Evidence of spontaneous selfing and disomic inheritance in Geranium robertianum

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Abstract
Knowing species’ breeding system and mating processes occurring in populations is important not only for understanding population dynamics, gene flow processes, and species’ response to climate change, but also for designing control plans of invasive species. Geranium robertianum, a widespread biennial herbaceous species showing high morphological variation and wide ecological amplitude, can become invasive outside its distribution range. A mixed-mating system may be expected given the species’ floral traits. However, autonomous selfing is considered as a common feature. Genetic variation and structure, and so population mating processes, have not been investigated in wild populations. We developed 15 polymorphic microsatellite markers to quantify genetic variation and structure in G. robertianum. To investigate whether selfing might be the main mating process in natural conditions, we sampled three generations of plants (adult, F1, and F2) for populations from the UK, Spain, Belgium, Germany, and Sweden, and compared open-pollinated with outcrossed hand-pollinated F2 progeny. The highly positive Wright’s inbreeding coefficient (FIS) values in adults, F1, and open-pollinated F2 progeny and the low FIS values in outcross F2 progeny supported autonomous selfing as the main mating process for G. robertianum in wild conditions, despite the presence of attractive signals for insect pollination. Genetic differentiation among samples was found, showing some western–eastern longitudinal trend. Long-distance seed dispersal might have contributed to the low geographic structure. Local genetic differentiation may have resulted not only from genetic drift effects favored by spontaneous selfing, but also from ecological adaptation. The presence of duplicate loci with disomic inheritance is consistent with the hypothesis of allotetraploid origin of G. robertianum. The fact that most microsatellite markers behave as diploid loci with no evidence of duplication supports the hypothesis of ancient polyploidization. The differences in locus duplication and the relatively high genetic diversity across G. robertianum range despite spontaneous autonomous selfing suggest multiple events of polyploidization.

Keywords
autonomous selfing, disomic inheritance, duplicate loci, Geranium robertianum, microsatellites

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1 | INTRODUCTION

The breeding system in Angiosperms can vary from autogamy (self-fertilization) to strict allogamy (obligate outcrossing). Strict allogamy may also evolve into a heteromorphic self-incompatibility system preventing selfing or into dioecy (Charlesworth, 2006; Richards, 1997). Autogamy can allow for purging deleterious recessive alleles by natural selection (Charlesworth & Charlesworth, 1987; Goodwillie et al., 2005) and facilitate colonization of new territories for pioneer species or occurrence in extreme or unpredictable habitats where pollinators are scarce or absent (Barrett, 2003; Hartfield et al., 2017; Kalisz & Vogler, 2003). However, it can reduce effective genome recombination and within-population genetic diversity (e.g., Bomblies et al., 2010; Jullien et al., 2019; Nordborg, 2000). Obligate outcrossing represents an advantage by mixing gene pools, increasing genetic diversity, and preventing inbreeding depression (Arista et al., 2017; Charlesworth, 2006), but it can require pollinating vectors, such as insects, birds, or bats, and a sufficient number of compatible mates or extensive gene flow between populations for ensuring reproductive success (Berjano et al., 2013; Menz et al., 2011). Retaining facultative self-pollination, in particular delayed autonomous selfing, can offer reproductive assurance when outcrossing has not occurred in case of limited pollinator service (Busch & Delph, 2012; Kalisz & Vogler, 2003). Pollinator service may be limited in fragmented habitats or in case of temporary unfavorable environmental conditions (Arista et al., 2017; Goodwillie & Weber, 2018). Therefore, a lot of species are characterized by a mixed-mating system to guarantee seed production despite a risk of inbreeding depression in the progeny (Goodwillie et al., 2005, 2010; Kalisz et al., 2004).

Outcrossing species usually possess attractive floral traits for pollinators, for example, a high number of colored flowers and nectar reward, whereas autonomous selfers often have reduced floral display and nectar reward (Bartoš et al., 2020; Goodwillie et al., 2010; Sicard & Lenhard, 2011). Knowing species’ breeding system and quantifying mating processes (outcrossing and selfing rates), which occur in populations, are important for understanding population dynamics, gene flow processes, and potential species’ response to climate change (Charlesworth, 2006; Razanajatovo et al., 2020). They are also important for designing conservation recovery plans of endangered species and control plans of invasive exotic species (Barrett, 2010; Dudash & Murren, 2008). For instance, small populations of species with a self-incompatibility system require a high number of compatible mates for successful demographic and genetic restoration, whereas inbreeding issues may be found for species with a mixed-mating system, requiring genetic rescue of small populations (e.g., Menges, 2008; Olivier et al., 2016; Van Rossum, Destombes et al., 2021). Autonomous selfers may easily produce seeds and naturalize, and may therefore become potentially invasive outside their distribution range (Antoñ & Denisow, 2018; Razanajatovo et al., 2016). Exclusion and pollination experiments can give insights on whether species are self-compatible or self-incompatible (e.g., Bartoš et al., 2020), but genetic studies using molecular markers can allow for quantifying outcrossing rates, inbreeding levels, pollen dispersal processes, and genetic diversity and structure in wild populations (e.g., Arista et al., 2017; Bomblies et al., 2010; Charlesworth, 2006; Gelmi-Candusso et al., 2017; Jacquemart et al., 2021).

