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RESEARCH ARTICLE

Intriguing small-scale spatial distribution of chloropastic and nuclear diversity in the endangered plant *Biscutella neustriaca* (Brassicaceae)

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Abstract *Biscutella neustriaca* is an isolated plant taxon with about three thousand known individuals distributed in several fragmented populations. Despite its status as an endangered plant subject to a European LIFE programme for its protection, no conclusive genetic analysis has been performed to help its conservation. We analysed the genetic variability and distribution of nuclear microsatellite markers in a large sample of the population, as well as of the *MatK* chloroplastic gene in a subsample. We showed, first, that both pollen and seed dispersal, as well as clonal reproduction are strongly limited, and the mating system is obligate outcrossing. Second, we detected two highly divergent chloroplast haplogroups, as well as two completely distinct nuclear gene pools suggesting an ancient isolation between two groups of populations. Intriguingly, a third group of populations appears to combine the nuclear gene pool of one group with the chloroplast haplotype of the other group, suggesting a more recent dramatic colonization and foundation event. Thanks to complementary geological and historical data, we propose a scenario for the evolutionary history of this metapopulation influenced by the dynamics of Seine meanders and human activities. Finally, we give some suggestions for future conservation actions.

Keywords Conservation genetics · Genetic structure · Admixture · Local adaptation

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Introduction

Molecular markers have become a standard tool, in association with demographic studies, to assess the conservation value of populations (Hedrick 2001; Oostermeijer et al. 2003), to help the management of rare or endangered species through inference on the biology of the species (Piggott and Taylor 2003), through the identification of management units (Moritz 1994; Palsbøll et al. 2007), and through guiding reinforcement procedures (Leinonen et al. 2008). Moreover, patterns of neutral polymorphism could also provide information to infer ecological and historical processes responsible for the currently narrow distribution of threatened species.

Biscutella neustriaca Bonnet, 1879 (Brassicaceae) is an herbaceous, perennial, diploid ($2n = 18$; Tremetsberger et al. 2002) plant taxon from the Seine river valley in Northern France (Fig. 1a). Its distribution currently comprises a handful of disconnected populations located within a very small area (the largest distance between two populations is less than 10 km; Fig. 1a), and has experienced the extinction of several populations since the 1930's (Fig. 1b). *B. neustriaca* belongs to the *B. laevigata* species complex which is widespread in central and southern

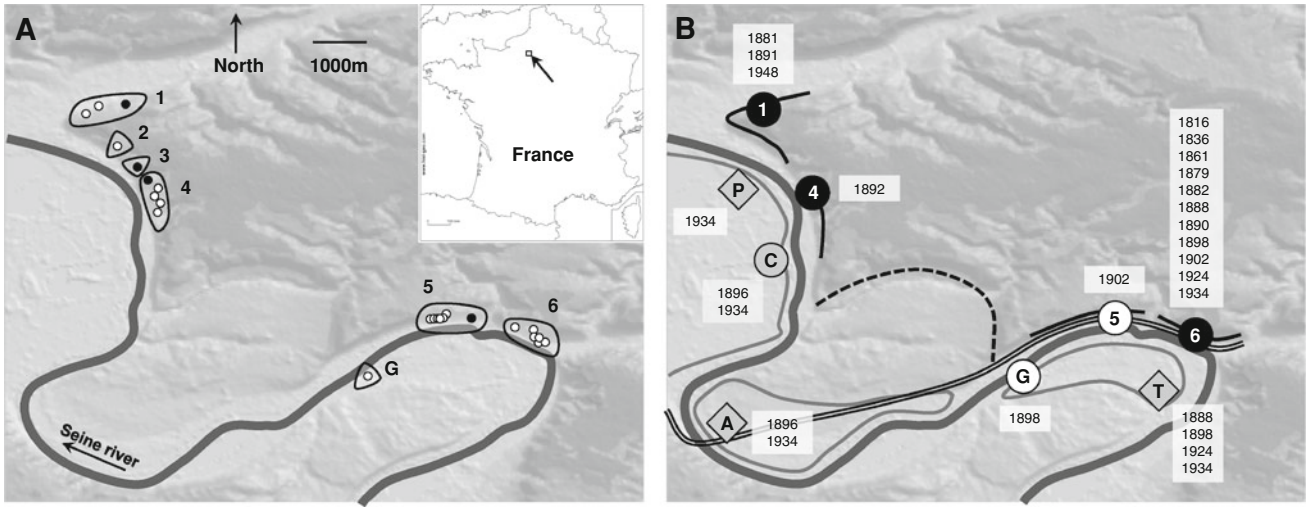

Fig. 1 **a** Geographical location of *Biscutella neustriaca* populations. *Small circles* represent subpopulations (subpopulations 1a, 3, 4a and 5a where seeds were sampled for paternity analysis are indicated by *filled circles*). Populations are surrounded by a *solid black envelope*. Populations 1–4 form the northern group, and populations 5 and 6 form the southern group. **b** Geological situation of *B. neustriaca* populations and historical reports of the taxa during the period 1816–1948 (see Table S4 for references). Following information is represented: calcareous hillsides (*black line*); slopes formed by the riverbed 1 million year ago (*dotted black line*); floodplains (*grey lines*); and the railway constructed in 1891–1896 (*double black line*). Populations are indicated by *circles coloured* according to the period the taxa was reported for the first time: before 1896 (*black*) and after 1896 (*white*). Population in *grey* were not considered in this study because plants were never reported after 1934 and supposed extinct (represented by *diamonds*; *P* Poses, *A* Andé, *T* Tosny) or not found during sampling (*C* Tournedos)

Europe (Olowokudejo 1992; Tremetsberger et al. 2002). The only previous study based on allozyme data from five individuals of *B. neustriaca* have suggested that it could be included in the subspecies *B. laevigata varia*, which is patchily distributed along the Rhine Valley (Tremetsberger et al. 2002). *B. neustriaca* might thus correspond to isolated populations of *B. laevigata* located in the margin of the species distribution area, as it is the case for many of the micro-endemic taxa described in the *B. laevigata* complex (Cook 2001). However, the closest populations of a related species within the genus *Biscutella* are located more than 200 km away. Moreover, the oldest descriptions of the local flora indicated that the distribution of *B. neustriaca* never exceeded the current area (Fig. 1b), enforcing the hypothesis that this metapopulation has been isolated for a long time from other members of the *B. laevigata* complex.

