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RESEARCH ARTICLE

Is hybridisation with non-native congeneric species a threat to the UK native bluebell *Hyacinthoides non-scripta*?

Markus Ruhsam[1] | Deborah Kohn[1] | Jeannine Marquardt[2,3] | Andrew R. Leitch[2] | Harald Schneider[4] | Johannes Vogel[5] | Spencer C. H. Barrett[6] | Philip E. Hulme[7] | Jane Squirrell[1] | Peter M. Hollingsworth[1]

[1]Royal Botanic Garden Edinburgh, Edinburgh, UK

[2]School of Biological and Behavioural Sciences, Queen Mary University of London, London, UK

[3]Department of Life Sciences, Natural History Museum, London, UK

[4]Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Yunnan, China

[5]Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung, Berlin, Germany

[6]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada

[7]Department of Pest Management and Conservation, Lincoln University, Lincoln, New Zealand

Correspondence
Markus Ruhsam, Royal Botanic Garden Edinburgh, Edinburgh, UK.
Email: m.ruhsam@rbge.ac.uk

Societal Impact Statement

Hybridisation is an important evolutionary force in plants, but it can potentially lead to genetic swamping and extinction of one or both parental species. The threat of extinction is of particular concern if hybridisation occurs between native and introduced species, especially when the native is of national importance. The widespread occurrence of non-native bluebells in the United Kingdom has raised concerns that the iconic native bluebell could be at risk due to extinction by hybridisation from introduced non-native bluebells. This study determines the taxonomic identity of non-natives and investigates the amount of hybridisation occurring in natural and semi-natural UK bluebell populations.

Summary

- The widespread occurrence of a non-native bluebell taxon in the UK has raised concerns that the iconic native bluebell *H. non-scripta* (Asparagaceae) could be at risk due to extinction by hybridisation from introduced non-native congeners. Understanding the nature of this threat requires quantification of the extent of hybridisation between the native and non-native taxa. An additional complication is taxonomic uncertainty regarding the identity of the non-native bluebells in the United Kingdom that are colloquially referred to as the 'Spanish' bluebell (*H. hispanica*).
- We collected 501 bluebell samples from 56 populations in the United Kingdom (*H. non-scripta* and non-natives) and the Iberian Peninsula (*H. hispanica*). The samples were assayed for variation at 1871 nuclear and 17 plastid single nucleotide polymorphisms.
- Our genetic analyses demonstrated that non-native bluebells in the United Kingdom are not *H. hispanica* but the hybrid between *H. hispanica* and *H. non-scripta*. Moreover, they supported the hypothesis that Portugal is the country of origin of the first *H. hispanica* introductions to the United Kingdom. The

frequency of hybrids was about 16%. Backcrosses between the (hybrid) non-native bluebell and the native *H. non-scripta* were primarily found in public parks. Of the sampled individuals for *H. non-scripta* from natural habitats, only 2% showed evidence of introgression.

- Although hybridisation might be frequent in locations where non-native bluebells have been introduced, we found no evidence of large-scale introgression in natural *H. non-scripta* populations. Therefore, our results do not support concerns of an 'extinction by hybridisation' scenario.

KEYWORDS
conservation, extinction, hybridisation, introgression, Spanish bluebell

1 | INTRODUCTION

Although hybridisation can be an important creative evolutionary force (Abbott et al., 2005; Arnold, 2006; Guo et al., 2006; Lexer et al., 2003), it might also lead to genetic swamping of one species by the other or even to the extinction of one or even both parental species (Hegde et al., 2006; Rhymer & Simberloff, 1996; Todesco et al., 2016). However, the threat of extinction is difficult to predict and is likely to depend on numerous factors such as the strength of reproductive barriers that isolate hybridising taxa, the vigour and fertility of hybrids, the size of the hybridising populations, habitat requirements, self-incompatibility alleles and herbivore and pathogen pressures (Carney et al., 2000; Ellstrand & Elam, 1993; Huxel, 1999; Levin, 2000; Todesco et al., 2016; Wolf et al., 2001). These considerations could have conservation implications if the hybridising species are both native taxa, as, for example, the existence of a locally rare species might be potentially threatened due to hybridisation with a more widespread congener (Ruhsam et al., 2015). However, the threat of extinction or genetic swamping is often perceived even more acutely if hybridisation happens between native and introduced plant taxa (Bleeker et al., 2007; Burgess et al., 2005; Moody & Les, 2002; Prentis et al., 2007; Py et al., 2017; Ruhsam et al., 2019; van Kleunen et al., 2015), especially when the native plant taxon is regarded as emblematic and/or of national importance. Additionally, hybridisation may serve as a stimulus for the evolution of invasiveness as progeny with a hybrid background may have one or more potential genetic benefits relative to their progenitors (Ellstrand & Schierenbeck, 2000). However, there is currently little evidence that hybridisation generally poses a major threat to native taxa as a recent global survey concluded that out of 870 invasive species (including animals and plants) there is only evidence for 16 (2%) species that might potentially threaten native taxa via hybridisation (Hirashiki et al., 2021).

Concerns over hybridisation and possible genetic swamping are relevant for the case of the British native bluebell *Hyacinthoides non-scripta* (L.) Chouard ex Rothm. (Asparagaceae), which hybridises with naturalised non-native bluebells in Britain (Figure 1) (Kohn et al., 2009; Pilgrim & Hutchinson, 2004; Rix, 2004; Stace et al., 2015). The non-native taxon, thought to be *H. hispanica*, was introduced to British gardens by 1683 (Preston et al., 2002), and the earliest wild UK *H. hispanica* specimen was collected in 1875 in Yorkshire (specimen held at the British Museum, BM). Although the non-native taxon is commonly referred to as the 'Spanish' bluebell in the United Kingdom, it is not clear whether it is truly the Iberian

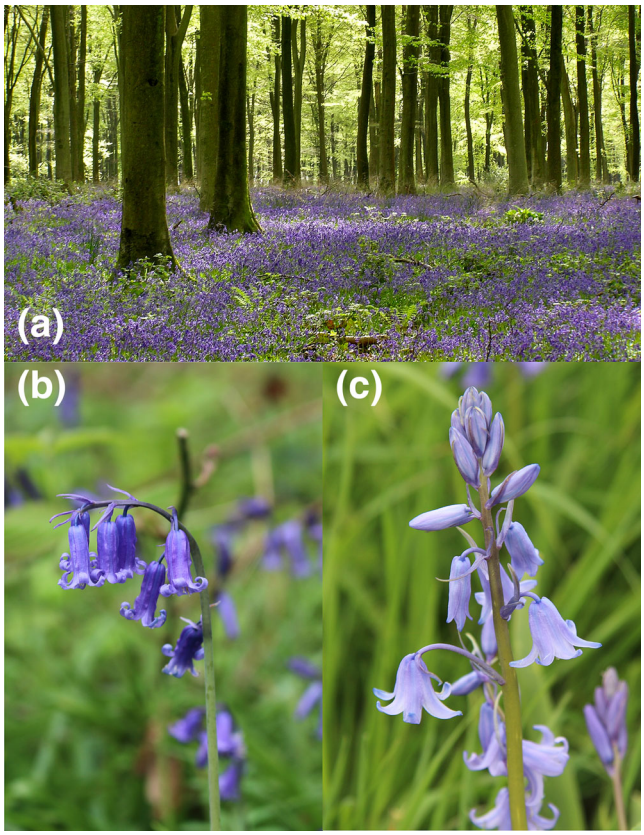

FIGURE 1 *Hyacinthoides* taxa in the United Kingdom. (a) Semi-natural woodland with the native *H. non-scripta* in England. (b) Typical morphology of the native *H. non-scripta* with one-sided tubular flowers on nodding inflorescences. (c) Typical morphology of the non-native *Hyacinthoides* taxon colloquially referred to as 'Spanish' bluebell with campanulate flowers on upright inflorescences. Photo credits: (a) SCHB, (b) DK and (c) MR.