Geranium robertianum L. (Geraniaceae) is a common, biennial–annual, ruderal herb and is highly variable morphologically. The species shows a wide ecological amplitude, mainly occurring in woodlands and hedge banks, but also in various open habitats, such as grasslands, wastelands, railway banks, skeletal soils, and walls, on calcareous and acidic soils (Tofts, 2004; Vandelook & Van Assche, 2010; Wierzbicka et al., 2014). It is widely spread in its native distribution area in Europe, and naturalized in temperate regions of many other continents, where it can become invasive (Tofts, 2004). Individual plants bear between 10 and 300 pink flowers (12–17 mm diameter), usually slightly protandrous, sometimes homogamous or protogynous (Bertin, 2001; Tofts, 2004). The dehiscing of the five inner anthers usually precedes the lengthening of the style and stigma receptivity. When the inner stamens wither, the five outer anthers move to the center of the flower around the style and dehisce (Knuth, 1908; Tofts, 2004). Flowers stay open for two to five days (Tofts, 2004; F. Vandelook, personal observation), which is similar to other Geranium species (e.g., Willson et al., 1979). Generally, five seeds per fruit are produced (Tofts, 2004). Flowers produce nectar and are visited by insects, in particular butterflies, Syrphid flies, wild bees, and honey bees (Endress, 2010; Tofts, 2004; Yeo, 1973), suggesting outcrossing. Self-fertilization is, however, possible, as stigmas during elongation can be covered with pollen of the inner whorl of stamens before possible outcrossing events, and when the stigmas standing above the dehiscing outer anthers recurve (Knuth, 1908; Tofts, 2004), allowing for prior and delayed autonomous selfing. Autonomous selfing has been considered as a common feature (Bertin, 2001; Yeo, 1973, 1985). Consequently, mixed mating likely occurs in G. robertianum. However, population mating processes have never been investigated in the field using codominant molecular markers to estimate genetic variation and inbreeding levels. Besides, plants only reproduce by seeds, which are dispersed not only at short distances by carpel projection but also at long distances by epizoochory (Tofts, 2004; Yeo, 1973). As a result, genetic variation and structure patterns may be contrasted according to mating processes and short- and long-distance seed dispersal (e.g., Bomblies et al., 2010; Gelmi-Candusso et al., 2017; Helsen et al., 2015; Jacquemart et al., 2021). Moreover, due to its wide distribution range combined with a wide ecological amplitude, G. robertianum appears as an interesting model for studying local adaptation and response to climate change (Hoffmann & Sgrò, 2011; Wierzbicka et al., 2014). Therefore, we developed polymorphic microsatellite markers to quantify genetic variation and structure in G. robertianum. To investigate whether selfing might be the main mating process in natural conditions, we sampled three generations of plants (adult, F1, and F2) for populations
from the UK, Spain, Belgium, Germany, and Sweden, and progeny obtained from outcrossed hand-pollinated were compared with progeny in open-pollinated conditions.

2 | MATERIALS AND METHODS

2.1 | Study populations and sampling

To cover a wide ecological amplitude and geographic range of *G. robertianum*, 43 populations were selected from various calcareous or acidic habitats (e.g., forests, forest edges, grasslands, railway banks, sandy and shingle beaches), from the UK, Spain, Belgium, Germany, and Sweden (Figure 1, Table 1). Populations varied in size, ranging from about 20 flowering individuals up to hundreds of individuals (F. Vandelook, unpublished data). Leaves were collected from a total of 191 individuals (adults, and F1 and F2 seed progeny) and dried in silica gel. Sampling occurred during the peak of flowering and pollinator activity, between mid-June and mid-August. F1 progeny was obtained from germination of seeds collected in wild populations on different maternal plants separated at least 1 meter from each other. F1 plants from eight populations from UK and Germany were grown (1) in the Botanical Garden of the Phillips-Universität Marburg (Germany) in 2013 until flowering and fruiting, with plants randomly distributed (but covered with shade nets, which might have reduced insect pollination); F2 seed progeny was obtained from germination of seeds collected on the F1 plants after open pollination (F2o); (2) in a nonheated greenhouse at Meise Botanic Garden (Belgium) in late May-June 2019, where F2 seed progeny was obtained after outcrosses (F2c) between F1 plants (Table 2) or selfing (F2s, but only for two maternal plants and four progenies in total). For the crossing experiment, flowers were emasculated before anthesis and bagged. Stigmas were hand-pollinated with pollen when they were receptive on two

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**FIGURE 1** Location of the 43 populations of *Geranium robertianum* (gray dot: open habitat; black dot: forest habitat) sampled in the UK, Spain, Belgium, Germany, and Sweden. For population codes, see Table 1. The approximate native distribution range is indicated in light gray (modified from Hultén & Fries, 1986)
### Table 1: Details for 43 populations of *Geranium robertianum*:

