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RESEARCH PAPER

Using genetic evaluation to guide conservation of remnant *Juniperus communis* (Cupressaceae) populations

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ABSTRACT

- Many critically endangered plant species exist in small, genetically depauperate or inbred populations, making assisted gene flow interventions necessary for long-term population viability. However, before such interventions are implemented, conservation practitioners must consider the genetic and demographic status of extant populations, which are strongly affected by species' life-history traits. In northwestern Europe, *Juniperus communis,* a dioecious, wind-pollinated and bird-dispersed gymnosperm, has been declining for the past century and largely exists in small, isolated and senescent populations.
- To provide useful recommendations for a recovery plan involving translocation of plants, we investigated genetic diversity and structure of populations in Belgium using four microsatellite and five plastid single-nucleotide polymorphism (SNP) markers.
- We detected no clonality in the populations, suggesting predominantly sexual reproduction. Populations exhibited high genetic diversity (H_e = 0.367–0.563) and low to moderate genetic differentiation ($F_{ST} \leq 0.133$), with no clear geographic structure. Highly positive inbreeding coefficients (F_{IS} = 0.221–0.507) were explained by null alleles, population substructuring and biparental inbreeding. No isolation by distance was observed among distant populations, but isolation at close geographic proximity was found. Patterns were consistent with high historical gene flow through pollen and seed dispersal at both short and long distances. We also tested four pre-germination treatments among populations to improve germination rates; however, germination rates remained low and only cold-stratification treatments induced germination in some populations.
- To bolster population regeneration, introductions of cuttings from several source populations are recommended, in combination with *in situ* management practices that improve seedling survival and with *ex situ* propagation.

INTRODUCTION

Habitat loss and fragmentation are among the greatest threats to biodiversity, leading to the isolation and decline of many plant populations (Frankham *et al.* 2004; Vanden Broeck *et al.* 2011). Small and isolated populations may suffer from restricted gene flow, pollination failure, Allee effects, genetic drift and high inbreeding levels (Frankham *et al.* 2004). As a consequence, populations may experience genetic erosion, increased genetic divergence and inbreeding depression, leading to reduced fitness, stunted evolutionary potential and ultimately population extinction (Frankham *et al.* 2004; Aguilar *et al.* 2006; Angeloni *et al.* 2011; Vranckx *et al.* 2012). Conservation of these small populations should not be neglected, as they may hold historical genetic diversity and evolutionary potential for population resilience or speciation, especially when they are ecologically marginal or disjunct from the main species distribution range (Van Geert *et al.* 2015; De Vriendt *et al.* 2017).

When restoring target populations that are depauperate and inbred by *in situ* ecological management practices, genetic variation will remain low, and inbreeding levels will increase in the newly established individuals if there is no possibility of gene flow from seed recolonization or from a persistent seed bank (Van Geert *et al.* 2008; Van Rossum 2008; Maschinski *et al.* 2013; Ottewell *et al.* 2016). Additional actions are necessary to restore connectivity between populations *via* the creation of biological corridors or through assisted gene flow such as plant translocations (McKay *et al.* 2005; Weeks *et al.* 2011; Van Rossum & Triest 2012; Berjano *et al.* 2013; Ottewell *et al.* 2016).

Plant translocations are increasingly common in species recovery plans. Translocations aim to bolster population size, increase genetic diversity and counteract genetic load in genetically impoverished populations through reinforcements.

Translocations can also (re)create new populations (Colas *et al.* 2008; Weeks *et al.* 2011; Betz *et al.* 2013; Zavodna *et al.* 2015; Orsenigo *et al.* 2017).

Optimizing the success of assisted gene flow interventions requires prior assessment of the genetic status of the potential recipient and source populations (Frankham *et al.* 2004; Maschinski *et al.* 2013). In particular, choice of the source material (provenance, number of populations) is crucial (Van Geert *et al.* 2015; Godefroid *et al.* 2016; Ottewell *et al.* 2016; Van Rossum & Raspé 2018). Indeed, locally sourced individuals are often preferred for reinforcing endangered populations to preserve local adaptations and/or evolutionary potential of single populations (Vergeer *et al.* 2004; Basey *et al.* 2015; Gentili *et al.* 2018). It has been suspected that the introduction of individuals from elsewhere might introduce maladaptive genes into the population, and the admixed progeny may suffer from outbreeding depression due to a disruption of co-adapted gene complexes (Hufford & Mazer 2003; McKay *et al.* 2005; Edmands 2007; Weeks *et al.* 2011).

However, long-term survival of populations requires sufficient genetic diversity to avoid inbreeding depression and to increase adaptive potential in the face of environmental changes (Sgrò *et al.* 2011; Maschinski & Albrecht 2017). When declining populations have lost genetic diversity or suffer from inbreeding depression, the source material used for reinforcements should not be limited to a single local population. Including a variety of genetic lines in introductions may increase the genetic diversity of the population through recombination among genomes, which may result in increased fitness due to heterosis (Weeks *et al.* 2011; Maschinski *et al.* 2013; Zavodna *et al.* 2015).

Species sensitivity to habitat fragmentation and subsequent restoration measures are highly dependent on life-history strategy (Aguilar *et al.* 2006; Angeloni *et al.* 2011). For instance, wind-pollinated species may have large pollen dispersal distances even if they remain vulnerable to habitat fragmentation (Aguilar *et al.* 2008; Vranckx *et al.* 2012). For seeds, wind- and animal-dispersed seeds are prone to long-distance dispersal (Aguilar *et al.* 2008). Bird-dispersed species are especially likely to maintain gene flow as birds may effectively connect distant populations through long-distance dispersal (Farwig *et al.* 2006). For clonally propagating plant species, recruitment may be the result of sexual reproduction or of clonal propagation. The appearance of new generations through sexual reproduction indicates successful population rejuvenation, and increases population evolutionary resilience (Menges 2008). Clonal propagation allows the maintenance of genotypes over several decades (Rozenfeld *et al.* 2007; Van Rossum *et al.* 2017; Van Rossum & Raspé 2018). However, effective population size may be much smaller than census population size observed in the field, and so population genetic status may be more critical than expected (Raabová *et al.* 2015; Van Rossum & Raspé 2018).