Because of this high level of isolation, *B. neustriaca* benefited from the two first E.U.-funded projects for plant conservation and protection in 1999–2003 and 2006–2012 (European LIFE Programmes LIFE99 NAT/F/6332 and LIFE06 NAT/F/000137 respectively). In 2008, at most 3,000 individuals in six main populations were counted. Within populations, individuals are generally aggregated in patches constituting subpopulations of less than ten to several 100 individuals. Populations of *B. neustriaca* are divided into two distinct geographic groups (northern and southern groups) that are separated from each other by an 8 km wide curl of the Seine, covered with woods and fields, both habitats being unsuitable for this lawn and meadow plant (Fig. 1). Because we previously found in experimental conditions that efficient pollen dispersal barely occurred beyond 10 meters in this insect-pollinated plant (Leducq et al. 2010), these two groups of populations may be partially genetically isolated from each other. In our previous experimental study, we also demonstrated that this plant possessed a functional self-incompatibility system, which could locally increase mate limitation in conditions of low individual density (Leducq et al. 2010). However, in highly fragmented populations of a self-incompatible plant, partial breakdown of self-incompatibility (SI) could evolve (Busch 2005; Mable et al. 2005), and thus we have no clear evidence about the extent of SI within populations of *B. neustriaca*.

Populations of *B. neustriaca* are mainly located on calcareous slopes on the right banks of the Seine (Fig. 1). Ecological habitat characteristics vary among and within locations from very unstable cliffs to grass slopes, with different levels of shrub cover and exposure to light (Table 1). Human activities have had a profound impact on the vegetation of these calcareous slopes: (1) a long history of grazing by sheep has maintained open habitats of calcareous grasslands over large areas; (2) in the last decade of the nineteenth century, the construction of a railway profoundly transformed the Seine banks around the southern populations (Fig. 1b); (3) in the second half of the twentieth century, the establishment of buildings on the banks of the Seine and the abandonment of sheep grazing

Table 1 Location (in italic), estimated size (2008) and properties of the sampled populations and subpopulations of *Biscutella neustriaca* (see Table S3 for additional subpopulation details)

Subpop. labels[a]	Subpop. size	Sampling		Disturbance (since XIX century)	Habitat	Management (since 2000)
		Nuclear markers	*MatK*			
Population 1 (NORTH): Romilly s/Andelle						
1a	>700	700	2	Grazing	Grass slope	Grazing
1bg	40	21	5			
1c	75	17	–	Crumbling	Chalk quarry	
Population 2 (NORTH): Val Pitant						
2a	74	18	5	Grazing	Road bank	Mowing
Population 3 (NORTH): Amfreville/ss-les-monts						
3	77	77	3	Grazing	Grass slope	–
Population 4 (NORTH): Val Hamet						
4 NN	120	107	3	Grazing	Road bank	Mowing/ grazing
4N	190	66	5		Grass slopes	–
4S	234	87	6			
4SS	302	74	–			
4C	330	43	6	Crumbling	Cliffs/chalk quarry	
Population 5 (SOUTH): Saint-Martin						
5f	>262	262	3	Railway	Railway banks	–
5 h	>16	16	2			
5i	>39	39	2			
5a	69	69	3			
5dg	>21	21	6			
5c	50	11	3	Crumbling	Cliffs/grass slope	–
Population 6 (SOUTH): Les Andelys						
6dhi	25	13	8	Railway	Railway banks	–
6a	160	55	3			
6 g	6	6	3	Crumbling/ grazing	Cliffs/grass slopes	
6f	39	35	3			
6bc	20	12	6			
Bernière sur Seine-Les Garennes (SOUTH)						
G	7[b]	3	3	Sand exploitation	Sand bank/quarry	–

[a] Subpopulation labels correspond to patch labels defined by conservation organizations. In some cases, for practical purpose, we considered several adjacent patches as a single subpopulation (e.g. subpopulation 6dhi corresponding to patches d, h and i from population 6)

[b] 216 individuals counted in 2009

leading to habitat closure have caused dramatic reductions of grasslands area; (4) over the last decade, the ecological management of habitat through grazing and mowing have safeguarded the remaining calcareous grasslands (Poudevigne et al. 2002; Dutoit et al. 2004); *Archives départementales de l'Eure*; Table 1).

In this study we used chloroplastic and nuclear markers in *Biscutella neustriaca* with three aims: (1) to gather information on the reproductive biology of this plant in natural populations (self-incompatibility, pollen dispersal, seed dispersal, clonal reproduction), to compare with our previous observations in experimental conditions; (2) to describe the population genetic structure and link this structure to the phylogeographic history of this isolated metapopulation; and (3) to apply our knowledge to orient conservation management practices for this plant. Specifically, we aimed at determining whether genetic differentiation occurred between the most spatially separated northern and southern groups of populations, and among local patches of individuals. For this purpose, multilocus genotypes at 10 microsatellite nuclear loci were obtained from individuals sampled from all known populations representing a sample of about 60 % of individuals. Additionally, we sequenced the chloroplast gene *MatK* in a representative sub-sample of 80 individuals in order to assess distribution and phylogeny of maternal lineages. Finally, we used paternity assignment on seedlings to determine the extent of SI in natural conditions, to identify

contemporaneous pollen migration among populations, and to link these observations to patterns of genetic structure.

Methods

Sample collection

We sampled leaf material from 1,751 individuals from all known populations of *Biscutella neustriaca* during spring and summer 2007 and 2008. Leaf material was taken in the least destructive possible way, so that most frail individuals were not sampled. Populations were numbered from 1 to 6, and an additional isolated population from the right riverbank was designated population G (Fig. 1). The populations form the elements of two large spatial groups: populations 1–4 belong to the northern group and populations 5, 6 and G, belong to the southern group (Fig. 1). Subpopulations were designated by their population number and an additional label defined by conservation organizations (Table 1; see Table S3 for more details). In population 4, the numerous subpopulations previously defined by conservation organizations were grouped into five subpopulations corresponding to well-defined geographical areas (4 NN, 4N, 4C, 4S and 4SS; Table S3). Four locations (1a, 3, 4a and 5a) were sampled exhaustively (with 4a included in subpopulation 4 NN). In other subpopulations, at least 20 % of observed individuals were independently and randomly sampled (Table 1). Only three individuals were found within population G during the sampling period, but it was reported that the size of this population strongly fluctuates between years (216 individuals were observed in 2009; Julien Buchet personal observation). Mapping of individuals was realized with a tachymeter (precision: 1 cm) within subpopulations 1a, 4a and 5a. Mapping was realized with a decametre (precision: 1 m) in population 3. In the remaining subpopulations, GPS coordinates of sampled individuals (populations 1, 2, 5 and 6) or small patches of individuals (population 4) were recorded (precision: 1–8 m).

