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SHORT NOTE

Preliminary report on cross-species microsatellite amplification for bumblebee biodiversity and conservation studies

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Additional keywords

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SUMMARY

The Iberian Peninsula holds a high diversity of bumblebees but there is a general lack of information about their biodiversity in this area. To overcome this and facilitate conservation studies, we present two novel multiplex assays for the amplification of six and five microsatellite loci respectively. Both assays successfully amplified for most of the studied species in the Iberian populations. Sibling workers and population genetic parameters were analysed in the managed species *B. terrestris* and in the wild species *B. monticola* and *B. mesomelas*, demonstrating the capability of these multiplex assays for biodiversity studies of both managed and wild bumblebee species.

Análisis preliminar de la amplificación de microsatélites en abejorros para estudios de biodiversidad y conservación

RESUMEN

La península Ibérica alberga una gran diversidad de abejorros, pero falta mucha información sobre su biodiversidad en esta zona. Para evitar esto y facilitar estudios de conservación, presentamos dos novedosos ensayos para la amplificación múltiple de seis y cinco loci de microsatélites respectivamente. Ambos ensayos funcionaron con éxito para la mayoría de las especies estudiadas en las poblaciones ibéricas. Se detectaron las obreras hermanas y se infirieron parámetros genético-poblacionales en la especie manejada *B. terrestris* y en las especies silvestres *B. monticola* y *B. mesomelas*, demostrando la capacidad de estos ensayos para estudios de biodiversidad de especies de abejorros tanto manejadas como silvestres.

Palabras clave adicionales

Genética de la conservación.
Apidae.
Bombus.
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INTRODUCTION

The need to conserve genetic diversity in wild bee populations and understanding its structure and function to design successful breeding and conservation programs has been highlighted recently (López-Uribe, Soro & Jha, 2017). Because of several potential causes, populations of both managed and wild bees have been reported to be declining during the last decade in different parts of the world (Biesmeijer *et al.* 2006). In this context, despite the large bumblebee species richness present in the Iberian Peninsula with 39 out of the 79 West-Palearctic species distributed in this area (Ornosa & Ortiz-Sánchez, 2004), few molecular studies have been undertaken to know the genetic diversity of Iberian bumblebee taxa. Such studies are becoming crucial to the conservation of these important pollinators given the reduction of the altitudinal distribution range towards better-preserved high areas observed in Pyrenean populations of several bumblebee species (Ornosa, Torres & De la Rúa, 2017). On the other hand, the distribution ranges of managed species as *B. terrestris* has changed due to escapes from agricultural facilities, especially in southern Spain, where

bumblebee breeding companies are located to supply pollinators to the many greenhouses (Ortiz-Sánchez 1992; Ornosa 1996; Cejas et al. 2018; Trillo et al. 2019).

An efficient approach for population genetic studies is to analyse the variation of microsatellite markers which allows genotyping different individuals with many loci, and thus elucidation of their genetic diversity and conservation status. Such markers are widely used in studies on the honeybee (Evans *et al.* 2013) and have been also implemented in stingless bees (see Hurtado-Burillo *et al.* 2014, as an example). Given the importance of the pollinating function of the bumblebees, and the economic benefits derived from its trade (Velthuis & Doorn, 2006), the validation of tools to explore the genetic diversity of *B. terrestris* are necessary to maintain the genetic diversity of commercial breeds.

In this work, we selected 11 microsatellite loci developed by Estoup *et al*. (1996) from *B. terrestris* and designed two novel multiplex assays of six and five microsatellites based on Wolf, Rhode and Moritz (2010). Multiplex assays were cross-amplified in ten wild *Bombus* species and one reference species (*B. terrestris*) to test their efficacy. The chosen species were *B. lucorum*, *B. hortorum*, *B. lapidarius*, *B. humilis*, *B. mesomelas*, *B. ruderarius*, *B. vestalis*, *B. pratorum*, *B. soroeensis* and *B. monticola*. These species have a conservation status of Less Concern (Nieto *et al*. 2014); however, some of them, like *B. mesomelas*, are in regression in its distribution range in Europe (Rasmont *et al*. 2015) and the Pyrenees (Ornosa, Torres & De la Rúa, 2017), while the managed *B. terrestris* is expanding its distribution range. To our knowledge, *B. mesomelas* and *B. monticola* are here microsatellite-genotyped for the first time.