| Population | Locality | Country | Latitude (N) | Longitude (W or E) | Habitat type | Soil pH | n  |
|------------|----------|---------|--------------|--------------------|--------------|--------|----|
| PAG        | Pagham   | UK      | 50°45'50"   | 0°44'35" W        | Shingle beach| 8.2    | 2  |
| LOS        | Littlestone-on-Sea, Kent | UK      | 50°58'18"   | 0°57'52" E        | Sandy beach  | 7.0    | 4  |
| ELH        | Elham    | UK      | 51°09'45"   | 1°06'24" E        | Hedge bank   | 6.8    | 2  |
| BRO        | Brockenhurst | UK      | 50°49'39"   | 1°34'17" W        | Hedge and forest | 7.0 | 2  |
| CAL        | Calne    | UK      | 51°25'07"   | 1°59'36" W        | Forest       | 3      | 4  | 5 |
| PO1        | Porlock  | UK      | 51°13'05"   | 3°37'31" W        | Shingle beach| 8.3    | 2  |
| PO2        | Porlock  | UK      | 51°13'06"   | 3°37'46" W        | Hedge and forest near the beach | 6.5 | 3  |
| SL1        | Slapton  | UK      | 50°17'17"   | 3°38'41" W        | Shingle      | 6.2    | 2  |
| SL2        | Slapton  | UK      | 50°17'29"   | 3°39'10" W        | Hedge bank   | 7.1    | 3  | 5  | 8 |
| MAL        | Mallorca | SP      | 39°45'37"   | 3°09'20" E        | Park near forest | 2  |    |
| AAL        | Aalter   | BE      | 51°05'04"   | 3°29'34" E        | Forest       | 1      |    |
| THO        | Thoricourt | BE      | 50°37'00"   | 3°57'11" E        | Forest fringe | 1  |    |
| PEP        | Pepingen | BE      | 50°44'41"   | 4°11'59" E        | Ruderal grassland | 1 |    |
| MEI        | Meise    | BE      | 50°55'42"   | 4°19'31" E        | Forest       | 4      |    |
| BOI        | Boistfort| BE      | 50°47'35"   | 4°24'57" E        | Ruderal grassland on roadside | 1 |    |
| WES        | Wespelaar| BE      | 50°57'41"   | 4°38'52" E        | Forest fringe | 1 |    |
| HE1        | Heverlee, Leuven | BE      | 50°51'56"   | 4°41'22" E        | Forest       | 2      |    |
| HE2        | Heverlee, Leuven | BE      | 50°51'48"   | 4°42'20" E        | Railway bank | 6.9 | 3  |
| DI1        | Molenstede| BE      | 51°00'38"   | 5°01'53" E        | Forest       | 1      |    |
| DI2        | Molenstede, Diest | BE      | 51°01'00"   | 5°01'59" E        | Forest       | 21     | 3  |
| DI3        | Diest    | BE      | 50°58'23"   | 5°02'02" E        | Ruderal grassland on roadside | 1 |    |
| DI4        | Diest    | BE      | 50°59'36"   | 5°02'53" E        | Railway bank | 8.1 | 3  |
| OLL        | Olloy-sur-Viroin | BE      | 50°04'08"   | 4°36'22" E        | Ruderal grassland on schists | 4.1 | 1  |
| DIN        | Dinant   | BE      | 50°16'29"   | 4°56'03" E        | Ruderal roadside on limestone | 7.5 | 1  |
| CHO        | Chokier  | BE      | 50°35'34"   | 5°26'30" E        | Forest on limestone | 7.6 | 1  |

(Continues)
| Population | Locality          | Country | Latitude (N) | Longitude (W or E) | Soil pH | A | F1 | F2c | F2o | F2s |
|------------|-------------------|---------|--------------|-------------------|---------|---|----|-----|-----|-----|
| BOM        | Bomal-sur-Ourthe  | BE      | 50°21′40″    | 5°31′02″          | E       |   |    |     |     |     |
| THE        | Theux             | BE      | 50°32′28″    | 5°49′51″          | E       |   |    |     |     |     |
| KEL        | Kemispens         | BE      | 50°41′43″    | 5°59′17″          | E       |   |    |     |     |     |
| MA2        | Marche-en-Frœs    | GE      | 50°46′29″    | 8°43′56″          | E       |   |    |     |     |     |
| MAL         | Marmor     | GE      | 50°46′21″    | 8°46′22″          | E       |   | 3  |     |     |     |
| FRA          | Fraurenberg, Giesenhöhe | GE | 50°46′49″    | 8°47′19″          | E       |   |    |     |     |     |
| REI          | Reiskirchen, Gisela | GE | 50°45′37″    | 8°50′11″          | E       |   | 8  | 3   | 4  | 10  |
| DRI          | Dreihausen, Eifel | GE | 50°45′44″    | 8°51′56″          | E       |   | 7  | 5   |     |     |
| RAU          | Rauischholzhausen | GE | 50°45′48″    | 8°52′07″          | E       |   |    |     |     |     |
| DR2          | Dreihausen, Eifel | GE | 50°45′38″    | 8°52′36″          | E       |   | 4  |     |     |     |
| BUR          | Burgbach-Kirchheim | GE | 50°52′30″    | 8°56′34″          | E       |   | 4  |     |     |     |
| NIE          | Nieder-Oleifden | GE | 50°46′49″    | 8°58′27″          | E       |   | 8  | 3   | 4  | 3   |
| DAN          | Dannenrod, Homberg | GE | 50°45′34″    | 13°13′35″         | E       |   | 5  | 4   | 3   | 9   |
| NOS          | Nossen           | GE | 51°13′17″    | 14°59′54″         | E       |   | 6  | 1   | 3   | 1   |
| KH1          | Königshain       | GE | 51°11′28″    | 14°59′58″         | E       |   | 6  | 7   | 2   | 2   |
| HOR          | Horndal          | SW | 60°19′14″    | 16°19′14″         | E       |   | 3  | 2   | 2   | 2   |
consecutive days and rebagged until ripe seeds could be collected, after three to four weeks.

2.2 | DNA analyses

2.2.1 | DNA extraction

DNA was isolated from ca. 15–25 mg of dried leaf material for 191 samples using a CTAB method (Doyle & Doyle, 1990). We estimated the concentration of genomic DNA extracts using the Qubit Quantitation Platform (Invitrogen), which was standardized to 2 ng/μl.