bluebell *H. hispanica* (Mill.) Rothm. or a hybrid between *H. non-scripta* and *H. hispanica* (*H. x massartiana* Geerinck), or a combination of both, as records in the Botanical Society of Britain and Ireland database suggest (https://database.bsbi.org/maps/). A recent morphological study that included *H. non-scripta*, *H. hispanica* and the non-native UK bluebell revealed that British non-native bluebells were morphologically similar to *H. hispanica* but occupied a separate phenotypic space (Ruhsam et al., 2020). These results were consistent with, but did not provide conclusive evidence for, the possible hybrid status of British non-native bluebells.

Despite the uncertainty regarding the taxonomic status of the non-native bluebell in the United Kingdom, it is clear that this taxon is widespread, occupying 36% (*H. hispanica*) to 48% (*H. x massartiana*) of the 10 km × 10 km ('hectad') UK recording grids (Ruhsam et al., 2020). However, it is uncertain how much of a threat the non-native bluebell poses to the native bluebell populations. *H. non-scripta* is also a widespread species in the United Kingdom, which often covers extensive areas, especially in woodlands, and is only absent from hectads in parts of the Scottish Highlands. It is estimated that the UK harbours up to half of the world's population of this species (Ingrouille, 1995); its worldwide range extends along the Atlantic coast of mainland Europe to Spain and Portugal (Grundmann et al., 2010).

Using an experimental open-pollinated setting with equal representation of taxa, Kohn et al. (2019) showed that the hybridisation rate (proportion of between-taxon offspring) between UK native and non-native bluebells was about 40%. The results indicated that *H. non-scripta* plants were more successful as both maternal (56.7% vs. 43.3% of seeds produced by natives and non-natives, respectively) and paternal parents (about 3 times more seeds were sired by natives) than non-natives. Additionally, seeds from native bluebells had a significantly higher germination rate than non-natives (40% vs. 28%, respectively), and pollen fertility of one native and one non-native population showed that the mean viability from native *H. non-scripta* pollen was significantly higher than from non-natives (Kohn et al., 2019). In northern Spain, where the native range of *H. non-scripta* and *H. hispanica* overlap, artificial pollination experiments involving intraspecific crosses resulted in more seeds per fruit than the interspecific (F_1-generation) crosses, whereby interspecific crosses with *H. non-scripta* as the pollen donor resulted in a small increase in seed per fruit compared with interspecific crosses with *H. hispanica* as the pollen donor (Marquardt et al., 2022). These results indicated preliminary evidence for constraints on the risk of genetic swamping of native bluebells by introduced bluebells (Prentis et al., 2007). Nevertheless, uncertainties remain as to the frequency with which non-natives/hybrids are found together with natives in the core woodland habitats of the native bluebell and the subsequent risk of introgression by pollinator-mediated cross-pollination; bluebells are insect pollinated, mainly by *Bombus* species and syrphid flies (Kohn et al., 2009).

The aim of this study was (1) to establish the taxonomic identity of UK non-native bluebells, that is, whether they are likely to be the Iberian bluebell *H. hispanica* or hybrids between *H. hispanica* and *H. non-scripta* and (2) to assess whether hybridisation between native and non-native bluebells is widespread in the United Kingdom by sampling populations in a range of habitats from garden like settings to woodlands. The primary goal of our work is to better understand the nature and extent of the threat to the native *H. non-scripta* from non-native bluebells from hybridisation.

2 | MATERIALS AND METHODS

2.1 | Sampling of plant material

Leaf samples of a total of 501 individuals were collected in silica gel from 56 populations in Spain, Portugal, and the United Kingdom as well as seven individuals from UK commercial garden centres (Figure 2). To investigate the taxonomic identity of UK non-native bluebells (i.e., whether they are likely to be *H. hispanica* or hybrids between *H. non-scripta* and *H. hispanica*), the sampling included 412 native (*H. non-scripta*, $n = 340$) as well as non-native bluebell samples ($n = 72$) from 38 populations located throughout the United Kingdom (Table 1). We used morphological characters following Kohn et al. (2009) to classify each assayed plant as either native (*H. non-scripta*) or non-native. We made a special effort to include individuals in typically native *H. non-scripta* populations, which displayed one or two atypical morphological traits for this species, that is, traits usually associated with non-natives such as pink flowers, wider leaves or flowers, thicker scapes or upright inflorescence but otherwise looked phenotypically native. These plants were classed as morphologically 'native-unusual' (Table 1) and were included to maximise the chance of detecting introgression from non-natives into native bluebell populations.

Of the 38 UK populations we sampled, seven were chosen because they had a very high proportion of non-native bluebells (highlighted as 'non-native' in Table 1, $n = 69$ plants). Fifteen populations were further selected to represent purportedly native bluebell woodlands, in order to assess current levels of introgression (first 15 entries of UK populations; AR to TM, Table 1). These 15 populations were sampled at a higher density with an average number of 19.2 samples per population ($n = 288$ plants). The remaining 16 native-looking populations ($n = 55$ plants) were sampled at low density with an average of 3.4 samples per population.

We also included samples from nine *H. non-scripta* populations in Spain ($n = 50$ plants, 'Iberian *H. non-scripta*') as well as five Spanish and four Portuguese *H. hispanica* populations ($n = 39$ plants, '*H. hispanica*'). A total of 87 samples was included from Marquardt (2017) (see Table 1). All samples from the Iberian Peninsula were collected from populations where the other species has not been recorded. Additionally, seven samples from seven commercial UK garden centres that were either sold as *H. non-scripta* ($n = 3$ plants) or as *H. hispanica* ($n = 4$ plants) were included (Table 1).

DNA was extracted using CTAB following Doyle (1990) but included a 10-min 1xTNE wash (200 mM Tris-HCl, 250 mM NaCl, 50 mM EDTA, 1 mL/sample) before starting the CTAB protocol.