MATERIAL AND METHODS

Sampling and DNA extraction

Individuals were collected during sampling campaigns in 2013-2015. DNA was extracted from one leg of each individual following Walsh, Metzger and Higuc (1991) or Ivanova, Dewaard and Hebert (2006).

Genotyping and amplification effectivity

Each amplification reaction contained 1X reaction buffer, 1.2 mM of $MgCl_2$, 0.3 mM of dNTPs, 0.2 µM of each primer (multiplex RB1: B10, B100, B11, B124, B126, B96 and multiplex RB2: B118, B119, B121, B131 and B132 with forward primers fluorescent-labelled, **Table I)**,

Table I. Summary information for microsatellite multiplex reactions. Observed (H_o) and expected (H_E) heterozygosity and null alleles frequency (F_{NULL}) are given for *B. terrestris* (reference species) and *B. mesomelas* and *B. monticola* (tested species) (Información resumida para reacciones multiplexas de microsatélites. Se proporcionan heterocigosidad observadas (HO) y esperadas (HE) y frecuencia de alelos nulos (FNULL) para *B. terrestris* (especie de referencia) y *B. mesomelas* y *B. monticola* (especies probadas).

Multiplex/ Locus	Primer sequence 5'–3'	Dye	*B. terrestris* (N=12)			*B. mesomelas* (N=13)			*B. monticola* (N=10)		
RB1			H_o	H_E	F_{NULL}	H_o	H_E	F_{NULL}	H_o	H_E	F_{NULL}
B10	5'- GTGTAACTTTCTCTCGACAG-3'	PET	0.875	0.900	0.036	-	-	-	0.846	0.722	0
	5'-GGGAGATGGATATAGATGAG-3'										
B100	5'-CGTCCTCCTATCGGGCTAAC-3'	VIC	0.750	0.771	0.062	0.308	0.260	0	0.462	0.355	0
	5'-CCTCGAAACCTCGTGACG-3'										
B11	5'-GCAACGAAACTCGAAATCG-3'	FAM	0.750	0.805	0.030	0.923	0.867	0	1	0.822	0
	5'-GTTCATCCAAGTTTCATCCG-3'										
B124	5'-GCAACAGGCGGGTTAGAG-3'	NED	0.875	0.846	0	0.692	0.675	0	0.385	0.556	0.110
	5'-CAGGATAGGGTAGGTAACAG-3'										
B126	5'-GCTTGCTGGTGAATTGTGC-3'	NED	0.938	0.840	0	0.769	0.749	0	0.615	0.530	0
	5'-CGATTCTCTCGTGTACTCC-3'										
B96	5'-GGGAGAGAAAGACCAAC-3'	VIC	0.563	0.611	0.022	0.846	0.781	0	0.385	0.746	0.207
	5'-GATCGTAATGACTCGATATG-3'										
RB2											
B118	5'-CCTAACTCCCTATATCITCG-3'	FAM	0.750	0.781	0.061	-	-	-	-	-	-
	5'-GAAACACCTATCTACATCTACAG-3'										
B119	5'-CATCGTGCTAGAAAAGGAAG-3'	NED	0.563	0.461	0	0	0	0	0	0	0
	5'-CCACACTGCAAAGITTCTG-3'										
B121	5'-GAACATGTGGAACGACGG-3'	NED	0.250	0.361	0.109	0.846	0.858	0.007	0.308	0.260	0
	5'-GAACAATCGATATGTCACCC-3'										
B131	5'-GATCGCCTATCTCITCTCGG-3'	FAM	0	0	0	0.462	0.604	0.133	0.923	0.793	0
	5'-GAGGCGCTCTCGACCTC-3'										
B132	5'-GAAATCGTGCCGAGGG-3'	VIC	0.667	0.840	0.062	0.153	0.145	0	0.923	0.754	0
	5'-CAGAGAACTACCTAGTGCTACGC-3'										

Primer sequences from Estoup *et al*. (1995, 1996).

Table II. Scoring efficiency of the cross-species amplification of 11 microsatellite loci in *Bombus* species. For each species is given the number of individuals (N), percentage of amplified individuals for each locus (%), locus size range (SR) and number of alleles (Na) and relative fluorescence intensity (RFI) of the amplification, ranked as low (L, fluorescence intensity <900 RFUs and high failure percentage), medium (M, fluorescence intensity ≈1000 RFUs) and high (H, fluorescence intensity >1900 RFUs) (Eficiencia de puntuación de la amplificación entre especies de 11 loci microsatélites en especies de Bombus. Para cada especie se proporciona el número de individuos (N), porcentaje de individuos amplificados para cada locus (%), rango de tamaño de locus (SR) y número de alelos (Na) e intensidad de fluorescencia relativa (RFI) de la amplificación, clasificado como bajo (L, fluorescencia intensidad <900 RPU y alto porcentaje de fallo), medio (M, intensidad de fluorescencia ≈1000 RPU) y alto (H, intensidad de fluorescencia >1900 RPU).