2.2.2 | Nuclear microsatellite primer development and multiplexing

Nuclear microsatellites were developed by Genoscreen (Lille, France) as described in Van Rossum, Destombes et al. (2021). Genomic DNA of 15 individuals was used (Table 1). A microsatellite library was developed using 1 μg from an equimolar DNA pool of 10 individuals through 454 GS-FLX Titanium pyrosequencing of a DNA library enriched for AG, AC, AAC, AAG, ACG, ACAT, and ATCT repeat motifs (Malausa et al., 2011). PCR products were purified and quantified, and GsFLX library was then constructed and sequenced on a GS-FLX PTP. The selection of sequences with target microsatellites was performed using QDD with the parameters set by default (Meglécz et al., 2010). The sequence reads were submitted to the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA694498. Among 38,206 raw sequence reads, 4,030 sequences comprised a microsatellite motif, from which 461 primer pairs were designed on flanking regions.

For biological validation, a total of 47 primer pairs showing a high number of repeats (at least 9) and covering a wide range of PCR product sizes (from 101 to 316 bp) were tested for amplification on eight DNA samples. PCR amplifications were performed with the same conditions than previously but with labeled primers (Di-repeat +tail Applied Biosystems). Each PCR product (diluted at 1:50 with dH2O) was mixed with Hi-Di™ Formamide (Life Technologies, Carlsbad, California, USA) and GeneScan™ 500 LIZ® Dye Size Standard (Applied Biosystems). Fragments were migrated on a 3730XL DNA capillary sequencer (Applied Biosystems). Alleles were scored using the microsatellite plugin in Geneious 11.1.2 (Biomatters). Finally, 15 polymorphic and interpretable markers (Table 3) were selected and three multiplexes were developed using Multiplex Manager v1.2 (Holleley & Geerts, 2009) and subsequently optimized. All individuals were genotyped using the same protocol.

2.3 | Data analysis

2.3.1 | Null alleles and independence of the loci

Each locus was checked for potential null alleles using INEST 2.2 (Chybicki & Burczyk, 2009). We conducted the Bayesian approach (IM) with 10^6 Markov Chain Monte Carlo iterations, of which the first 10^5 were discarded as burn-in phase to test two models: a full model (nfβ, including null alleles, inbreeding, and genotyping failures) and a model (nb) where there was no inbreeding. The best fitting model corresponded to the lowest value of the deviation information criterion (DIC). INEST also estimated null allele frequencies for each locus with their 95% highest posterior density intervals (HPDI). To assess the independence of the loci, a test for genotypic disequilibrium was performed between pairs of loci with sequential Bonferroni-type correction (Rice, 1989) on adults and on F1 progeny using FSTAT version 2.9.4 (Goudet, 2003).
TABLE 3 Characteristics of 15 microsatellite markers developed in *Geranium robertianum*. For each marker (and duplicate loci in GER17, GER35, GER42, GER45, and GER47 indicated as A and B), the forward and reverse sequences, repeat type, size of the original fragment (bp), number of alleles (An), allele size range, multiplex number, fluorescent dye, primer amount used in the multiplex PCR (pmol), and null allele frequency (with their 95% highest posterior density intervals) are given.

| Locus name | Primer sequence (5’– 3’) | Repeat motif | An | Size range (bp) | Multiplex number | Dye | Primer amount (pmol) | Null allele frequency |
|------------|--------------------------|--------------|----|----------------|------------------|-----|---------------------|----------------------|
| GER07      | F: AGTGGCCTTTACCGAACACG  | (tc)13       | 16 | 96–153         | 1                | 6-FAM | 1.6                | 0.010 (0.000–0.024)   |
|            | R: TGAAGGTGTGGACGGCAACA  |              |    |                |                  |       |                     |                      |
| GER29      | F: CTTTGTGTTTTGATAGCATTAA | (c tt)10     | 7  | 93–117         | 1                | VIC   | 4                  | 0.019 (0.000–0.039)   |
|            | R: AAATTGACCGTGTCGATA    |              |    |                |                  |       |                     |                      |
| GER08      | F: ATATAAACCCCCAAAGACGCG | (c tt)12     | 13 | 260–296        | 1                | NED   | 1                  | 0.026 (0.000–0.048)   |
|            | R: TCCCTCGAAATGACGCTCTGT |              |    |                |                  |       |                     |                      |
| GER45      | F: CGA AAACCTAGAAACCGACA | (aga)9       | 6  | 113–128        | 1                | NED   | 1                  | 0.010 (0.000–0.025)   |
|            | R: CATGGTGTCGTTCAGTTTG   |              |    |                |                  |       |                     | 0.028 (0.003–0.056)   |
| GER17      | F: GGGTCAATTTTGCACCTTCA  | (ag)11       | 10 | 142–166        | 1                | PET   | 1.6                | 0.015 (0.000–0.033)   |
|            | R: AGACGATGGGTGATGGAAG   |              |    |                |                  |       |                     | 0.031 (0.006–0.060)   |
| GER26      | F: CTTTCTCTTTGTGTCTTCGT  | (ag)10       | 4  | 151–157        | 2                | 6-FAM | 4                  | 0.133 (0.087–0.184)   |
|            | R: GATTTAAAGCTTCTGCCC    |              |    |                |                  |       |                     |                      |
| GER30      | F: AGAAATGACCAATCCAAACCC | (c tt)10     | 7  | 96–120         | 2                | 6-FAM | 4                  | 0.017 (0.001–0.037)   |
|            | R: CTTTGTAGCCAATGAGGCC   |              |    |                |                  |       |                     |                      |
| GER05      | F: ATCTTAGCTTTCCTTCCCC   | (ct)13       | 11 | 168–194        | 2                | VIC   | 1.8                | 0.013 (0.000–0.029)   |
|            | R: TCCGAACTGCGCTCCATT    |              |    |                |                  |       |                     |                      |
| GER42      | F: AATGCTGAAGCTGTCCCCCTA | (tc)9        | 5  | 119–137        | 2                | NED   | 1.6                | 0.016 (0.000–0.037)   |
|            | R: CCCAAGAACAAGTAGAAAGAGATTGG |          | 3  | A: 119–133     |                  |       |                     | 0.017 (0.000–0.041)   |
|            |                            |              |    | B: 135–137     |                  |       |                     |                      |
| GER23      | F: AAGTCACAACGCTGGTCAATAGC| (tc)10       | 5  | 199–220        | 2                | PET   | 2.24               | 0.004 (0.000–0.017)   |
|            | R: GTGGATTCTGGAAAGCTGAA  |              |    |                |                  |       |                     |                      |
| GER41      | F: TCGTCTGGAGGAAAGACGCT  | (ttc)9       | 13 | 138–174        | 2                | PET   | 1.26               | 0.007 (0.000–0.022)   |
|            | R: CATGCTCGAGAGTAGGCTT    |              |    |                |                  |       |                     |                      |