Seed sampling

Seeds for paternity analysis were collected in May–July 2008 from about 10–30 % of individuals of subpopulations 1a, 3, 4a and 5a. Seeds were sown at 22 °C in the greenhouse and seedlings were grown until sufficient leaf material was available. A very high variance in number of seedlings per mother was obtained in these subpopulations (1–88 seedlings per mother, Table 2). In subpopulation 1a, most individuals produced a low number of seeds; in subpopulation 3, many individuals produced no seeds; and in subpopulation 5a, the germination rate of seeds was very low ($\approx$40 %) compared to other subpopulations (>75 %). Overall, we obtained leaf material from 1,630 seedlings (272–563 per subpopulation, Table 2).

Molecular analysis

Collected leaf material from adults and seedlings was dried for 24 h at 55 °C. DNA was extracted from 10 to 15 mg of dried leaf material using the extraction kit NucleoSpin® 96 Plant from Macherey–Nagel®.

Table 2 Results of analyses of clonality, paternity, and outcrossing rate within four subpopulations of *B. neustriaca*

Subpopulation	1a	3	4a	5a
Clonality				
N = # of ramets (G = # of genets)	700 (642)	77 (73)	82 (79)	69 (69)
R = Index of clonal diversity	0.917	0.947	0.963	1
P_{sex} (range)	$(2.88 \times 10^{-4}) - (5.52 \times 10^{-39})$	$(1.50 \times 10^{-10}) - (1.61 \times 10^{-8})$	8.46×10^{-9}	–
Clone size (m)	0.2 (0.0–1.8)	1.1 (0.3–2.3)	1.1 (0.2–1.6)	–
Paternity analysis				
Sampled mothers	65	17	23	21
Seedlings per sampled mother	8.1 (1–53)	22.8 (4–86)	17.5 (1–37)	11.9 (1–88)
Total seedlings	563	390	405	272
Proportion assigned (95 %)	25.7 %	25.5 %	40.7 %	69.8 %
Outcrossing rate (t_m : mean ± S.E.)	0.977 ± 0.022	0.975 ± 0.039	0.952 ± 0.018	0.982 ± 0.031

Number of ramets sampled (N) and number of genets (G, indicated within parentheses). Index of clonal diversity [$R = (G - 1)/(N - 1)$]. Range of probabilities that each repeated genotype originated from distinct sexual reproductive events (P_{sex}). Clone size, estimated as the mean distance between different ramets of a same genet (range in parenthesis). Details of paternity analysis: number of individuals on which seedlings were collected (sampled mothers), mean number of seedlings analyzed per mother (range in parenthesis), total number of seedlings analyzed and proportion of seedlings assigned at the 95 % confidence level. Multilocus outcrossing rate (t_m)

We developed PCR primers for ten polymorphic microsatellite loci (Table S1). Microsatellite markers were developed using a microsatellite-enriched genomic library that we obtained from an individual of *B. neustriaca* from subpopulation 4 NN following an enrichment procedure with Dynabeads (Glenn and Schable 2005). The procedure followed Frérot et al. (2010). In brief, we performed in silico analysis of the sequences with an in-house PERL script that uses the software MREPS (Kolpakov et al. 2003) to find microsatellite patterns and alignment procedures to eliminate sequence redundancy. Primer sequences were designed in flanking regions using the Primer3 software (http://frodo.wi.mit.edu/). We genotyped all individuals for these ten microsatellite loci. Forward primers (F) were labelled with either Applied Biosystems® FAM or VIC dyes. Several loci were amplified simultaneously using a multiplex PCR procedure (Table S1) as described in (Frérot et al. 2010). After amplification, PCR multiplex products were loaded two at a time (multiplex 1 with multiplex 3 and multiplex 2 with multiplex 4) on a 16-capillary ABI 3130 sequencer. Each sample contained 1 µl of each PCR multiplex product, 9.7 µl of formamide and 0.3 µl of size marker 500Liz Applied Biosystems®. Genotypes were determined with the software GENEMAPPER™ v3.7 (Applied Biosystems®).

We amplified and sequenced a 2,495-bp (base pair) region of the chloroplast genome including the *MatK* gene for a subsample of 80 individuals (Table 1). To do so, we used a set of three pairs of PCR primers producing three overlapping fragments of about 1,000 bp (Table S2, Figure S1). These primers were obtained after a first round of sequencing performed with primers K1F and K2R delimiting the total *MatK* sequence (Johnson and Soltis 1995; Fénart et al. 2006). PCR products were sequenced with the BigDye3.1 sequencing kit (Applied Biosystems), loaded on an ABI 3130 capillary sequencer. Sequences were edited using MEGA5 (Tamura et al. 2011) and the complete sequence of *MatK* was reconstructed for each individual from the three overlapping fragments using the software SeqScape® (Applied Biosystem).

Divergence at the chloroplastic gene MatK

We estimated pairwise nucleotide divergence among *MatK* sequences using the maximum composite likelihood method (Tamura et al. 2004). All codon positions were included, and positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. We reconstructed the haplotype network including a partial sequence from the congeneric species *B. didyma* (GQ424575, Couvreur et al. 2010), which is external to the species complex *B. laevigata* (Olowokudejo and Heywood 1984; Tremetsberger et al. 2002).

We estimated the time since divergence between haplotype groups I and II with a 1,476 bp dataset from 33 published brassicales *MatK* sequences and one Malvale outgroup (Supplemental Table S5). We first estimated the tree topology via a maximum likelihood analysis using PHYML (Guindon et al. 2010). The obtained topology was then used as a starting tree in the software BEAST v1.7.2 (Drummond and Rambaut 2007). Two fossil calibrations were used in the analyses: *Dressiantha bicarpellata*, 89.3 Mya (Million years) (Gandolfo et al. 1998), representing the oldest known Brassicales (Couvreur et al. 2010) and the oldest known Brassicaceae pollen fossil dated at 16 Mya (Yavuz-Iska and Demirci 2009). A uniform distribution as prior to the calibration nodes was applied and the age of the two fossils was used as lower hard bound of the specific calibrated nodes (i.e. the brassicales and the brassicaceae). The age of the root was constrained by an upper hardbound set to the age of the oldest eudicot pollen (125 Mya; Brenner 1996). We assumed a birth–death model of speciation. In these analyses, each codon position was assumed to follow an independent model of evolution. Analyses were undertaken by sampling every 1,000 iterations from a run of 20 million generations (the two first million iterations was discarded as burn-in). Tracer 1.5 was used to check for stationarity of the model likelihood and parameters (Lemey et al. 2010). The effective sampling size of all parameters was above 200 and therefore considered reliable.