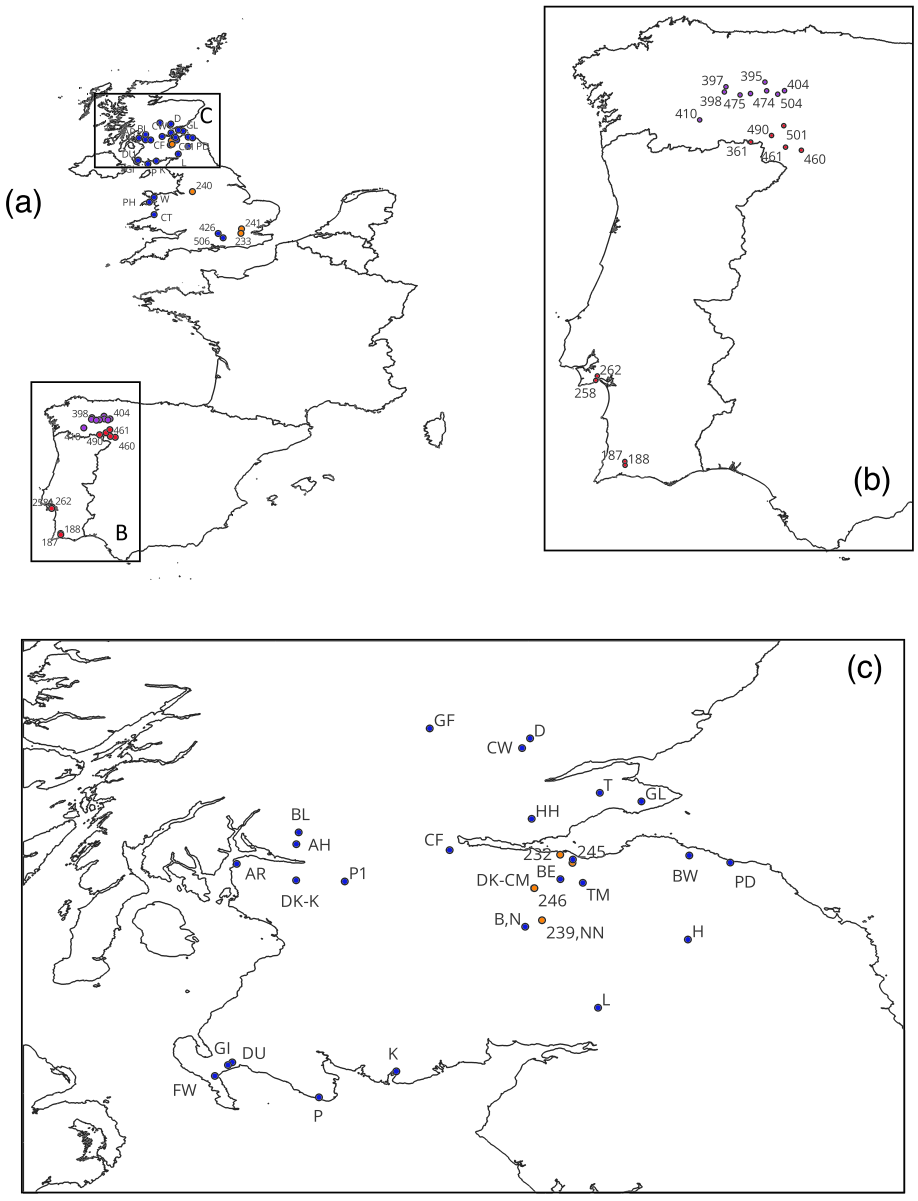

FIGURE 2 (a) Map of *Hyacinthoides* sampling locations in the United Kingdom and the Iberian Peninsula. (b) Detailed view of locations on the Iberian Peninsula. (c) Detailed view of sampling locations in Scotland. Colour codes: red denotes *H. hispanica*, purple Iberian *H. non-scripta*, blue UK *H. non-scripta* and orange UK non-native populations.

2.2 | Re-sequencing and processing of reads

Genome-wide markers have recently been developed by sequencing the transcriptomes of three European bluebell species, that is, *H. hispanica*, *H. non-scripta* and *H. paivae* (Marquardt, 2017; Marquardt et al., 2022). All samples apart from the 87 taken from Marquardt (2017) (see Table 1) were re-sequenced for 164 nuclear regions as well as 12 organelle regions comprising eight plastid and four mitochondrial genes (each about 150–200 bp long, Dataset S1). PCR amplification and paired-end library preparation were carried out using the 48.48 Access Array Fluidigm workstation at the Bart's and the London Genome Centre, UK. All 164 amplicons were barcoded by sample, pooled and sequenced on an Illumina MiSeq (250 bp, paired end).

Trimmomatic v 0.33 (Bolger et al., 2014) and cutadapt v 1.8.1 (Martin, 2011) were used to filter reads according to their quality, to remove primer sequences and trailing bases with an average quality below Q30 (across four bases), and read pairs shorter than 100 bp. The trimmed reads were aligned to the 176 target genes for each sample separately using bwa v 0.7.15 (Li & Durbin, 2009) and SAMtools v 1.5 (http://samtools.sourceforge.net). Variant discovery and genotype calling were carried out using GATK v 3.5 (McKenna et al., 2010) and vcftools 0.1.16 (Danecek et al., 2011), removing indels (–remove-indels) and filtering SNPs with a quality of less than 60 (–minQ 60) and less than 500 coverage (–DP 500). Hard filtering was applied to exclude variants with missing information in more than 30% of the samples, and samples with more than 20% missing data were excluded.

2.3 | Genetic analyses

To visualise the genetic relationships between populations, a discriminant analysis of principal components (DAPC) was carried out using

TABLE 1 Sampling locations for bluebell sites and the number of individuals used for the single nucleotide polymorphism (SNP) array.

