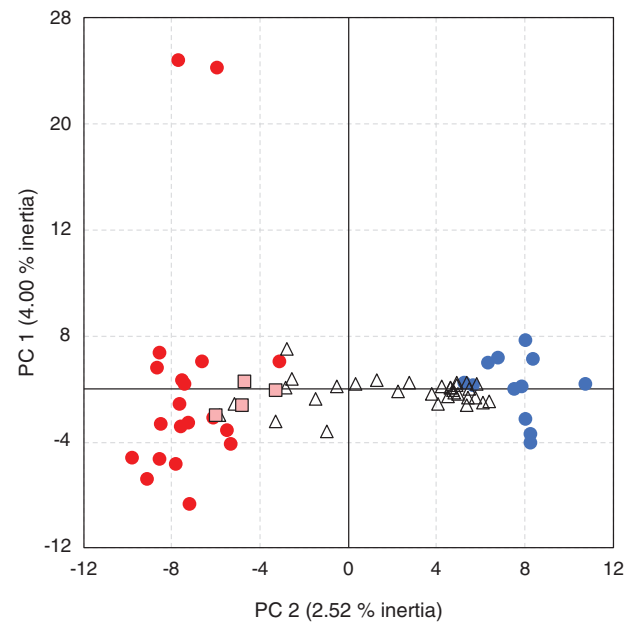

FIGURE 2 Principal component analysis (PCA) of genomic variation for 68 individuals of the bumblebee *Bombus terrestris*. Dots represent individuals assigned to the native (blue) and non-native (red) genetic clusters and open triangles indicate hybrids between them according to STRUCTURE analyses considering a threshold q-value of .95. Light red squares indicate the four specimens sampled from commercial colonies

inspection of the specimens collected in the field clearly shows individuals with *B. t. lusitanicus* phenotypes and individuals with commercial phenotypes or mix characteristics (Figure 1). Accordingly, one genetic cluster corresponded to the phenotypes of the native *B. t. lusitanicus* (hereafter, native genetic cluster) and the other to commercial phenotypes (hereafter, non-native genetic cluster) (Figure 1b; see Section 3.3 for more details). PCA also supported a clear separation along PC1 between the two genetic clusters identified by STRUCTURE analyses, with hybrid/introgressed individuals placed at an intermediate position (Figure 2). Accordingly, posterior probabilities of assignment (q) yielded by STRUCTURE were highly correlated with scores obtained for the first principal component (PC1) (Pearson's correlation, $r = .98$, $P < .001$).

Considering a threshold q-value of .95 to classify genotypes as purebreds or hybrids, 14 individuals were assigned with a high probability to the native genetic cluster, 20 individuals were assigned to the non-native genetic cluster, and 30 individuals were hybrids with different levels of genetic admixture (Figure 3). Around 31% of wild-caught specimens were assigned with a high probability to the non-native genetic cluster, indicating that they represent either commercial individuals foraging in natural or seminatural areas or the presence of colonies established in the wild from naturalized individuals. As expected, most of these individuals were sampled nearby the main greenhouse areas. Interestingly, the four individuals sampled from colonies of the two companies operating in the area were assigned with a probability of 14–29% to the native genetic cluster. This indicates that commercial lines are complex breeds
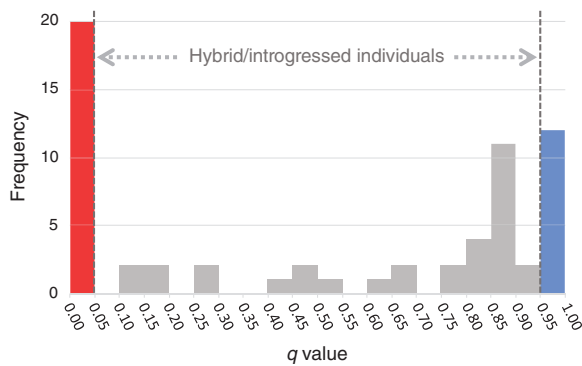
FIGURE 3 Frequency distribution of posterior probabilities of assignment (*q*-values) inferred by STRUCTURE analyses for our dataset of 64 wild-caught individuals of the bumblebee *Bombus terrestris*. Dashed vertical lines indicate threshold values of posterior probabilities (*q*-value = .95) used to classify individuals as purebred or hybrids. Blue and red bars indicate the frequency of purebred individuals assigned to the native and non-native genetic clusters, respectively. Grey bars indicate the frequency of individuals with different degrees of admixed ancestry (i.e. hybrid/introgressed individuals)