Analyses of genetic diversity and population genetic structure using microsatellite data

We analyzed patterns of clonal reproduction with the software GenClone 2.0 (Rozenfeld et al. 2007). For each multilocus genotype found in more than one ramet, we estimated the probability that the repeated genotypes originated from distinct sexual reproductive events (P_{sex}), according to Arnaud-Haond et al. (2007). The index of clonal diversity was computed as $R = (G - 1)/(N - 1)$, with N the number of ramets sampled, and G the number of distinct multilocus genotypes (genets). Spatial coordinates of individuals were used to compute clone size. Subsequent genetic analyses were performed after removing all but one ramet per genet. We computed the following statistics of genetic variation at microsatellite loci within each subpopulation using FSTAT 2.9.3.2 (Goudet 1995): allelic richness (A), estimated by the rarefaction method based on nine diploid individuals; Nei's gene diversity (H_e); and Wright's inbreeding coefficient (F_{is}). We also tested for differences in A and H_e between northern and southern populations (1,000 permutations). We performed a hierarchical analysis of genetic structure at microsatellite loci by computing multilocus estimates of hierarchical F-statistics using the

R-package HIERFSTAT (Goudet 2005). Four nested levels were considered: (1) total population; (2) group (northern vs. southern populations); (3) population and (4) subpopulation (see Fig. 1). We estimated 95 % confidence intervals for these *F*-statistics using bootstrapping over loci.

We performed Bayesian-based clustering using the software STRUCTURE 2.3.2 (Falush et al. 2003), allowing us to test for the existence of homogenous genetic subsets within the metapopulation at microsatellite loci, independent of population and subpopulation geographical structuring. Analyses were carried out under the admixture model over 1,000,000 iterations of the MCMC chain with a 100,000 burn-in period. To test for the most likely number of homogeneous genetic subsets, designated *K*, we used the $\Delta(K)$ method developed by Evanno et al. (2005) based on the estimate of the likelihood by performing 20 runs of each *K* value in the range 1–20.

Inference of recent immigration rates

We used a Bayesian method to estimate recent immigration rates among pairs of subpopulations based on microsatellite multilocus genotypes, as implemented in the software BIMr (Faubet and Gaggiotti 2008). This Bayesian method uses the gametic disequilibrium signal generated by immigrant individuals or their descendants to infer rates of recent immigration. Subpopulations with sample size lower than 20 were discarded. We performed five different MCMC runs to check for congruence, with 500,000 iterations per run. The first 20,000 iterations consisted of short pilot runs used to tune up the proposal distributions to obtain reliable acceptance rates. The next 10,000 iterations were discarded as burn-in and the remaining observations were sampled every 50 iterations, giving a sample size of 9,400 for each analysis.

Paternity analysis: inference of contemporaneous pollen migration

We performed paternity analysis on seedlings from four fully sampled subpopulations (1a, 3, 4a and 5a) grouped together with the software Cervus 3.0. (Marshall et al. 1998). We first estimated allelic frequencies from all known potential parents, i.e. 1,751 individuals sampled through all populations of *B. neustriaca*. Then, we simulated paternity analysis for multilocus genotypes of 100,000 offspring based on the estimated allelic frequencies. We assumed that 60 % of candidate fathers were sampled, which approximately corresponded to our global sample. We chose the 95 % confidence level and assumed the proportion of mistyped loci was 1 %. We considered all sampled individuals among all known populations as candidate fathers. Seedlings and individuals genotyped for less than seven loci were removed from the analysis. We based paternal assignments on the best delta value, i.e. for a given seedling, when the LOD score value of the most probable candidate father was significantly higher than the second candidate father's highest LOD-score value (Marshall et al. 1998). Seedling multilocus genotypes, grouped by maternal families, were also used to estimate the outcrossing rate in subpopulations 1a, 3, 4a and 5a with the software MLTR 3.3 (Ritland 2002).

Results

Variation in MatK chloroplast sequences

Five haplotypes of *MatK* were found within *B. neustriaca* (Genbank accessions numbers JN987156–JN987160). Three were frequent (haplotypes Ia, Id and II) and two were very rare (haplotype Ic in three copies and Ib in one copy). Each subpopulation sample was fixed for a single haplotype, with the exception of subpopulation 1a who showed two haplotypes (Ia and Ib). The distribution of the three most frequent haplotypes showed strong geographic clustering: haplotype Ia was present in northern populations only (populations 1, 2 and 4), haplotype Id in populations 5 and G only, and haplotype II in subpopulation 5a and population 6 only. Rare haplotypes Ib and Ic were found in subpopulation 1a and population 3 respectively. The haplotype network indicated that the *B. neustriaca* haplotypes strongly diverged from the closest outgroup *B. didyma* (Fig. 2). Within *B. neustriaca*, two groups of haplotypes, separated by five nucleotide differences and an insertion/deletion of 35 bp, were revealed (Figure S1). The first group comprised the most common haplotype Ia, as well as three haplotypes (Ib, Ic and Id) differing by one to three nucleotides from Ia. The second group contained a single haplotype (the common haplotype II). The estimated time of divergence between haplotype groups I and II was 2.53 Mya (0.58–5.19 Mya).

Clonality

Overall, our ten microsatellite loci allowed us to discriminate efficiently all genotyped individuals with a low overall unique genotype probability ($p_{gen} < 0.001$, i.e. the probability that the same multilocus genotype is derived from distinct sexual reproductive events). In all cases where several ramets shared the same multilocus genotype, we obtained very low values of the probability that the repeated genotypes originated from distinct sexual reproductive events (p_{sex}), indicating that these correspond to the same genet. The index of clonal diversity (*R*), measuring the proportion of samples of pairs of ramets that belong to different genets, or more simply the genotype diversity, was high overall (0.96) but varied somewhat among the