Species	Population	Code	Lat.	Long.	Country	SNP	Morphology		
							Native	Native-unusual	Non-native
Iberian Peninsula (IP)									
H. hispanica	Monchique	187	37.313	−8.546	Portugal	1	1	n/a	n/a
	Parq de Mina[1]	188	37.258	−8.542	Portugal	2	2	n/a	n/a
	Quinta de Anjou[1]	262	38.557	−8.946	Portugal	2	2	n/a	n/a
	Setubal	258	38.492	−8.969	Portugal	2	2	n/a	n/a
	Calabor*	361	41.959	−6.715	Spain	5	5	n/a	n/a
	Tabara*	460	41.840	−5.978	Spain	6	6	n/a	n/a
	Ferreras*	461	41.884	−6.208	Spain	7	7	n/a	n/a
	Lanseros*	490	42.054	−6.412	Spain	7	7	n/a	n/a
	Tomeros*	501	42.197	−6.234	Spain	7	7	n/a	n/a
IP Total	**9**					**39**	**39**	**0**	**0**
H. non-scripta	Susane de Sul*	395	42.831	−6.508	Spain	7	7	n/a	n/a
	Castelo*	397	42.763	−7.075	Spain	2	2	n/a	n/a
	Pacios*	398	42.687	−7.098	Spain	5	5	n/a	n/a
	Pobladura*	404	42.706	−6.223	Spain	5	5	n/a	n/a
	As Corrainzas*	410	42.281	−7.457	Spain	3	3	n/a	n/a
	Toreno*	472	42.704	−6.483	Spain	7	7	n/a	n/a
	Villabuena*	474	42.664	−6.718	Spain	7	7	n/a	n/a
	Trabadelo*	475	42.644	−6.870	Spain	7	7	n/a	n/a
	Folgoso*	504	42.655	−6.322	Spain	7	7	n/a	n/a
IP Total	**9**					**50**	**0**	**0**	**0**
United Kingdom (UK)									
Native	Ardgowan[S]	AR	55.919	−4.864	Sco	20	10	10	n/a
Native	Ashfield House	AH	56.022	−4.559	Sco	20	10	10	n/a
Native	Balmaha	BL	56.084	−4.547	Sco	20	10	10	n/a
Native	Beeslack[S]	BE	55.839	−3.207	Sco	22	10	12	n/a
Native	Brock Wood	BW	55.963	−2.547	Sco	13	8	5	n/a
Native	Broughton	B,N	55.590	−3.387	Sco	26	14	12	n/a
Native	Callendar[S]	CF	55.991	−3.774	Sco	17	8	9	n/a
Native	Carlops 1	DK-CM	55.792	−3.342	Sco	24	12	12	n/a
Native	Gillingshill	GL	56.246	−2.792	Sco	17	7	10	n/a
Native	Glentyan Ho[S]	DK-K	55.833	−4.560	Sco	20	10	10	n/a
Native	Lochore Meadows	HH	56.155	−3.354	Sco	18	9	9	n/a
Native	Pease Dean	PD	55.926	−2.337	Sco	19	10	9	n/a
Native	Pollok[B]	P1	55.827	−4.311	Sco	12	3	9	n/a
Native	Tarvit[S]	T	56.291	−3.005	Sco	19	10	9	n/a
Native	Temple	TM	55.820	−3.092	Sco	21	10	11	n/a
Native	Balgreggan	FW	54.809	−4.976	Sco	3	3	0	0
Non-native	Carlops 2	246	55.792	−3.340	Sco	6	0	0	6
Native	Carreg Ti-pw	CT	52.315	−4.149	Wal	3	3	0	0
Native	Court Wood	CW	56.525	−3.403	Sco	3	3	0	0
Non-native	Craigmillar	CM	55.924	−3.145	Sco	16	0	0	16
Native	Beenham Woods	426	51.426	−1.136	Eng	2	2	0	0
Native	Darroch Wood	D	56.576	−3.362	Sco	4	4	0	0
Native	Dunragit Wood	DU	54.880	−4.887	Sco	2	2	0	0

(Continues)

TABLE 1 (Continued)

Species	Population	Code	Lat.	Long.	Country	SNP	Morphology		
							Native	Native-unusual	Non-native
Non-native	Edinburgh-RBGE[B]	232	55.967	−3.208	Sco	10	0	0	10
Non-native	Epping[B]	241	51.642	0.038	Eng	14	0	0	14
Native	Gennoch Mains	GI	54.866	−4.909	Sco	3	3	0	0
Native	Glassie Farm	GF	56.628	−3.876	Sco	3	3	0	0
Native	Harestane	H	55.523	−2.554	Sco	5	5	0	0
Native	Holyrood[B]	245	55.942	−3.143	Sco	6	5	0	1
Native	Kirkudbright[B]	K	54.833	−4.047	Sco	3	1	0	2
Native	Langholm	L	55.166	−3.014	Sco	5	5	0	0
Non-native	Manchester[B]	240	53.399	−2.347	Eng	3	0	0	3
Native	Molash*	506	51.223	0.903	Eng	3	3	0	0
Native	Physgill House[S]	P	54.697	−4.443	Sco	2	2	0	0
Native	Plas Hendre[S]	PH	52.904	−4.373	Wal	4	3	1	0
Non-native	Stobo	239,NN	55.624	−3.301	Sco	15	0	0	15
Non-native	Sydenham[B]	233	51.436	−0.067	Eng	6	1	0	5
Native	Ty-newydd	W	53.127	−4.152	Wal	3	3	0	0
UK Total	**38**					**412**	**192**	**148**	**72**
UK + IP Total	**56**					**501**			
Commercial *H. non-scripta*	n/a	CB	n/a	n/a	UK	3	n/a	n/a	n/a
Commercial *H. hispanica*	n/a	CB	n/a	n/a	UK	4	n/a	n/a	n/a
Grand Total						**508**			

Note: Iberian populations with a superscript denote number of samples (1 = one sample, * = all samples), which are from Marquardt (2017). 'n/a' not applicable, superscript for UK populations denotes populations from park-like grounds of stately homes ([S]) or large built-up areas ([B]). Country abbreviations: 'Sco' Scotland, 'Eng' England, 'Wal' Wales. 'SNP' number of samples used for the SNP array; 'Morphology', UK samples were classified as 'native' or 'non-native' based on their morphology. Putative *H. non-scripta* plants that displayed one or two unusual morphological traits (see Methods) were classed as 'native-unusual'.

adegenet v 2.1.2 (Jombart, 2008) in R (R Core Team, 2018). DAPC is a multivariate method that focuses on the between-group variability, while minimising within-group variation.

STRUCTURE v 2.3.4 (Pritchard et al., 2000) was used to explore the genetic structure and identify the most likely number of distinct genetic groups. STRUCTURE uses a Bayesian algorithm to cluster samples into *K* distinct genetic groups by minimizing deviations from Hardy–Weinberg and linkage equilibrium within each cluster. The analyses were carried out for $K = 1$ to 10 using 100,000 MCMC iterations after a burn-in of 20,000 steps and were repeated 10 times for each *K*. If genetic clusters have widely different sample sizes (unbalanced sampling), STRUCTURE has been shown to yield poor estimates of both individual ancestry and *K*, if the default settings are used (Wang, 2017). We therefore followed Wang's (2017) recommendation and used the alternative option allowing a separate α, which is a measure of the relative admixture level between populations (option 'Separate α for each Population' ticked). To identify the most likely number of distinct genetic groups (*K*), the parsimony index PI, which aims to identify the number of populations (*K*) that consistently yields the minimal admixture estimates of sampled individuals, was calculated using the software Kfinder2 (Wang, 2019). The parsimony index has been shown to be consistently more accurate for various population structures and sampling scenarios than the other commonly used methods such as the Δ*K* (Evanno et al., 2005) or Pr(X|K) (Pritchard et al., 2000) statistics (Wang, 2019).

To assess the potential hybrid nature of samples, we used the estimated individual membership coefficients from STRUCTURE (Pritchard et al., 2000). These are the proportions P for each of the identified (*K*) genetic clusters that have contributed to an individual's genomic makeup. There is no objective threshold for P that can be used to define pure and hybrid samples but a P value ≥ 0.05 to 0.2 from a different genetic cluster has been generally applied in the literature (Feurtey et al., 2017; Larsen & Kjær, 2009; Ruhsam et al., 2019; Vähä & Primmer, 2006). Ruhsam et al. (2019) compared the effect of three different P thresholds (0.1, 0.15 and 0.2) on levels of introgression in *Malus sylvestris* in Northern Britain, and although this generated uncertainty for a few individuals, the overall picture and interpretation of the data stayed the same regardless of the chosen P threshold. In this study, we classify *H. non-scripta* individuals with a P ≤ 0.1 of the *H. hispanica* genome as pure individuals.