(Continues)
2.3.2 | Genetic variation within generations

Expected heterozygosity ($H_e$) and Wright’s inbreeding coefficient ($F_{IS}$), corrected for small sample size, were calculated for each locus and for each generation (adults, F1 progeny, and F2 progeny) using FSTAT. We tested the significance of the $F_{IS}$ values (for each locus and over all loci) by randomization tests and sequential Bonferroni-type correction. Differences in $H_e$ and $F_{IS}$ between F1, open-pollinated, and outcross F2 progeny were tested for the eight populations used for the crossing experiment (Table 2) by the Wilcoxon matched-pairs tests by locus using STATISTICA version 12.

2.3.3 | Population genetic structure at a wide geographic scale

To investigate population genetic structure patterns, we performed a principal coordinate analysis (PCoA) based on a standardized distance matrix using GenAlEx 6.5 (Peakall & Smouse, 2012) and Bayesian clustering analyses using STRUCTURE version 2.3.4 (Pritchard et al., 2000) on adults, F1 progeny, and open-pollinated F2 progeny. For STRUCTURE analyses, we computed 15 runs for $K$ = 1 to 10 clusters, using an admixture ancestry model with correlated allele frequencies and no prior population information, run length of burn-in period of $10^6$ iterations, and 2 $10^6$ Markov Chain Monte Carlo replications. The most likely number of $K$ clusters was inferred as described in Evanno et al. (2005) after running STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The most likely estimated membership ($Q$) values of the 15 independent runs computed with CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) were visualized as a bar plot.

3 | RESULTS

3.1 | Loci and scored alleles

Out of the 15 primer pairs, 10 could be interpreted to amplify diploid loci. Five primer pairs (GER17, GER35, GER42, GER45, and GER47) showed two to four peaks ascribed to different alleles (Figure S1). From the genotyping of the F2 progeny obtained by outcrosses (F2c) and of their maternal and paternal plants, the amplified regions for each primer pair could be interpreted as corresponding to two duplicate loci (Table 3), not overlapping for GER42, but overlapping for the four other markers (Figure S1). However, the higher size of the peak allowed us to identify when two overlapping alleles occurred in both loci. For GER35, only one (rare) allele was found in both loci, and separating the two loci was easy. For GER17, GER45, and GER47, it can be difficult to distinguish both loci in some genotypes without data on maternal and paternal plants together with their progeny, and so we recommend not using them unless performing paternity analyses or
from open pollination of randomly distributed plants from eight clustering was not related to habitat differences (Figure 1, Table 1). Except for (F2c) progeny, for which expectations, with a deficit of heterozygotes for almost all loci, expected disequilibrium between 15 and 6 of the 190 pairs of loci after sequential Bonferroni correction (p < .05) for adults and F1 progeny, respectively.

3.2 | Genetic variation within generations

Expected heterozygosity (H_e) values per locus ranged from 0.901 with a mean of 0.550 for adults and F1 progeny collected in wild populations (Table 4), and were similar between F1, open pollination (F2o), and outcross (F2c) F2 progeny (Wilcoxon test Z = 0.50–1.72, p s .085). Wright's inbreeding coefficient (F_IS) values significantly (p < .05) differed from the Hardy–Weinberg expectations, with a deficit of heterozygotes for almost all loci, except for (F2c) progeny, for which F_IS values were not significant or significantly negative (Table 4), and significantly lower than F1 and F2o progeny (Z = 3.72, p < .001). F_IS values were slightly but significantly lower (Z = 2.33, p = .020) for the F2o progeny obtained from open pollination of randomly distributed plants from eight populations, compared with F1 progeny collected in the same eight wild populations (mean F_IS = 0.668 and 0.768, respectively), suggesting the occurrence of some, however, limited, outcrossing. The IIM analysis indicated that inbreeding contributed to the positive F_IS values (lowest DIC for the nbf model). Population D12, for which 21 adults were sampled across the whole population, showed 11 different multilocus genotypes, with 1–9 individuals sharing the same multilocus genotype, H_e = 0.318 and F_IS = 0.656 (significantly positive, p < .05).