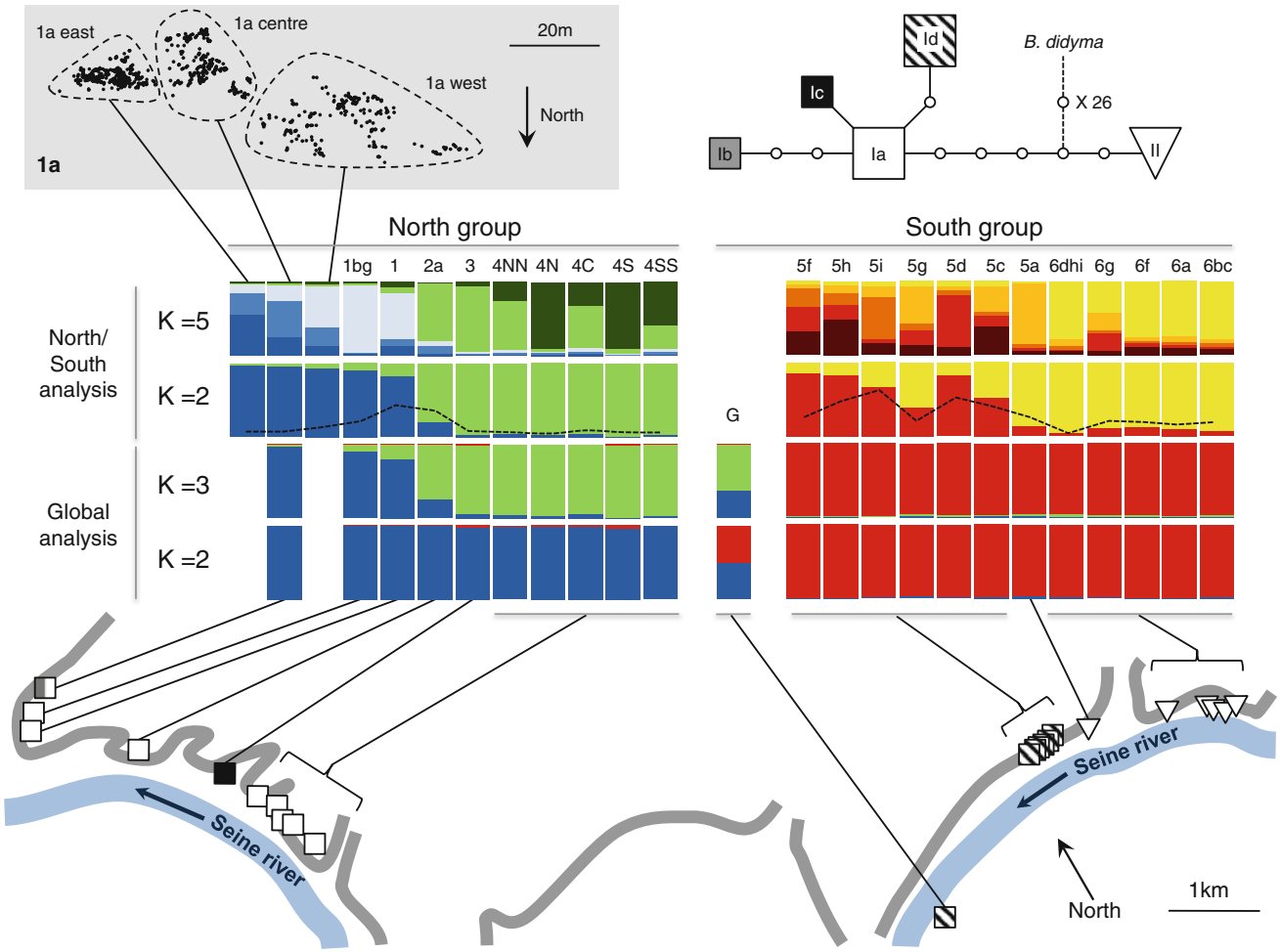

Fig. 2 Spatial distribution and phylogeny of chloroplast and nuclear genetic diversities in *B. neustriaca* subpopulations. *Grey lines* indicate calcareous slopes. Locations of subpopulations are represented by *symbols* according to observed *MatK* haplotypes I (*squares*) or II (*triangles*). Haplotype *Ib* is labelled in *grey*, *Ic* in *black*, *Id* hatched. *Top right MatK* haplotype network including *B. didyma* as outgroup (GQ424575). The network was manually inferred from *MatK* sequences. Five haplotypes are indicated by their respective symbols. *Lines* indicate one-step mutations, deletions or insertions. *Circles* indicate intermediate and hypothetical haplotypes. *Middle* results from Bayesian-based clustering analyses (STRUCTURE) represented by diagrams indicating the proportion of each cluster in a given subpopulation. Northern and southern populations were either analysed together (global analysis, assuming $K = 2$ and 3) or separately (north and south analyses assuming $K = 2$ and 5 in both cases). *Dotted lines* in north and south analysis for $K = 2$ represent the proportion of mixed genotypes in each subpopulation (i.e. individual assigned to at least 20 % of all clusters). *Top left* for north analysis, results are detailed for three spatial patches of subpopulation *1a*

four exhaustively sampled subpopulations (from 0.917 to 1; Table 2). Most clones consisted of only two ramets, and the distance between ramets of the same genet ranged from 0.01 to 2.30 m (Table 2). Subpopulation 1a showed the highest extent of clonality, but with the lowest average clone size (0.2 m), whereas subpopulation 5a showed no evidence of clonality. Subpopulations 3 and 4a had intermediate levels of clonality, but with a higher average clone size (1.1 m).

Genetic diversity at nuclear microsatellite loci

We found a high proportion of missing genotypes at locus B45 in populations 5 (14.4 %) and 6 (59.5 %), as compared to other populations (0–4.5 %), indicating the occurrence of null alleles at high frequency in these southern populations. Hence, all analyses including populations 5 and 6 omitted locus B45. Table 3 reports estimates of genetic diversity at microsatellite loci for each subpopulation. Genetic diversity was significantly lower within southern populations (5 and 6) than in northern populations (1 to 4) when estimated as the allelic richness [$p < 0.001$; $A = 4.81$ (3.95–5.43) in northern vs. 3.79 (3.11–4.35) in southern subpopulations] or Nei's gene diversity [$p < 0.001$; $H_e = 0.65$ (0.57–0.73) in northern vs. 0.52 (0.38–0.59) in southern subpopulations]. Allelic richness and genetic diversity were found to be significantly positively correlated to the log of the estimated subpopulation size ($r^2 = 0.239$, $p < 0.05$; and $r^2 = 0.244$,

Table 3 Statistics of genetic diversity (mean among loci ± standard deviation) within populations of *Biscutella neustriaca* at nine nuclear microsatellite loci (locus 45 removed from analysis): allelic richness, estimated by the rarefaction method based on nine diploid individuals; Nei's gene diversity (H_e); Wright's inbreeding coefficient (F_{is})