Organelle haplotypes and frequencies were calculated using the Excel add-in HAPLOTYPE-ANALYSIS v 1.05 (Eliades & Eliades, 2009).

3 | RESULTS

3.1 | SNP array

A total of 1871 high-quality SNPs comprising 1788 bi-allelic and 83 multi-allelic SNPs from 164 nuclear regions was identified and used for further analysis (Dataset S1). On average, 1.35% (median 0.59%) of the samples failed to amplify per locus, and 0.96% (median 0.69%) of data was missing per sample.

One (atp6) of the 12 organelle regions failed to amplify in about 5% of the samples and was therefore excluded. A total of 17 SNPs from eight plastid and three mitochondrial regions resulted in five haplotypes (HT), with substantial frequency differences between groups (Dataset S2). HT1 mainly occurred in *H. hispanica* (87%) and UK non-natives (60%), was absent in Iberian *H. non-scripta* and had a low presence (9%) in the 15 purportedly native UK *H. non-scripta* populations. All native populations with the HT1 haplotype occurred in the grounds of stately homes or very close (<500 m) to urban areas. In contrast, HT5 was absent in *H. hispanica* but fixed in Iberian *H. non-scripta* and occurred in 90% of UK *H. non-scripta* and 8% of UK non-natives (Dataset S2). The other three haplotypes were rare with HT2 and HT4 only occurring in one UK non-native and one *H. hispanica* individual, respectively, and HT3 was restricted to *H. hispanica* (3%) and UK non-natives (30%). There were also frequency differences between Spanish and Portuguese *H. hispanica* populations as HT1 was fixed in Spanish samples but only occurred in 29% of the Portuguese samples, where HT3 was the most frequent haplotype (57%, Dataset S2). Commercial bluebell samples from UK garden centres were fixed for HT5 regardless of species.

3.2 | Population structure

The DAPC was carried out using 80 principal components explaining 71.9% of the variance and identified four distinct genetic groups

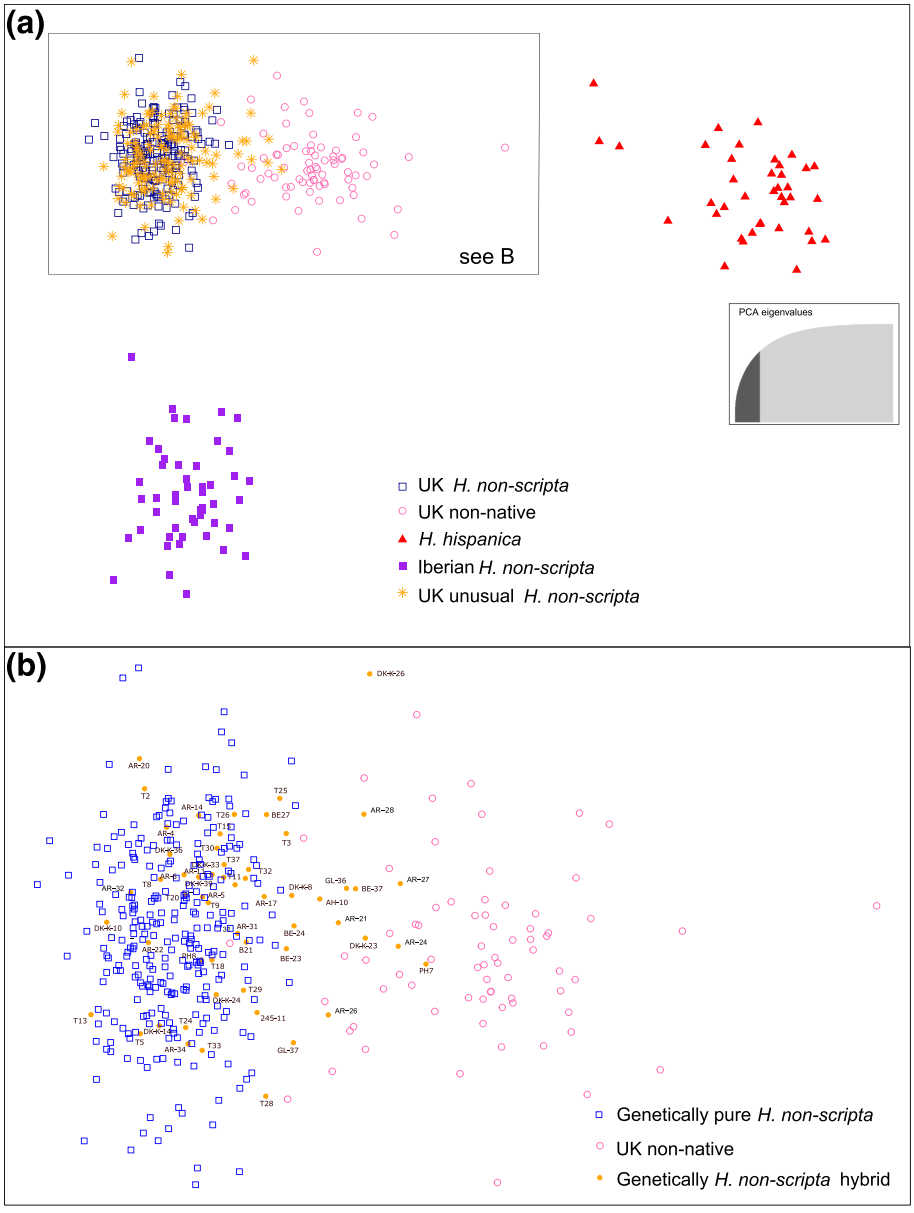

FIGURE 3 Discriminant analysis of principal components (DAPC) of *Hyacinthoides* samples to determine genetic similarity between samples. (a) DAPC analysis of 508 bluebell samples based on 1871 single nucleotide polymorphisms (SNPs) using 80 principal components (71.9% of the variance explained) grouped into five a priori categories based on morphology. (b) Detailed view of UK *H. non-scripta* and UK non-native samples. Samples that were classified as *H. non-scripta* in the field but according to the STRUCTURE results had more than 10% contribution from *H. hispanica* gene pools and/or a *H. hispanica* chloroplast haplotype, suggesting hybrid ancestry ('Genetically *H. non-scripta* hybrid') are named. Samples highlighted in yellow denote plants that are hybrids only because of a presumably non-native HT1 plastid type, that is, have native nuclear contributions of more than 90%.

comprising a UK *H. non-scripta*, a UK non-native, a *H. hispanica* and an Iberian *H. non-scripta* group (Figure 3). UK non-natives were intermediate between UK *H. non-scripta* and *H. hispanica* samples, albeit closer to the UK *H. non-scripta* group. The majority of samples from the 'UK unusual native' category that had some atypical features grouped with UK *H. non-scripta* samples, compared to only a few that grouped with the UK non-native samples. The Iberian *H. non-scripta* samples formed their own group and were well separated from the UK *H. non-scripta* group. Only one commercial bluebell sample that was sold as '*H. non-scripta*' grouped with the UK *H. non-scripta* group whereas all other six samples grouped with the UK non-native cluster regardless of whether they were sold as '*H. non-scripta*' or '*H. hispanica*'.