Species		B10	B100	B11	B124	B126	B96	B118	B119	B121	B131	B132
B. lucorum	%	100	100	100	90	100	100	100	100	70	100	80
N= 10	SR (Na)	186-230 (13)	132-182 (10)	157-171 (5)	238-252 (5)	166-202 (11)	233-235 (2)	210-218 (5)	128-130 (2)	157 (1)	121 (1)	145-185 (9)
	RFI	H	H	H	H	H	H	H	H	H	H	H
B. terrestris	%	100	100	100	100	100	100	100	100	100	100	94
N= 16	SR (Na)	186-222 (14)	152-170 (7)	154-172 (8)	232-260 (10)	116-202 (12)	234-11246 (5)	208-222(7)	128-132 (3)	158-166 (3)	121 (1)	157-175 (9)
	RFI	H	H	H	H	H	H	H	H	H	H	H
B. hortorum	%	40	83	100	100	100	100	0	80	100	100	60
N=5	SR (Na)	178-180 (2)	146-162 (4)	140-158 (5)	260-280 (7)	148-204 (7)	238-248 (5)	-	124 (1)	155-181 (6)	122-136 (5)	149-175 (6)
	RFI	L	M	M	M	M	M	-	L	H	H	L
B. lapidarius	%	100	100	100	100	100	100	100	100	100	100	100
N=10	SR (Na)	208-240 (10)	156-174 (5)	160-186 (6)	266-278 (5)	139-159 (8)	244-256 (6)	207-223 (7)	124 (1)	157-165 (2)	136-152 (6)	153-161 (5)
	RFI	H	H	H	H	H	H	H	H	H	H	H
B. humilis	%	100	100	100	100	100	100	100	100	100	100	100
N=8	SR (Na)	178-186 (4)	136 (1)	130-132 (2)	248-252 (2)	134-144 (2)	224-236 (4)	212-216 (2)	124-128 (2)	158-164 (2)	121-125 (3)	143-155 (5)
	RFI	M	H	H	H	H	H	H	H	H	H	H
B. ruderarius	%	100	100	100	100	100	100	100	0	100	100	100
N=6	SR (Na)	174-194 (8)	136 (1)	136-144 (4)	246-258 (4)	136-142 (3)	241-251 (6)	212-216 (3)	-	158-164 (2)	121 (1)	145-151 (4)
	RFI	H	H	H	H	H	H	H	-	H	H	H
B. mesomelas	%	0	100	100	100	100	100	0	94	100	100	94
N=16	SR (Na)	-	148-154 (2)	156-202 (11)	256-264 (5)	162-182 (8)	238-250 (5)	-	124 (1)	140-174 (12)	121-125 (3)	143-155 (5)
	RFI	-	H	M	H	H	M	-	M	H	H	L
B. vestalis	%	100	100	100	100	100	100	100	100	0	100	100
N=3	SR (Na)	196 (1)	144-154 (1)	138 (1)	234 (1)	147-161 (3)	254-256 (2)	230-248 (2)	136 (1)	-	126-128 (2)	153-163 (3)
	RFI	L	L	M	M	L	L	L	H	-	M	M
B. pratorum	%	100	86	100	100	100	100	0	86	100	100	86
N=7	SR (Na)	194-196 (2)	148-154 (2)	132-138 (2)	232-234 (2)	136 (1)	237 (1)	-	124 (1)	149-165 (3)	117-127 (3)	176-180 (3)
	RFI	M	M	H	H	H	M	-	H	M	H	L
B. soroeensis	%	100	86	86	100	100	100	0	0	0	100	100
N=7	SR (Na)	200-212 (4)	150-168 (6)	160-176 (6)	250-258 (4)	194-224 (8)	251-265 (7)	-	-	-	137-153 (6)	169-179 (5)
RFI	-	M	L	M	M	L	M	-	-	-	M	L
B. monticola	%	100	100	100	100	100	100	0	100	100	100	100
N=13	SR (Na)	212-226 (5)	154-156 (2)	137-157 (8)	232-236 (3)	160-164 (3)	245-253 (4)	-	124 (1)	151-153 (2)	129-143 (7)	164-182 (8)
	RFI	H	H	H	H	H	H	-	H	H	M	M