3.3 | Genetic structure at wide geographic scale

The PCoA distinguished Spanish samples from the other populations that showed some continuous variation, although UK samples tended to be separated from Belgian, German, and Swedish samples that overlapped (Figure 2). Within each region, adult, F1, and/or F2o generations overlapped, suggesting similar mating processes in the three generations. The Bayesian clustering analysis gave an optimal number of clusters at K = 2. The UK samples showed high membership (Q) values for cluster 1 (≥80%) for 91% of the individuals) and clustered together with a few German and Belgian samples (e.g., AAL, BOI, DIN, DR2, HE2, NIE, and RAU) (Figure 3a). A second peak was found for DeltaK at K = 4, further distinguishing the D12 population from Belgium (from which there were 21 samples) and some longitudinal trend for the continental populations (Figure 3b). The clustering was not related to habitat differences (Figure 1, Table 1).

4 | DISCUSSION

The highly positive inbreeding coefficient (F_IS) values found for adults, F1, and F2o progeny supported the former hypothesis (Bertin, 2001; Yeo, 1985) that autonomous self-pollination is the main mating process contributing to seed production in wild populations of G. robertianum, and that outcross pollination was limited, despite the presence of attractive signals for insect pollination such as nectar production (Endress, 2010) and reporting of pollinator visitations (Bertin, 2001; Tofts, 2004). However, given the high number of flowers per plant, geitonogamous self-pollination might also be possible in case of pollinators visiting several flowers on the same plant (Goodwillie et al., 2010; Richards, 1997). Moreover, crosses between closely related individuals, such as full siblings with the same multilocus genotype, resulting in biparental inbreeding, might also contribute to high F_IS values (Bombolies et al., 2010). This needs to be verified by investigating within-population genetic variation with more samples (Leipold et al., 2020). Spontaneous autonomous selfing is often observed in annuals, weeds, and pioneer species such as G. robertianum, whereas outcrossing is more common in perennials and species occurring in stable vegetation communities (Barlós et al., 2020; Charlesworth, 1992). For predominantly selfing species, outcrossing rates can also vary along the flowering season, depending on pollinator and resource availability (Julien et al., 2021).

Some genetic differentiation among samples was found, but with no pronounced geographic pattern except for the UK and Spanish (Mallorca) samples and some western–eastern longitudinal trend. Long-distance seed dispersal (Tofts, 2004) might have contributed to the low geographic structure, as found for the bird seed-dispersed Juniperus communis (Jacquemart et al., 2021) and for species showing epizoocchorous seed dispersal, such as Anthyllis vulneraria (Helsen et al., 2015) and Dianthus carthusianorum (Rico & Wagner, 2016), as well as accidental introduction of seeds along with anthropogenic activities and infrastructures (Wierzbicka et al., 2014). Moreover, no evidence of reproductive isolation was found between the UK and German populations assigned to separate clusters as viable seeds and healthy plants were obtained from outcrosses (F. Vandelook, unpublished data). Local genetic differentiation between populations may have resulted not only from genetic drift effects promoted by spontaneous selfing, but also possibly from local ecological adaptation (Bombolies et al., 2010; Hartfield et al., 2017; Wierzbicka et al., 2014). To get a comprehensive view of genetic structure patterns and of their shaping factors, we need to expand the sampling within populations and across species' distribution range.

The presence of duplicate loci suggests that the species might be of polyploid origin, which is consistent with the hypothesis that G. robertianum is an allotetraploid resulting from hybridization between G. purpureum and another unknown parental species, based on chromosome numbers, morphological similarities, cytological observations, and nectar composition (Baker & Baker, 1976; Widler-Kiefer & Yeo, 1987; Yeo, 1973, 2004). Tetrasomic inheritance, that is, random pairing of four homologous chromosomes, leading to all possible
Table 4: Within-population genetic estimates per locus and per generation (wild: samples from wild populations, grouping adults and F1 progeny; A: adults; F1: F1 seed progeny; F2o, F2s, and F2c: F2 seed progeny, obtained from open, self, and outcross pollination, respectively) for Geranium robertianum at 20 nuclear microsatellite loci: expected heterozygosity ($H_e$) and Wright's inbreeding coefficient ($F_{IS}$)