The STRUCTURE analysis was generally consistent with the DAPC analysis but revealed additional sub-structure within the UK *H. non-scripta* samples (Figure 4). The most likely number of distinct genetic groups in the data set according to the Parsimony index was $K = 6$. These comprised two *H. hispanica* pools subdivided by the geographic origin of the samples from Portugal ('red') and Spain ('pink') and four *H. non-scripta* pools that could be divided into a mainly Iberian *H. non-scripta* gene pool ('orange') and three mainly UK *H. non-scripta* gene pools (Figure 4, Dataset S3). UK non-natives were a mixture of the Portuguese *H. hispanica* and the three UK *H. non-scripta* pools. Fifty-six samples from 9 populations out of a total of 340 samples (16%, Table 2) classified as native *H. non-scripta* based on morphology had more than 10% contribution from *H. hispanica* gene pools and/or a *H. hispanica* plastid haplotype, suggesting hybrid ancestry (Figure 4).

A total of 12% (23 out of 192) of the samples morphologically classified as 'UK *H. non-scripta*' had more than 10% contribution from *H. hispanica* gene pools and/or a *H. hispanica* plastid haplotype, suggesting hybrid ancestry (Table 2). Out of 148 samples identified as 'UK native unusual' in the field, 22.3% ($n = 33$) turned out to be hybrids (Table 2).

4 | DISCUSSION

The primary objectives of this investigation were to determine the taxonomic identity of the UK non-native bluebell and assess whether hybridisation with the native UK bluebell *Hyacinthoides non-scripta* is extensive and likely to pose a threat to its integrity in the United Kingdom. As discussed below, our results provide no compelling evidence that there is large-scale gene exchange between native and non-native bluebells that poses a threat to the genetic integrity of native bluebells in the United Kingdom. We also illustrate why the correct taxonomic identification of introduced species is of relevance to conservation because of taxonomic confusion in the commercial trade of bluebells.

4.1 | The taxonomic identity of the UK non-native bluebells

Non-native bluebells are often referred to as 'Spanish' bluebells, while morphologically unlike the Iberian native, their identity and provenance have been uncertain. Ruhsam et al. (2020) reported that non-native bluebells were morphologically close to *H. hispanica* but occupied a distinct phenotypic space based on principal component analysis. Although this is consistent with hybridisation, it does not provide conclusive evidence for the hybrid status of British non-native bluebells as they could also represent descendants of the originally introduced *H. hispanica* populations, which have adapted morphologically and ecologically to UK environmental conditions.

Our genetic analyses demonstrate that the assayed UK non-native bluebells are not pure *H. hispanica* but rather the hybrid *H. x massartiana* formed by crosses between *H. hispanica* and *H. non-scripta*. The DAPC plot demonstrates that there is clear separation without overlap between *H. hispanica* and UK non-native bluebells samples (Figure 3). UK non-natives form a largely separate cluster from *H. non-scripta* but are closer to this group than to *H. hispanica* and intermingle with samples classified in the field as 'UK native unusual'. The STRUCTURE plot (Figure 4) is consistent with this interpretation as most UK non-native samples have a mixed genomic background consisting of various proportions from the *H. hispanica* and *H. non-scripta* genomes.

H. hispanica has been recorded from 35.7% of 10 km × 10 km recording grids (1492 out of 4174) in the United Kingdom (Ruhsam et al., 2020). The vast majority of these records overlaps with grids where the hybrid *H. x massartiana* has also been recorded (47.5% of 10 km × 10 km squares [1983 out of 4174], Ruhsam et al., 2020). Preston et al. (2002) stated that *H. hispanica* has long been confused with *H. x massartiana* and probably has been over-recorded in error of the hybrid, a statement that is supported by our results and own field observations. Individuals of the diverse non-native group very rarely matched descriptions or photographs of *H. hispanica* in its native range, and the variety of combinations of key characters such as anther colour, scape habit, leaf size and flower shape defied simple dichotomy.

The STRUCTURE analysis revealed that there is substantial genetic differentiation between the assayed *H. hispanica* populations from Spain ('pink' group) and Portugal (mainly 'red' group). The genomic composition of all UK non-native samples consisted of substantial proportions of the 'red' Portuguese group but lacked contributions from the 'pink' Spanish group. *H. hispanica* was introduced to British Gardens by 1683 (Preston et al., 2002), and our results strongly suggest that these original introductions came from Portugal (Figure 4). This was also supported by the high frequency of HT3 in the UK non-native group, which only occurred in Portuguese *H. hispanica* populations. Nearly 90% of UK non-native samples had a *H. hispanica* plastid type suggesting that the hybridisation event involved a *H. hispanica* maternal and *H. non-scripta* paternal parent. This is consistent with the experimental findings of Kohn et al. (2019), which showed that *H. non-scripta* plants were about three times more likely to sire seeds than non-natives.

The genetic analyses also revealed that there is substantial nuclear genetic differentiation between UK *H. non-scripta* and Iberian *H. non-scripta* samples (Figures 3 and 4). This is likely due to the post-glacial migration of this species from the Iberian Peninsula further