FIGURE 4 Genetic admixture scores (mean $\pm$ *SE*) obtained with (a) STRUCTURE (*q*-values) and (b) principal component analysis (PCA) (scores along PC1) for individuals that were tentatively identified as natives, non-natives, and hybrids based on their phenotype

probably originated from a mix of different lineages that might involve either *B. t. lusitanicus* or a third lineage genetically closer to it and for which we do not have reference genotypes. However, the most striking result is that only 19% of analysed individuals were assigned with a high confidence to the pure native genetic cluster and 50% of sampled specimens were first-generation hybrids or backcrosses between native and commercial genotypes, indicating that genetic introgression is pervasive in southern Spain (Figure 3).

3.3 | Correspondence between genotypic and phenotypic assignments

Genetic assignment scores inferred with either STRUCTURE (one-way ANOVA: $F_{2,63} = 86.80$, $P < .001$) or PCA (along PC1) (one-way ANOVA: $F_{2,63} = 90.13$, $P < .001$) were significantly different among individuals tentatively identified as natives, non-natives, and hybrids based on their phenotype ($P < .02$ for all post hoc Tukey's tests) (Figure 4). No individual assigned to the non-native genetic cluster was phenotypically identified as *B. t. lusitanicus* and 75% (nine out of 12) of individuals assigned to the native genetic cluster were tentatively classified as *B. t. lusitanicus* according to their phenotype (Figure 1). Remarkably, 63% of the individuals tentatively identified as native based on their phenotype had some degree of introgression from the primary commercial lineage (Figure 1b).

3.4 | Introgression spread

We found clear evidence that commercial bumblebee genotypes are spreading into native populations. The proportion of the non-native
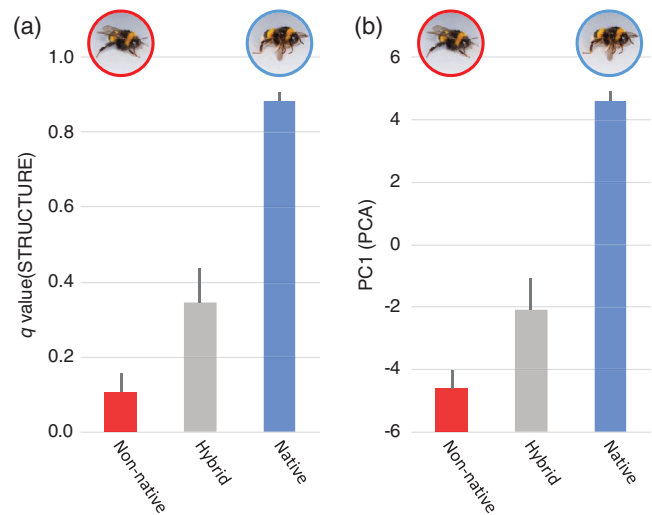
genotype decreased non-linearly with the distance to the main greenhouse areas either considering all individuals (exponential function: $F_{1,26} = 35.59$, $P < .001$, $R^2 = .58$; Figure 5a) or only those assigned with a probability >30% to the native lineage (i.e. conservatively excluding potential commercial individuals with admixed ancestry at a high confidence; logarithmic function: $F_{1,16} = 12.86$, $P = .002$, $R^2 = .45$; Figure 5b). Although the frequency of commercial genotypes sharply declined with the distance to main greenhouse areas, non-native alleles have introgressed into native populations inhabiting protected natural parks >60 km away from commercial bumblebee release points (Figure 1a).