1.2 mg/ml of BSA, 1.5 U of Kapa Biosystems Taq and ~40 ng of DNA. The same PCR conditions were used for both assays: initial denaturation at 95 ºC for 5 min, followed by 30 cycles of 30 s at 92 ºC, 30 s at 54 ºC and 30 s at 72 ºC, with a final elongation of 30 min at 72 ºC. Alleles were separated using capillary electrophoresis on an ABI Prism 3700 (Applied Biosystems) and scored with Genemapper 4.8 software (Applied Biosystems). The fluorescence intensity (measured in relative fluorescent units, RFU) was ranked as low (L, fluorescence intensity <900 RFUs and high failure percentage), medium (M, fluorescence intensity ≈1000 RFUs) and high (H, fluorescence intensity >1900 RFUs) (Appendix 1) and used as a measure of amplification effectivity. GenAlEx 6.5 (Peakall & Smouse, 2012) was used to estimate allele size range (SR) and number of alleles (Na) per locus.

Population genetic analysis

Population parameters were only analysed in the managed species *B. terrestris* (reference species) and in the wild species *B. monticola* and *B. mesomelas* (tested species) to study the efficacy of our multiplex assays to obtain genetic diversity parameters. Sibling workers from the same colony inferred with Colony 2.0.6.2 (Wang, 2012) were excluded from further analyses. Micro-Checker 2.2.3 (Van Oosterhout *et al*. 2004) was used to calculate the frequency of null alleles. Observed (Ho) and expected heterozygosity (He) values were obtained with GenAlEx 6.5. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated with Genepop on the web (Rousset, 2008). Bonferroni correction was applied to LD results to avoid type I errors.

RESULTS AND DISCUSSION

Of the 121 species-locus combinations, 91.7% amplified correctly (based on the relative fluorescence intensity observed), showing B118 the lowest amplification success (**Table II**). In total 82% of combinations were polymorphic, although this rate could be bias by the number of individuals analysed per species (i. e. only three *B. vestalis* individuals could be sampled). The obtained results show that both multiplexes presented here are suitable for population studies of the analysed species since even datasets with only eight microsatellite loci (as in Maebe *et al.* 2015) provide appropriate genetic information for conservation purposes.

In the reference species (*B. terrestris*), the number of alleles ranged from 1 (B121) to 14 (B10) (mean: 7.18) (**Table II**). Four sibling workers were excluded for population analysis (final N=12). Heterozygosity and null allele frequency results were similar to those obtained by Moreira *et al*. (2015) at European level, confirming the efficacy of the multiplex assays in the Iberian population. Neither significant deviation from HWE nor LD were found. In the tested species *B. mesomelas*, two (B10 and B118) of the 11 loci did not amplify (**Table II**). Number of alleles varied from 1 (B119) to 11 (B11) (mean: 5.7). Three sibling workers were removed from population analysis (final N=13). No significant deviations from HWE, LD or null alleles were found. In *B. monticola*, one marker (B118) did not amplify, while in the other ten loci, the percentage of amplification achieved 100% and the number of alleles ranged from 1 (B119) to 8 (B11) (mean: 4.3). Three sibling workers were removed from population analysis (final N=10). Significant deviation from HWE was found in B96, which might be due to the existence of null alleles, observed at a frequency of 0.207. LD test revealed no significantly linked loci after applying Bonferroni correction.

CONCLUSIONS

In conclusion, the obtained results showed that the amplification of these 11 microsatellite loci optimized in two multiplex assays might be useful to genotype both wild Iberian bumblebee populations of many of the studied species and the commercial breeds of *B. terrestris*. Moreover, they could potentially be used in populations with other origin. As demonstrated by the results obtained in the population analysis of *B. mesomelas* and *B. monticola*, the use of these loci might be helpful to depict the population structure and genetic differentiation of bumblebee populations and for future assessments of their conservation status. This is especially urgent in mountain habitats as the Pyrenees since an upward trend towards better-preserved high areas has been observed in the bumblebee populations (Ornosa, Torres & De la Rúa, 2017). On the other hand, multiplex assays have shown a good resolution to infer the genetic parameters of the managed species *B. terrestris*. This result provides a suitable tool to value the genetic diversity of the bumblebee breeds in companies producing nests for pollination of crops, as well as to determine the gene flow between managed and wild populations of this important species (Kraus et al. 2011).

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