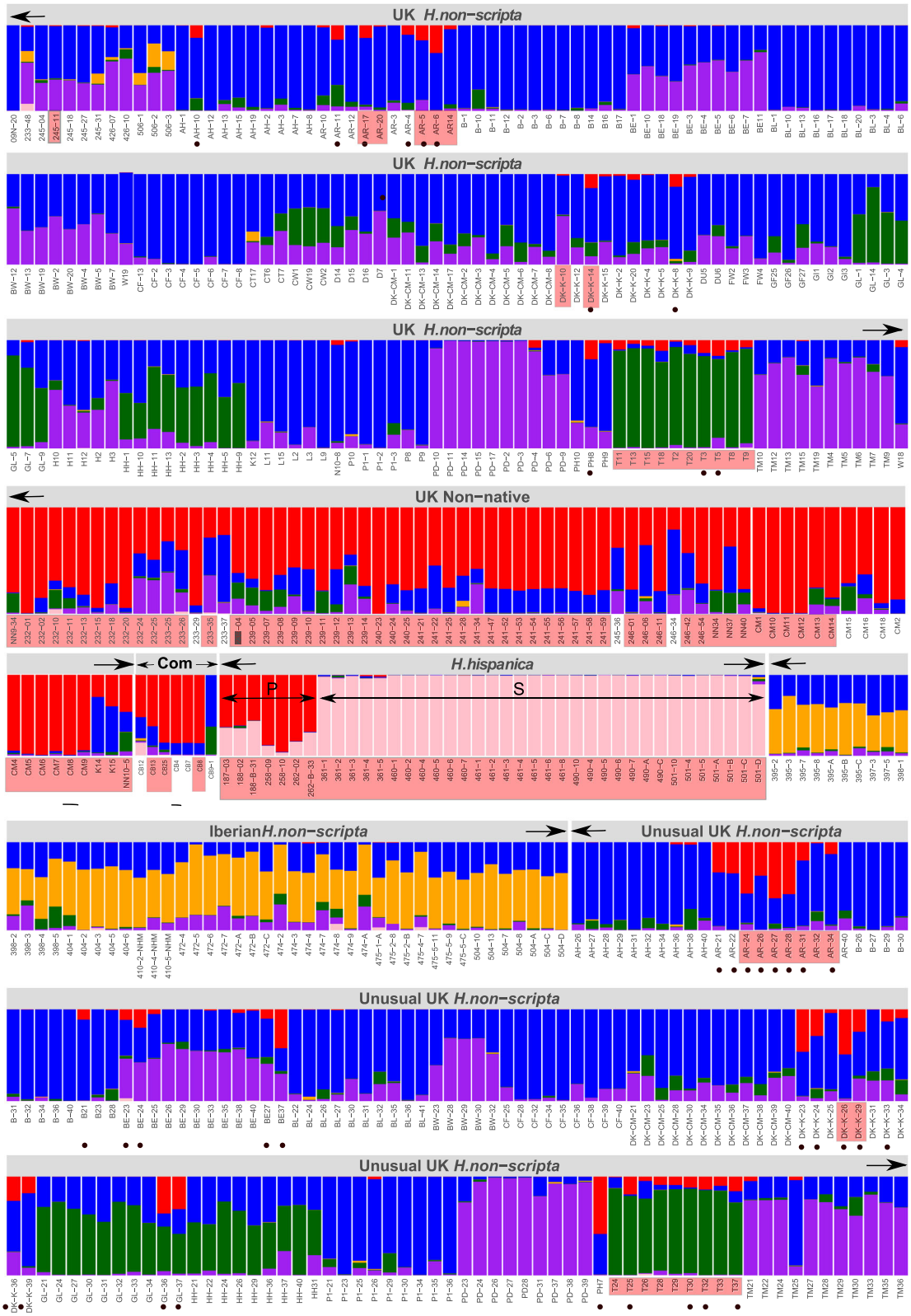

FIGURE 4 Barplot of STRUCTURE membership coefficients for $K = 6$ of 508 *Hyacinthoides* samples using 1871 single nucleotide polymorphisms (SNPs). Grey bars indicate the five a priori groups based on morphology, and 'Com' indicates bluebell samples from UK commercial garden centres. 'P' and 'S' highlight *H. hispanica* samples from Portugal and Spain, respectively. Sample names with a deep pink box have a *H. hispanica* chloroplast type and with a black dot have more than 10% *H. hispanica* contribution and are therefore considered to be hybrids.

TABLE 2 Genetic identification based on 1871 nuclear and 17 plastid SNPs of UK *Hyacinthoides non-scripta* samples that were identified in the field as native samples ('Field ID native') or unusual native samples ('Field ID native-unusual').

Field ID 'native'	192	
Genetic ID native	169	88.0%
Genetic ID hybrid	23	12.0%
Field ID 'native-unusual'	148	
Genetic ID native	115	77.7%
Genetic ID hybrid	33	22.3%

north into the United Kingdom resulting in isolation with no or very restricted subsequent gene flow between the source and founder populations (Comes & Kadereit, 1998).

Our results also suggest that there is considerable confusion and mislabelling of bluebell plants in the horticultural trade. Although based on a small number of seven samples from seven different nurseries, only one bluebell plant was correctly labelled as *H. non-scripta*. All other samples labelled either as *H. hispanica* or *H. non-scripta* were genetic hybrids. This finding is reminiscent of those by Ruhsam et al. (2022) in which only 42.5% of trees labelled as *Malus sylvestris* sold by 13 UK nurseries could be classified as pure *M. sylvestris*. This confusion may be of little consequence to customers who are only interested in buying ornamental bluebells for their garden but is concerning for those who would like to plant native species. Recommendations against and concerns about planting 'hispanica' from the international conservation charity Plantlife and other public conservation bodies such as the Wildlife Trust, the Woodland Trust, The Conservation Volunteers and others could be undermined by hybrids being mislabelled as natives.

4.2 | Is hybridisation between native and non-native bluebells widespread in the United Kingdom?

About 12% (23 out of 192) of the samples that were identified as pure *H. non-scripta* plants in the field had a contribution of more than 10% from the *H. hispanica* gene pool and/or the HT1 plastid haplotype, suggesting hybrid ancestry. Although it cannot be excluded that HT1 naturally occurs at a low frequency in UK *H. non-scripta*, this seems unlikely. HT1 is the dominant haplotype in *H. hispanica* samples (87%) and does not occur in the Iberian *H. non-scripta* group, which is fixed for HT5. HT5 occurs at a frequency of 91% in UK *H. non-scripta* where 6 out of 18 samples with HT1 were identified as hybrids based on the contributions from the *H. hispanica* genome. Additionally, all *H. non-scripta* samples with HT1 occurred within the grounds of stately homes or very close to built-up areas. It is therefore more probable that HT1 is an introgressed *H. hispanica* haplotype rather than a low frequency natural haplotype of UK *H. non-scripta*, especially due to the maternal inheritance of organelles in bluebells (Sears, 1980) with limited seed dispersal distances (van der Veken et al., 2007). About 22% (33 out of 148) of the samples classified as 'native-unusual' UK plants, that is, plants with some traits usually associated with non-natives (such as pink flowers, wider leaves or flowers, thicker scapes or upright inflorescence) but that otherwise looked native and/or occurred in large bluebell populations, were hybrids.

Collectively, our results indicate a hybrid frequency of 16% (56 out of 340) in the group of samples targeting large, purportedly native populations which were identified as either *H. non-scripta* or 'unusual' in the field. However, hybrids were not randomly dispersed between populations but were largely restricted to the following six populations: Ardgowan (AR), Beeslack (BE), Glentyan House (DK-K), Gillingshill (GL), Tarvit House (T) and Plas Hendre (PH). The common feature of these populations is that they are located within the grounds of (sometimes former) stately homes usually built in the 19th century or earlier. Many stately homes have extensive park like grounds and gardens associated with them where non-native plants have been planted. However, not all populations within in the grounds of stately homes had hybrids, for example, Callandar House (CF) or Physgill House (P). It is therefore likely that the frequency of bluebell hybrids in such locations depends on whether non-native bluebells were planted either close to or even within native populations.