Population	Sub-population	Allelic richness	H_e	F_{is}^{a}
1	1a	4.226 ± 1.509	0.646 ± 0.142	0.026***
	1bg	3.945 ± 1.628	0.609 ± 0.152	−0.100
	1c	4.866 ± 2.239	0.627 ± 0.197	0.062
2		4.085 ± 2.145	0.570 ± 0.252	−0.033
3 and 4	3	4.981 ± 1.859	0.670 ± 0.149	−0.016
	4 NN	5.430 ± 1.921	0.725 ± 0.112	0.056**
	4 N	5.260 ± 2.194	0.661 ± 0.141	0.091***
	4 C	5.262 ± 2.248	0.651 ± 0.154	0.014
	4 S	4.953 ± 1.828	0.626 ± 0.152	0.090***
	4 SS	5.068 ± 1.935	0.651 ± 0.150	0.102***
5	5f	3.508 ± 1.086	0.501 ± 0.184	0.091***
	5h	3.111 ± 1.616	0.375 ± 0.218	0.036
	5i	3.544 ± 1.418	0.536 ± 0.187	0.066*
	5 g	3.668 ± 1.381	0.477 ± 0.200	0.068
	5d	n.a.[b]	0.586 ± 0.249	0.056
	5c	4.060 ± 1.747	0.486 ± 0.191	0.169*
	5a	3.731 ± 1.570	0.536 ± 0.258	0.010
6	6dhi	3.883 ± 1.160	0.568 ± 0.198	0.073
	6g	n.a.[b]	0.548 ± 0.108	−0.014
	6f	4.074 ± 1.802	0.556 ± 0.241	0.061*
	6a	3.940 ± 1.363	0.536 ± 0.194	0.147***
	6bc	4.345 ± 1.999	0.570 ± 0.262	0.034
G		n.a.[2]	0.731 ± 0.277	0.241*

[a] Results of the test of deviation from Hardy–Weinberg genotypic proportions: P value <0.001 (***); <0.01 (**); <0.05 (*)

[b] *n.a.* not applicable because the sample size was too small

$p < 0.05$, respectively). However, for equivalent sizes, diversity remained lower in southern populations, especially in population 5 (Figure S2). Population G showed high gene diversity (0.72) compared to the small current population size, which is in agreement with strong fluctuations in population size suspected in this population. F_{is} values were low, as expected for a self-incompatible plant, but predominantly positive, with a significant overall excess of homozygotes in several subpopulations, which could be due to local biparental inbreeding.

Population genetic structure

F_{ST} among subpopulations at the global scale was surprisingly high (0.163), taking into account the strictly outcrossing mating system, and the restricted geographic distribution. However, within the northern and southern groups, the values of F_{ST} among subpopulations were much lower (0.067 and 0.081, respectively), suggesting strong differentiation between the geographic groups. This was confirmed by the results of the hierarchical analysis of genetic structure (Table 4). The strongest pattern of differentiation occurred between the northern and southern groups ($F_{\text{group within total}} = 0.0903$), whereas differentiation at the population ($F_{\text{pop within group}} = 0.0405$) and subpopulation ($F_{\text{subpop within pop}} = 0.0409$) levels were significantly lower (non-overlapping 95 % confidence intervals as determined by bootstrapping over loci).

We performed non-parametric genetic clustering using the STRUCTURE software in two steps. The analysis was first performed at the global scale, considering all subpopulations together, and repeated for a range of clusters number (K) lying between 1 and 22 (Figure S3a). Altogether, our results showed the existence of two large clusters ($K = 2$), separating northern populations (populations 1–4) and southern populations (populations 5 and 6), or three clusters ($K = 3$), separating additionally population 1 from the other northern populations (Fig. 2). Population G was the exception with all individuals showing admixture between northern and southern clusters. For higher values of K ($K > 3$), the clustering between north and south still remained strongly supported, but likelihood estimates varied greatly among the 20 independent replicates and, for a given replicate, were found to depend strongly on the number of clusters (1–3) identified in the southern group (Figure S4). Hence, we decided to perform further analyses by separating the data into two a priori groups, corresponding to northern and southern populations, excluding population G.

For the northern group analysis, the highest $\Delta(K)$ were found for $K = 2$ and 5 (Figure S3b). For $K = 2$, we found

Table 4 Hierarchical analysis of genetic structure at nine nuclear microsatellite loci (locus 45 removed) in *Biscutella neustriaca*

	Hierarchical level			
	$F_{\text{Group within total}}$	$F_{\text{Pop within group}}$	$F_{\text{Subpop within pop}}$	$F_{\text{ST}} = F_{\text{Subpop within total}}$
Total	0.0903	0.0405	0.0409	0.1625
	[0.0727–0.1094]	[0.0169–0.0666]	[0.0239–0.0587]	[0.1239–0.1994]
North[a]	–	0.0335	0.0359	0.0672
		[0.0112–0.0545]	[0.0196–0.0588]	[0.0409–0.0996]
South[b]	–	0.0403	0.0465	0.0805
		[0.0096–0.0750]	[0.0226–0.0743]	[0.0401–0.1414]

Values presented are multilocus estimates of hierarchical F-statistics and their 95 % confidence intervals (square brackets) determined by bootstrapping over loci. Four levels were considered: total population; group (northern vs. southern populations); population; and subpopulation

[a] Analyses were restricted to the northern populations, so the group level was removed

[b] Analyses were restricted to the southern populations, so the group level was removed

similar results to the global analysis performed with $K = 3$, with a first cluster corresponding to subpopulation 1a, a second cluster corresponding to populations 3 and 4, and a group of admixed subpopulations showing a north–south cline between both clusters (Fig. 2). For $K = 5$, three highly mixed clusters segregated within population 1, with a clear east–west geographic cline, and two other clusters segregated within populations 2, 3 and 4 (Fig. 2). For the southern group analysis, the highest $\Delta(K)$ were found for $K = 2$, 3 and 5 (Figure S3c). In all cases, the two most distant subpopulations (5f and 6bc) were strongly differentiated and intermediate subpopulations showed admixture with an east–west cline (Fig. 2).

Overall, nuclear microsatellite data clearly indicated strong differentiation between the northern and southern groups, and clines of allelic frequencies within each group, suggesting isolation by distance. The chloroplast genetic data also showed strong geographic structure, but the deepest genetic break occurred between population 6 and all other populations (Fig. 2).

Bayesian analysis of recent immigration rates

The results from five different runs with the software BimR were concordant and estimated that immigration rates (proportion of individuals exchanged between two subpopulations) between each pair of subpopulations were lower than about 10^{-8} per generation, suggesting that the sampled subpopulations did not experience successful migration in the last two generations.

Paternity analysis: detection of contemporaneous pollen migration

Paternity analysis was carried out on a pool of seeds collected in subpopulations 1a, 3, 4a and 5a. The 1,751 adult individuals sampled in the whole population were all considered as potential fathers. The proportion of seeds assigned to a given father ranged from 25 to 70 % depending on the subpopulations (Table 2). Among the offspring with assigned paternity, the majority (72.3–97.7 %) was assigned to fathers located within the same subpopulation (Table 5). The proportion of fathers detected outside subpopulations was higher in subpopulations 5a (27.7 %) than in other subpopulations (1.3–5.5 %). Most of immigrating pollen came from within the same population or from subpopulations located within 2 km. The maximum distance between a mother plant and an identified father was about 4 km; we detected no pollen migration among northern and southern populations (Table 5). Overall, the high proportion of immigrating pollen from neighboring subpopulations detected through paternity analysis was inconsistent with the very low migration rates estimated through the indirect method (BimR, see above). Estimates of multilocus outcrossing rate were close to 1.0 in all four subpopulations investigated (Table 2).