4 | DISCUSSION

The consequences of hybridization are hard to predict, but displacing the locally adapted genotype is likely to reduce individual's fitness and have a negative impact on the performance of local populations (Prentis et al., 2007; Rhymer & Simberloff, 1996; Todesco et al., 2016). In fact, there have been observations that native subspecies densities decline nearby greenhouses, where commercial subspecies are more prevalent, indicating a potential competitive displacement (Trillo et al., 2019). This is not surprising as the newly arrived genotypes are maintained by a huge propagule pressure; hence, their populations are subsidized and do not depend on their performance in natural conditions. As commercial lines are selected for non-random characteristics, including big colonies and fast generation times, the introgressed alleles may be suboptimal and non-locally adapted (Rhymer & Simberloff, 1996). For instance, if alleles associated with adaptation to warmer temperatures from southern Spain are lost, the effects may be deleterious in a context of populations that occur in the southern tip of the species distribution with an increasing pressure from ongoing
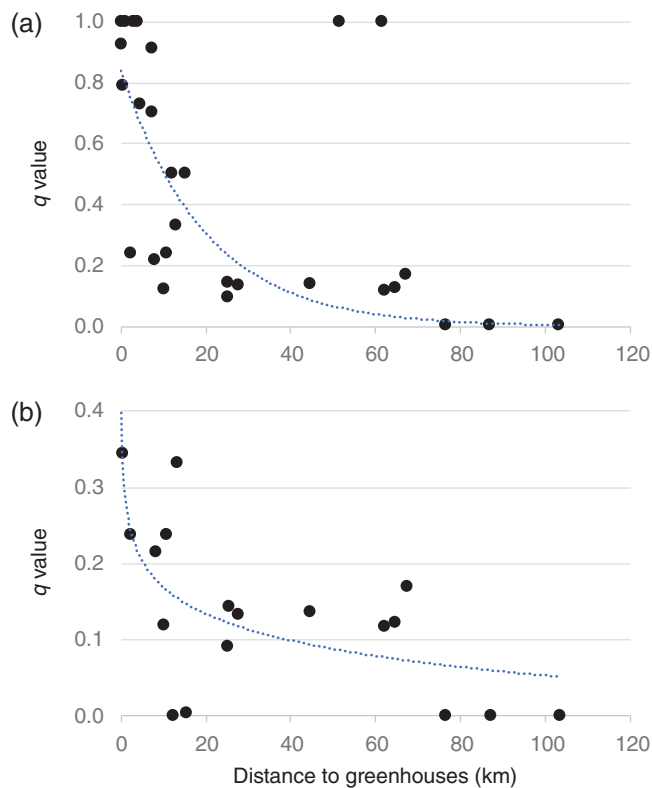
FIGURE 5 Introgression spread. Association between probability of assignment to the non-native genetic cluster and distance to main greenhouse areas considering (a) all analysed individuals or (b) only those assigned with a probability >30% to the native genetic cluster (i.e. conservatively excluding with a high confidence potential commercial individuals with admixed ancestry). Regression lines represent the function yielding the highest fit to observed data (panel a, exponential; panel b, logarithmic)

climate warming (Kerr et al., 2015). Our results support that genetic introgression is more widespread than initially thought. The few previous studies aiming to document hybridization events among bumblebee subspecies have focused on methodological aspects that allow hybrid detection and, indeed, have reported the presence of potential hybrids (Cejas et al., 2018; Cejas et al., 2020; Kraus et al., 2011). The most robust evidence so far comes from a recent study from Portugal, where the occurrence of hybridization events is confirmed using RAD-seq data (Seabra et al., 2019). However, the extent of hybridization reported in that study is moderately low, with <16% of wild-caught individuals (six out of 38 individuals) presenting genomic signatures of hybridization (see Figure 4 in Seabra et al., 2019). In contrast, we show a striking generalized process of genetic introgression in our study area, with >45% of wild-caught individuals (30 out of 65 individuals) presenting signs of hybridization between native and commercial lineages (Figure 1b). Remarkably, only 19% of our wild-caught individuals were assigned as genetically pure native individuals (vs. 79% in the study by Seabra et al., 2019) and the signatures of introgression in our study area expanded >60 km from the release sources (Figure 1a). This level of genetic introgression has the potential to put at serious risk the genetic identity of native pollinator populations. However, we lack robust tests of the magnitude and extent that this problem may cause elsewhere. For example in the United Kingdom, some colonies of the native *B. t. audax* subspecies, which historically hibernate during winter, have expanded their life cycle into the winter since early 2000s, a date matching the introduction of commercial lines of *B. t. dalmatinus* (Stelzer, Chittka, Carlton, & Ings, 2010). The possibility of genetic introgression in this context has been suggested but never evaluated. We fear that the Spanish case study is representative of what is occurring in several agricultural areas of EU.