| Locus  | $H_e$ Wild | $H_e$ A | $H_e$ F1 | $H_e$ F2o | $H_e$ F2s | $H_e$ F2c | $F_{IS}$ A | $F_{IS}$ F1 | $F_{IS}$ F2o | $F_{IS}$ F2s | $F_{IS}$ F2c |
|--------|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| GER07  | 0.901      | 0.827  | 0.907  | 0.869  | 0.667  | 0.868  | 0.184* | 0.888* | 0.560* | 1.000 ns | -0.045 ns |
| GER29  | 0.410      | 0.403  | 0.398  | 0.596  | 0.000  | 0.500  | 0.809* | 0.815* | 0.753* | -        | -0.162 ns |
| GER45A | 0.236      | 0.051  | 0.328  | 0.478  | 0.000  | 0.498  | 1.000 ns| 0.912* | 0.761* | -        | -0.120 ns |
| GER45B | 0.597      | 0.665  | 0.451  | 0.427  | 0.667  | 0.317  | 0.807* | 0.934* | 0.933* | 1.000 ns | -0.101 ns |
| GER08  | 0.746      | 0.616  | 0.799  | 0.646  | 0.667  | 0.628  | 0.792* | 0.886* | 0.646* | 1.000 ns | 0.074 ns |
| GER17A | 0.507      | 0.471  | 0.528  | 0.487  | 0.000  | 0.506  | 0.782* | 0.833* | 0.413 ns| -        | -0.057 ns |
| GER17B | 0.712      | 0.656  | 0.720  | 0.700  | 0.667  | 0.702  | 0.844* | 0.829* | 0.633* | 1.000 ns | 0.039 ns |
| GER30  | 0.582      | 0.504  | 0.490  | 0.477  | 0.000  | 0.431  | 0.949* | 0.790* | 0.461* | -        | -0.025 ns |
| GER26  | 0.637      | 0.492  | 0.705  | 0.759  | 0.667  | 0.713  | 0.844* | 0.902* | 0.830* | 1.000 ns | 0.380* |
| GER05  | 0.729      | 0.541  | 0.798  | 0.823  | 0.750  | 0.753  | 0.763* | 0.889* | 0.722* | 0.667 ns | -0.080 ns |
| GER24A | 0.182      | 0.148  | 0.202  | 0.056  | 0.000  | 0.131  | 1.000* | 0.781* | -0.015 ns| -        | -0.063 ns |
| GER24B | 0.037      | 0.100  | 0.000  | 0.000  | 0.000  | 0.000  | 1.000* | -        | -        | -        | -        |
| GER41  | 0.755      | 0.619  | 0.806  | 0.681  | 0.667  | 0.726  | 0.793* | 0.892* | 0.748* | 1.000 ns | -0.088 ns |
| GER23  | 0.585      | 0.520  | 0.620  | 0.563  | 0.667  | 0.467  | 1.000* | 0.908* | 0.848* | 1.000 ns | 0.254 ns |
| GER27  | 0.716      | 0.686  | 0.659  | 0.574  | 0.667  | 0.487  | 0.888* | 0.935* | 0.751* | 1.000 ns | -0.242 ns |
| GER47A | 0.523      | 0.491  | 0.546  | 0.611  | 0.667  | 0.552  | 0.843* | 0.892* | 0.952* | 1.000 ns | -0.263 ns |
| GER47B | 0.587      | 0.534  | 0.620  | 0.638  | 0.000  | 0.651  | 1.000* | 0.953* | 0.585* | -        | 0.107 ns |
| GER38  | 0.735      | 0.537  | 0.774  | 0.678  | 0.000  | 0.659  | 1.000* | 0.943* | 0.826* | -        | -0.306* |
| GER35A | 0.106      | 0.101  | 0.109  | 0.000  | 0.000  | 0.000  | 1.000 ns| 1.000* | -        | -        | -        |
| GER35B | 0.723      | 0.584  | 0.783  | 0.714  | 0.667  | 0.715  | 0.868* | 0.909* | 0.618* | 1.000 ns | -0.269 ns |
| Mean   | 0.550      | 0.477  | 0.562  | 0.539  | 0.371  | 0.515  | 0.866* | 0.891* | 0.703* | 0.966* | -0.049 ns |

Note: Departure from Hardy–Weinberg expectations: ns: not significant; * $p < .05$ after Bonferroni correction.

Figure 2: Principal coordinate analysis (PCoA) plot for 144 samples from 43 populations of Geranium robertianum. Axes 1 and 2 explained 13.4% and 11.7% of the total variation, respectively. Country of origin: BE: Belgium; GE: Germany; SP: Spain, SW: Sweden. Generation: A: adults; F1: F1 seed progeny; F2o: F2 seed progeny, obtained from open pollination.

Figure 3: Results of Bayesian clustering (modal $K = 2$ and 4) for 144 samples from 43 populations of Geranium robertianum (ordered by increasing longitude). Each horizontal bar in the bar plot represents one individual and shows the probability of membership to each of the two or four clusters. Country of origin: BE: Belgium; GE: Germany; SP: Spain, SW: Sweden. Generation: A: adults; F1: F1 seed progeny; F2o: F2 seed progeny, obtained from open pollination. For population codes, see Table 1.
et al., 2014). The differences in locus duplication and the relatively high genetic diversity (Table 1) across the range of G. robertianum despite spontaneous autonomous selfing suggest multiple events of polyploidization (Soltis et al., 2014).

Further testing of developed molecular markers on G. purpureum and a comprehensive study of population genetic structure of both species might contribute to shed light on speciation processes and possible relationships between population genetic structure based on molecular markers, and morphological and environmental variation across species’ distribution range.

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CONFICT OF INTEREST
No conflict of interest.

AUTHOR CONTRIBUTION
Fabienne Van Rossum: Conceptualization (supporting); Formal analysis (lead); Methodology (supporting); Visualization (equal); Writing—original draft (lead). Olivier Raspé: Conceptualization (supporting); Formal analysis (supporting); Methodology (supporting); Validation (supporting); Writing—original draft (supporting); Writing—review & editing (equal). Filip Vandelook: Conceptualization (lead); Formal analysis (supporting); Methodology (lead); Validation (equal); Visualization (equal); Writing—original draft (equal); Writing—review & editing (equal).

DATA AVAILABILITY STATEMENT
Individual multilocus genotypes are available at Zenodo (https://doi.org/10.5281/zenodo.4698869).