Discussion

Clonal reproduction and mating system

Our results showed that some level of clonal reproduction occurs, through rhizomes, but most clones consisted of only two ramets, so that the overall genotypic diversity was very high ($R = 0.96$ in *B. neustriaca*, whereas $R = 0.42$ for an average over 195 clonal taxa as reported by Vallejo-Marin et al. 2010). The maximum distance between ramets of the same genet was 2.30 m, which was consistent with our observations that in some populations long rhizomes connecting several rosettes cropped out of the soil. Estimates of the multilocus outcrossing rate were higher than 0.95 in all four subpopulations, confirming that

Table 5 Contemporaneous pollen migration among four targeted subpopulations (first column) and all possible source populations estimated from CERVUS at 95 % confidence level

Origin of seedlings		Origin of pollen						
		Within subpopulation	Source population					
			North				South	
			1	2	3	4	5	6
North	1a	97.7%	0.8%[a]	–	0.8%	0.8%	–	–
	3	96.9%	–	–	–	3.1%	–	–
	4a	94.5%	0.6%	–	1.8%	3.0%[b]	–	–
South	5a	72.3%	–	–	–	–	23.1%[c]	4.6%

[a] 1a excluded

[b] 4a excluded

[c] 5a excluded

Proportions of pollination events identified within the targeted subpopulations are indicated in second column. Grey cells indicate pollen migration between northern and southern subpopulations

the SI system detected under experimental conditions (Leducq et al. 2010) is fully functional within natural populations. Hence, despite an observed reduction in fruit-set in some subpopulations, it does not appear that a breakdown of self-incompatibility, leading potentially to reproductive assurance through selfing, occurred in this plant, as was also reported e.g. for the endangered and strictly self-incompatible species *Brassica insularis* (Glémin et al. 2008) and *Rutidosis leiolepis* (Young et al. 2002).

Patterns of gene flow

Information from nuclear and chloroplast markers, as well as from direct (paternity analysis) versus indirect (geographic distribution of multilocus genotypes) approaches, allowed us to infer patterns of gene flow in *Biscutella neustriaca*.

Several lines of evidence suggest that seed dispersal is highly restricted spatially and does not contribute to gene exchange between neighboring populations. First of all, chloroplast haplotypes were fixed within most subpopulations, and were highly structured spatially among subpopulations. Particularly in the southern group, very divergent haplotypes were found when comparing a group comprising all subpopulations of population 5 except subpopulation 5a, and a second group comprising population 6 together with subpopulation 5a. Secondly, indirect estimates of current migration rates between pairs of subpopulations, based on a Bayesian inference with the software BimR, showed virtually no evidence of recent genetic exchange.

Results from the paternity analysis also suggested spatial restriction in pollen dispersal. Indeed a majority of assigned paternities were attributed to fathers within the same subpopulation as the mother, and the largest fraction of inter-subpopulation paternity assignments corresponded to a neighbouring subpopulation, whereas no successful pollination was detected between the northern and southern populations. Detection of successful pollination between neighbouring subpopulations seems to contradict the results from the Bayesian analysis of recent immigration rates that showed no signature of recent gene flow between any pair of subpopulations. We suggest that the latter result indicates the absence of realized gene flow due to combined effects of potentially low germination rates and low seedling survival in natural populations, whereas paternity analysis estimations are likely biased by enforced germination of the sampled seeds. In this scenario, the direct approach to estimate contemporaneous gene flow (paternity analysis) would estimate potential migration rate, and the indirect method (Bayesian method) would estimate realized (effective) migration. In contrast, the results from the paternity analysis seem to be consistent with results from the population genetic structure analysis, which systematically showed the occurrence of geographic clines in allelic frequencies at nuclear markers across neighbouring subpopulations. The absence of evidence for pollen exchange between the northern and southern populations could result from the substantial distance separating both groups.

Scenarios for the distant and recent history of *Biscutella neustriaca*

Our results showed a puzzling spatial distribution of the nuclear and chloroplast DNA diversity, especially at such a small geographical scale, which cannot be explained by contemporary demographic events only. Indeed, there exist two gene pools for the nuclear DNA and two diverged chloroplast haplotype groups, but they are not fully concordant spatially: the two gene pools of the nuclear genome are distributed

between the northern populations 1–4 and the southern populations 5 and 6, while the two chloroplast haplotype groups are distributed between populations 1–5, and population 6 (Fig. 2). Hence, individuals from populations 5 and G show evidence of genomic admixture with the chloroplast genome of the northern populations and the nuclear genome of the other southern population 6. Finally, it is intriguing that the divergence between haplogroups I and II is very large, leading to an estimate of the divergence time of 2.38 My (0.53–4.77 My).

To explain this distribution, we looked for independent geological and historical events that occurred in this geographical zone. First, the dynamics of the river Seine could have played a role, since hillsides where *B. neustriaca* populations are located were formed by the excavation of the calcareous plateau by the river during the last million years (Antoine et al. 2007). During this period, geological data show strong dynamics of the Seine meanders, one of the most obvious pieces of evidence being the meander separating the northern and southern groups (Lécolle 1989). Indeed, this meander underwent an important inversion, as attested by ancient calcareous hillsides that mark the position of the riverbed 1 million years ago (Fig. 3a). Given the restricted seed dispersal in this metapopulation, this geological event may have caused the geographical isolation of northern and southern populations. Another ancient scenario that could explain the co-occurrence of two divergent maternal lineages involves the last glaciation from −120,000 to −10,000 years. During this period, climatic changes led to profound perturbations of European flora, transforming deciduous forests in tundra (Goni et al. 2005). These changes probably had a dramatically impact on *B. neustriaca*, which potentially had a more widespread distribution in the Seine valley before glaciation, since some populations were already attested in floodplains surrounding the river (Fig. 1b). Calcareous slopes and cliffs however represent the most suitable habitats for *B. neustriaca*, and thus likely served as refuges for this plant during the glaciation. Then, the two maternal lineages could have been isolated in two disconnected refuges during this period (Fig. 3b). These two scenarios are not exclusive and both can explain the geographical separation of haplotypes. Interestingly, the time period for the meander change is also concordant with the estimated divergence age between chloroplast haplotypes, and this event thus potentially promoted the divergence between haplotypes. We also compiled data on the botanical reports of *B. neustriaca* in this geographical zone (summarized in Table S4 and Fig. 1b). Population 5 is mainly located on artificial slopes established by a railway construction in 1896 (Table 1). Materials used for slopes were extracted in local quarries (*Archives Départementales d'Evreux* and personal observations). Although