In the past years, the regulation of commercial introductions of exotic species has advanced considerably, although international coordination is still needed (Aizen et al., 2019). However, the regulation on the use of local subspecies is falling behind. For example *B. terrestris* colonies are deployed in Europe without restrictions on the subspecies used with the exception of the Canary Islands (Spain), Norway, and since 2015, the United Kingdom. Imports of non-local bumblebees are also restricted in some West Asian countries such as Turkey and Israel. The case of the Canary Islands is exemplar, as only colonies of the locally native *B. t. canariensis* have been used since 1994. In contrast, the United Kingdom only recently tightened regulations to prevent the use of non-native subspecies, which were previously allowed to be purchased. In response, commercial producers have since 2015 only supplied the U.K. sub-species *B. t. audax*.

While stronger regulations may be seen as a threat to farmers depending on such resources, we do not believe it will be problematic. Two examples are illustrative here. First, the Norwegian authorities do not allow the importation of colonies from outside the country and required the local production of the endemic subspecies *B. t. terrestris*. As the Norwegian market is small, the three big companies dominating the market refused to supply this service, but two local companies emerged to fill the gap. In the Middle East, the Israeli authorities do not allow the importation of bumblebee colonies from outside the country. As the Israeli market is big, it was profitable for one of the leading international companies to move production operations within the country in order to supply the local market. In both cases, the trade of colonies is minimized, with the increased benefit of avoiding the spread of novel pathogens (Graystock et al., 2013).

As the market expands, more countries in North Africa and West Asia will turn to commercial sources of bumblebees for pollination. Strong regulations from day one are necessary to ensure preserving genetic diversity and local adaptations of native populations (Rhymer & Simberloff, 1996; Todesco et al., 2016). Beyond bumblebees, the demand for the development of other commercial pollinators is increasing. Mason bees (*Osmia* spp.) are already being sold as pollinators in Europe and the United States with no clear regulations on their shipment. For example *Osmia bicornis* from Germany can be bought and released elsewhere in Europe with no restrictions. There are three distinctive subspecies of *O. bicornis* (*O. b. bicornis*, *O. b. cornigera*, and *O. b. fractinoris*) and the above-exposed evidence with bumblebees teaches us that extra care is needed when moving them outside their respective native ranges. A similar scenario happens in the United States, where *Osmia lignaria* has two distinct subspecies (*O. l. propinqua* and *O. l. lignaria*) which should not be moved beyond their native ranges. As far as

we know, trade of *O. lignaria* subspecies outside its distribution range is not happening, because the leading *Osmia* seller in the United States (CrownBees) has regulated this on a personal decision, but no legislation is in place preventing this to happen when the market expands.

We urge countries to issue regulations enforcing the use of local genetic lines. The demand for commercial pollination services will keep increasing in parallel with the demand for pollinator-dependent crops (Aizen, Garibaldi, Cunningham, & Klein, 2008) and the technification of cropping systems around the world. As new pollinator taxa will be domesticated in the future to fulfil this demand, the time is ripe to recognize the high risks commercial pollinators entail for the genetic diversity of local native pollinators and act consequently. Breeding of local genetic lines has also the potential to minimize transport and spread of pathogens and to create opportunities in the local markets. We cannot allow commercial arguments to overrule ecological considerations.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

I.B. and J.O. designed the study. F.P.M. and A.H.-G. conducted field and laboratory work. J.O. analysed the genomic data. I.B. drafted the manuscript. All authors contributed to writing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/2688-8319.12012

DATA AVAILABILITY STATEMENT

Raw Illumina reads have been deposited at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA626483: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA626483 (Bartomeus, Molina, Hidalgo-Galiana, & Ortego, 2020a). Input files for all analyses are available for download at FigShare: https://doi.org/10.6084/m9.figshare.12154662 (Bartomeus, Molina, Hidalgo-Galiana, & Ortego, 2020b).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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