This finding might also explain the widespread nature of the non-native hybrid taxon in the United Kingdom despite the selective disadvantages it seems to have compared to the native bluebell (Kohn et al., 2019). Rather than hybrid vigour and enhanced competitive ability, it is likely that the widespread planting of the non-native taxon in private and public gardens as well as mislabelled nursery stock might have facilitated its country-wide distribution. This is supported by our field observations that the non-native taxon is largely restricted to residential areas with urban and landscaped park like settings. This can be likened to the spread of the Oxford ragwort, *Senecio squalidus*, in the United Kingdom, a hybrid that likely originated in the Oxford Botanic Garden in the late 17th century via hybridisation between two introduced plants from Mt Etna (Italy), *Senecio aethnensis* and *S. chrysanthemifolius* (Nevado et al., 2020). By the second half of the 18th century, the species was growing on college walls outside the Oxford Botanic Garden and over the next 200 years spread throughout the United Kingdom aided by the establishment of the railway system, roads and motorways (Abbott et al., 2009; Nevado et al., 2020). In both cases, it is likely that human intervention rather than superior genetic benefits substantially and efficiently spread the hybrids throughout the country, more or less deliberately via planting in bluebells, and inadvertently via the establishment of a modern transportation infrastructure in ragworts.

Our results highlight that the frequency of hybrids in natural populations that are not close to extensive human settlements, or are unlikely to have experienced large-scale human interference, is very low, as nearly all the plants in the 'native' group from these populations turned out to be pure *H. non-scripta*. As our sampling strategy also specifically targeted natives that featured not exclusively 100% typical morphologies, this is likely to overestimate rather than underestimate hybridity. Only in 3 of 25 populations (excluding the populations with hybrids from the six stately homes AR, BE, DK-K, GL, T, PH

and population '245' from Holyrood Park/Edinburgh adjacent to a large residential area) were four hybrids identified (AH-10, GL-36, GL-37 and B21; 2%, 4 out of 257 samples), although B21 was just over the (arbitrary) 10% threshold for pure samples.

4.3 | Role of demography in the likelihood of hybridisation

Our findings suggest that the very large population sizes in semi-wild places are an effective buffer against hybridisation with the much less frequent non-native bluebells and is consistent with the theoretical analyses of Wolf et al. (2001). In their model investigating the contribution of various demographic parameters to the risk of extinction of hybridising taxa, they reported that the sensitivity of each parameter varied dramatically across parameter sets but that the initial population size of the native species relative to that of the invader, as in our case, was one of the important parameters.

The importance of population size effects and frequency of parental species on hybridisation is also supported by the study of Carney et al. (2000) on asymmetric introgression between a local population of the rare *Helianthus bolanderi* and the more common *H. annuus* over a 50-year period in California, USA. The population, which at the start of the study consisted of *H. bolanderi*, is now almost entirely composed of *H. annuus*-like hybrids and the more common parent, *H. annuus*. Similarly, a study of hybridisation between the rare native *Morus rubra* and the more widespread non-native *M. alba* in Ontario, Canada, found evidence that introgression was bidirectional but asymmetrical and related to the relative frequency of individuals of the parental taxa (Burgess et al., 2005). In a study on hybridisation between the native *Senecio pinnatifolius* and non-native *S. madagascariensis* in Australia, Prentis et al. (2007) demonstrated that a high frequency (range 8.3%–75.6%) of hybrids was produced in open-pollinated seeds of both species but that mature hybrids were absent from sympatric populations. *S. madagascariensis* had a hybridization advantage as significantly more progeny than expected were sired based on proportional representation of the two species in sympatric populations. Calculations indicated that *S. pinnatifolius* would produce fewer viable seed than *S. madagascariensis*, if the latter species reached a frequency of between 10% and 60% and that the native would appeared to be under threat if the non-native increased numerically in areas of contact (Prentis et al., 2007). Thus, in assessing hybridisation probabilities for native and non-native species, demographic information associated with population sizes is clearly critical.

4.4 | Morphological variation

In their morphological analysis of native and non-native UK bluebells, Ruhsam et al. (2020) reported that *H. non-scripta* morphologies extended beyond the 'typical' British bluebell phenotype of narrow leaves and one-sided tubular flowers on nodding inflorescences. As this study was entirely based on morphology, it cannot be excluded that some unusual *H. non-scripta* plants were actually hybrids, potentially biasing the results. However, in our study, the large proportion of samples that were genetically pure *H. non-scripta* in the 'native-unusual' group support the findings of Ruhsam et al. (2020). For example, many plants with unusually wide leaves (>2 cm), wide scapes (>4 mm) or upright inflorescences were genetically pure *H. non-scripta*. Similarly, a pink tepal colour that is considered very rare in *H. non-scripta* (nearly exclusively blue coloured) could be considered a sign of introgression from pink coloured non-natives. However, only 5 pink flowered plants (all from the stately home populations BE and T) out of 10 were genetically hybrids. These results suggest that the morphological variability in native bluebells is considerably larger than previously thought (see Ruhsam et al., 2020, for the morphological range detected in UK bluebells) but that this variation should not necessarily be inferred to be the consequence of hybridization with non-native bluebells.

4.5 | Conclusions

The key message that emerges from our study is that although hybridisation might be frequent in locations where non-native bluebells have been introduced on a large scale, such as in the grounds of stately homes or residential areas, gene exchange does not appear to spread beyond the immediate contact zone. Hybridisation is therefore generally absent or minimal in *H. non-scripta* populations in semi-natural habitats such as woodlands and hedgerows in rural areas. Our own field observations in residential areas that border natural bluebell populations suggest that hybrids rarely spread out of the contact zone. Considering the frequently enormous size and density of native compared to non-native populations (based on our field observations), the opportunity for hybridisation is largest in the immediate contact zone but will quickly diminish further away. Natives have higher pollen fertility, about three times higher mating success as paternal parents and are likely to produce more seeds with a significantly higher germination rate than non-natives (Kohn et al., 2019). Our analysis of natural *H. non-scripta* populations therefore supports Kohn et al.'s (2019) view that the higher reproductive performance of natives together with their massive numerical advantage over non-natives represents a substantial barrier to introgression and the earlier concern of 'extinction-by-hybridization' of *H. non-scripta* in the United Kingdom.

AUTHOR CONTRIBUTIONS

Peter M. Hollingsworth, Deborah Kohn, Markus Ruhsam, Jane Squirrell, Harald Schneider, Johannes Vogel and Philip E. Hulme created the project; Deborah Kohn, Jane Squirrell, Markus Ruhsam and Jeannine Marquardt procured samples; Jeannine Marquardt and Andrew R. Leitch designed the genetic assay; Markus Ruhsam carried out the genetic work and analyses and drafted the manuscript with major contributions from Spencer C. H. Barrett, Peter M. Hollingsworth and Deborah Kohn; all authors commented and agreed on it.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Additional data supporting the findings of this article are available in the supporting information of this article.

ORCID

Markus Ruhsam https://orcid.org/0000-0002-8457-345X
Deborah Kohn https://orcid.org/0000-0001-6731-6884
Jeannine Marquardt https://orcid.org/0000-0002-4898-5668
Andrew R. Leitch https://orcid.org/0000-0001-8574-302X
Harald Schneider https://orcid.org/0000-0002-4548-7268
Spencer C. H. Barrett https://orcid.org/0000-0002-7762-3455
Philip E. Hulme https://orcid.org/0000-0001-5712-0474
Peter M. Hollingsworth https://orcid.org/0000-0003-0602-0654

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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