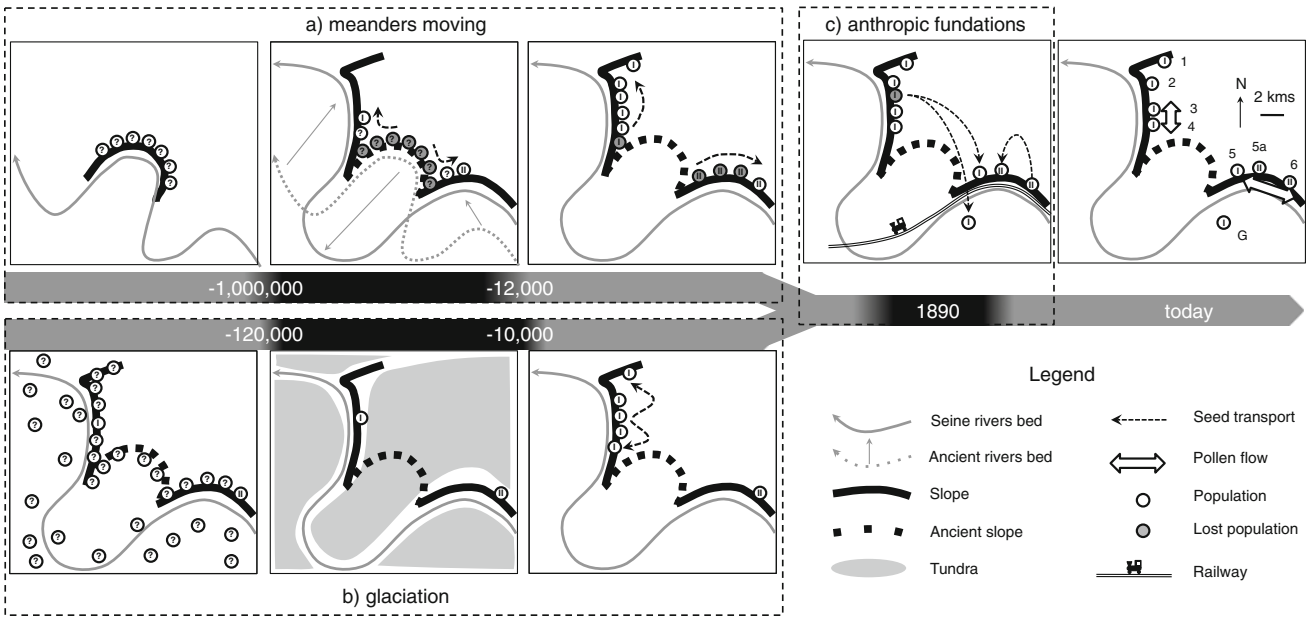

Fig. 3 Three non-exclusive scenarios (*dotted frames*) of the history of *B. neustriaca* populations, supported by genetic analysis (legend on *bottom right*). The *central arrow* indicates time in years. Each map represents a hypothetical past situation of populations. *Circles* represent hypothetical location of populations, labelled with corresponding maternal haplotype (I and II or unknown (?); see Fig. 2). **a** A unique group of populations was restricted to a meander of the paleo-seine river and underwent a progressive habitat perturbation after the meander inversion. **b** Populations initially widespread in the Seine valley, with several haplotypes (including I and II), underwent a drastic reduction during the last glaciation. After glaciation, only populations with haplotypes I and II colonized slopes from two distinct refuges. Scenarios **a** and **b** lead to the geographical separation and possibly to the divergence of two maternal lineages I and II. **c** Recent population foundations resulting from anthropic seed transports, possibly during railway construction. The current situation (*top right*) results from successive scenarios and contemporaneous pollen flows

precise locations of these quarries are unknown, many were found on northern slopes (e.g. subpopulations 1c and 4c) and on floodplains where populations were already attested (Fig. 1b). Moreover, the presence of *B. neustriaca* in floodplains and population 5 were reported since 1896 and 1902 respectively, i.e. after the railway construction, whereas populations 1 and 6 were reported at least 15 years before (Fig. 1b; Table S4). In light of these historical events, we propose that material transports from north to south, during the railway construction, were the vector of numerous seed migration events that led to foundation of some populations in the late nineteenth century, including populations G and 5 (Fig. 3c). Because we have only few elements about subpopulation G, and none about other subpopulations that were reported in floodplains, the origin of their situation, out of the usual habitat of *B. neustriaca*, remain however highly speculative. Indeed, many other perturbations, as frequent flood events, were reported in the valley and thus seeds transports by water could not be excluded in these cases (Poudevigne et al. 2002). Finally, according to our scenario represented in Fig. 3, pollen migration likely occurred since the railroad construction, leading to the admixture of chloroplast and nuclear genomes from different groups in these populations.

Implications for the conservation of *Biscutella neustriaca*

Biscutella neustriaca is an endangered plant subject to a protection program (European Life Program). The occurrence of a fully functional self-incompatibility system, as suggested by our results, may have a negative impact on seed production in small or low-density populations as a consequence of a mate-finding Allee effect (the "S-Allee" effect, Wagenius et al. 2007; Leducq et al. 2010). Also, the existence of different spatially non-matching chloroplast and nuclear gene pools suggests that the management of this metapopulation should be handled with particular care if reinforcement is planned in the future. Indeed, the large divergence between chloroplast haplotypes as well as the existence of two clearly different nuclear gene pools in northern and southern populations over a very small spatial scale may indicate the existence of local adaptation or of genetic incompatibility. Local adaptation is possible because the habitats between southern and northern populations are ecologically different, especially regarding the soil (population 5 is located on artificial embankments) and exposure (southern populations are south-facing while northern populations are north or west-facing). Even if there is no local adaptation, the historical separation and divergence between chloroplast haplotypes, and possibly between nuclear multilocus genotypes, may have resulted in fixation of genetic incompatibilities between northern and southern populations by genetic drift. Hence, future conservation plans such as reinforcement and hybridization will first require experiments to test for genetic incompatibilities and local adaptation (Frankham et al. 2011). Translocations in natural populations, as well as hybridization in controlled conditions, are necessary to determine which populations can be mixed and which cannot.

Our study shows that even at very small geographic scale, high genetic divergence can exist in an isolated plant taxon, and should thus be taken into account for conservation purpose. Our results also illustrate the necessity of comparing both chloroplastic and nuclear genetic data since they can show different diversity and distribution patterns, which cannot be overlooked for conservation management.

Data accessibility

- DNA sequences of five chloroplastic haplotypes: Genbank accessions numbers JN987156, JN987157, JN987158, JN987159 and JN987160.
- Supplementary document: Microsatellite data for structure, diversity and paternity analyses

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