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REFERENCES
Antoń, S., & Denisow, B. (2018). Pollination biology and breeding system in five nocturnal species of Oenothera (Onagraceae): Reproductive assurance and opportunities for outcrossing. Plant Systematics and Evolution, 304, 1231-1243. https://doi.org/10.1007/s00660-018-1543-y
Arista, M., Berjano, R., Viruel, J., Ortiz, M. Á., Talavera, M., & Ortiz, P. L. (2017). Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible Hypochaeris salzmanniana (Asteraceae). Annals of Botany, 120(3), 447–456. https://doi.org/10.1093/aob/mcx059
Baker, I., & Baker, H. G. (1976). Analysis of amino acids in flower nectars of hybrids and their parents, with phylogenetic implications. New Phytologist, 76, 87–98. https://doi.org/10.1111/j.1469-8137.1976.tb01441.x
Barrett, S. C. H. (2003). Mating strategies in flowering plants: The outcrossing-selfing paradigm and beyond. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 358(1434), 991-1004. https://doi.org/10.1098/rstb.2003.1301
Barrett, S. C. H. (2010). Why reproductive systems matter for the invasion biology of plants. In D. M. Richardson (Ed.), Fifty years of invasion ecology (pp. 195–210). Blackwell Publishing Ltd. https://doi.org/10.1002/9781444329988.ch15
Bartoš, M., Janeček, Š., Janečková, P., Padyšáková, E., Tropek, R., Götzenberger, L., Klomberg, Y., & Jersáková, J. (2020). Self-compatibility and autonomous selfing of plants in meadow communities. Plant Biology, 22, 120–128. https://doi.org/10.1111/plb.13049
Berjano, R., Gauthier, P., Fisogni, A., Doblas, D., Pons, V., & Thompson, J. D. (2013). Mate limitation in populations of the endangered Convolvulus lineatus L.: A case for genetic rescue? Journal of Nature Conservation, 21, 334–341. https://doi.org/10.1016/j.jnc.2013.05.001
Bertin, R. I. (2001). Life cycle, demography, and reproductive biology of Herb Robert (Geranium robertianum), Rhodora, 103, 96–116.
Bombles, K., Yant, L., Laitinen, R. A., Kim, S.-T., Hollister, J. D., Warthmann, N., Joffrey Fritz, J., & Weigel, D. (2010). Local-scale patterns of genetic variability, outcrossing, and spatial structure in natural stands of Arabidopsis thaliana. PLoS Genetics, 6(3), e1000890. https://doi.org/10.1371/journal.pgen.1000890
Busch, J. W., & Delph, L. F. (2012). The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. Annals of Botany, 109, 553–562. https://doi.org/10.1093/aob/mcr219
Charlesworth, D. (2006). Evolution of plant breeding systems. Current Biology, 16, R726–R735. https://doi.org/10.1016/j.cub.2006.07.068
Charlesworth, D., & Charlesworth, B. (1987). Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics, 18, 237-268. https://doi.org/10.1146/annurev.es.18.110187.001321
Chybicki, I. J., & Burczyk, J. (2009). Simultaneous estimation of null alleles and inbreeding coefficients. Journal of Heredity, 100, 106–113. https://doi.org/10.1093/jhered/esn088
Doyle, J. J., & Doyle, J. L. (1987). Isolation of plant DNA from fresh tissue. Focus, 12, 13–15.
Dudash, M. R., & Murren, C. J. (2008). The influence of breeding systems and mating systems on conservation genetics and conservation decisions. In S. P. Carroll, & C. W. Fox (Eds.), Conservation biology: Evolution in action (pp. 68–80). Oxford University Press.
Earl, D. A., & vonHoldt, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources, 4, 359–361. https://doi.org/10.1007/s12686-011-9548-7
Endress, P. K. (2010). Synorganisation without organ fusion in the flowers of Geranium robertianum (Geraniaceae) and its not so trivial obdiplostepomony. Annals of Botany, 106, 687–695. https://doi.org/10.1093/aob/mcq171
Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Molecular Ecology, 14, 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x
Gelmi-Candusso, T. A., Heymann, E. W., & Heer, K. (2017). Effects of zoochory on the spatial genetic structure of plant populations. Molecular Ecology, 26, 5896–5910. https://doi.org/10.1111/mec.14351
Goodwillie, C., Kalisz, S., & Eckert, C. G. (2005). The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. Annual Review of Ecology, Evolution, and Systematics, 36, 47–79. https://doi.org/10.1146/annurev.ecolsys.36.091704.175539
Goodwillie, C., Sargent, R. D., Eckert, C. G., Elle, E., Geber, M. A., Johnston, M. O., Kalisz, S., Moeller, D. A., Ree, R. H., Vallejo-Marín,
Widler-Kiefer, H., & Yeo, P. F. (1987). Fertility relationships of Geranium (Geraniaceae): Sectt. Ruberta, Anemonifolia, Lucida and Unguiculata. Plant Systematics and Evolution, 155(1–4), 283–306. https://doi.org/10.1007/BF00936305

Wierzbicka, M., Galera, H., Sudnik-Wójcikowska, B., & Wilkomirski, B. (2014). Geranium robertianum L., plant form adapted to the specific conditions along railway: “railway-wandering plant”. Plant Systematics and Evolution, 300, 973–985. https://doi.org/10.1007/s00606-013-0936-1

Willson, M. F., Miller, L. J., & Rathcke, B. J. (1979). Floral display in Phlox and Geranium: Adaptive aspects. Evolution, 33, 52–63. https://doi.org/10.1111/j.1558-5646.1979.tb04662.x

Yeo, P. F. (1973). The biology and systematics of Geranium, sections Anemonifolia Knuth and Ruberta Dum. Botanical Journal of the Linnean Society, 67(4), 285–346. https://doi.org/10.1111/j.1095-8339.1973.tb02551.x

Yeo, P. F. (1985). Hardy Geraniums. Croom-Helm.

Yeo, P. F. (2004). The morphology and affinities of Geranium sections Lucida and Unguiculata. Botanical Journal of the Linnean Society, 144, 409–429. https://doi.org/10.1111/j.1095-8339.2003.00258.x

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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