Previous studies evaluating the population status of endangered species for restoration have mainly focused on herbaceous and insect-pollinated species (*e.g.* Betz *et al.* 2013; Rascle *et al.* 2018; Van Geert *et al.* 2015; Van Rossum & Raspé 2018). There have been few studies on long-lived woody species with potentially high pollen and seed dispersal distances (Aguilar *et al.* 2008; Vranckx *et al.* 2012). For such species, genetic diversity loss due to a reduction in population size may not be apparent for several generations, a phenomenon termed 'diversity debt' (Bowles *et al.* 2015). Wind-pollinated coniferous tree species that exhibit obligate outcrossing due to dioecy are assumed to have high levels of intra-population genetic diversity and low levels of genetic differentiation among populations, even if populations are declining as a result of habitat fragmentation (García *et al.* 1999; Thomas *et al.* 2007; Provan *et al.* 2008; Vranckx *et al.* 2012; Vilcinskas *et al.* 2016). Optimal restoration measures for such species may thus differ from what is usually recommended for insect-pollinated herbaceous species.

Common juniper, *Juniperus communis* L., is a wind-pollinated and bird-dispersed tree widely distributed but strongly declining in North Atlantic and Central European countries (Thomas *et al.* 2007; Ward 2007; Vanden Broeck *et al.* 2011; Vilcinskas *et al.* 2016) due to poor regeneration by seeds, even after habitat restoration (Oostermeijer & De Knegt 2004; Vanden Broeck *et al.* 2011). Assisted gene flow interventions may therefore be necessary to rescue remnant juniper populations. Results from previous assessments of juniper population genetic structure have varied, with significant population differentiation within Ireland (Provan *et al.* 2008) but low levels of differentiation in the Netherlands and Germany (Oostermeijer & De Knegt 2004; Reim *et al.* 2016). Inbreeding, which is recognized as a long-term threat for population viability (Keller & Waller 2002; Edmands 2007; Weeks *et al.* 2011), has not been consistently investigated for this species (Michalczyk *et al.* 2006; Provan *et al.* 2008; Reim *et al.* 2016). These data may be particularly important given that junipers span diverse ecological contexts (*e.g.* wet acidic moors *versus* dry calcareous grasslands) and therefore may express differential adaptation to different climate and soil conditions (Provan *et al.* 2008; Vanden Broeck *et al.* 2011; Vilcinskas *et al.* 2016). Information on how genetic diversity is partitioned among the remnant populations may thus be helpful for designing recovery programmes.

In Belgium, populations of the common juniper are highly fragmented and characterized by very low regeneration rates, low seed production and high seed abortion rates (Gruwez *et al.* 2013, 2014; Delescaille 2015; Rassart 2019). Populations also experience pre-dispersal seed predation, low germination rates and high seedling mortality (Delescaille 2015). In the present paper, we investigate genetic diversity, genetic structure and seed germination of remnant juniper populations from calcareous and acidic soils to evaluate the genetic status of the populations. We addressed the following questions: (i) does this species show low genetic diversity, high inbreeding levels and high genetic differentiation associated with fragmentation and small population size; (ii) what is the extent of clonal propagation and does clonality influence the genetic status of the populations; and (iii) is germination rate associated with population size, population isolation, inbreeding or genetic diversity?

Our results will contribute to the design of recovery programmes for woody species involving plant translocations.

MATERIAL AND METHODS

Plant species

Juniperus communis L. (Cupressaceae, Gymnosperms) is a slow-growing and long-lived coniferous evergreen small tree

species. It is dioecious and wind-pollinated. Its dark fleshy fruits are dispersed by birds (García 2001; Thomas *et al.* 2007; Ward 2007; Vilcinskas *et al.* 2016). This species has a circumpolar distribution, reflecting its wide distribution during the Holocene and the last glaciation. Common juniper grows in various habitats, at elevations up to 2400 m, with one subspecies (*J. communis* subsp. *nana*) even occurring above the tree line in the Euro-Siberian mountains (Thomas *et al.* 2007; Vilcinskas *et al.* 2016). Typical juniper habitats include dry calcareous grasslands, acidic siliceous soils in heathlands and peat substrates in wet heathlands and bogs, but also sand dunes and coastal or inland woodlands (Thomas *et al.* 2007). These habitats have been prioritized for conservation and restoration in many European countries (*e.g.* through EU LIFE projects, Habitat Directive 92/43/EEC 1992). This pioneer species is tolerant to poor soils, drought and low temperatures, and is intolerant to shade, high temperatures, nitrogen deposition and competition from other species (Thomas *et al.* 2007; Ward 2007; Vanden Broeck *et al.* 2011; Pers-Kamczyc *et al.* 2020).

Abandonment of traditional agro-pastoral practices and subsequent reforestation, as well as climate change, have led to the decline of this species in several European countries in the past century (Thomas *et al.* 2007; Ward 2007; Vanden Broeck *et al.* 2011; Vilcinskas *et al.* 2016; Broome *et al.* 2017). Many extant populations consist of a few ageing individuals with low seed production and poor health status. The lack of natural regeneration in these habitats is attributed to poor seed quality, pre- and post-dispersal seed predation and a lack of favourable conditions for germination and establishment (García 2001; Thomas *et al.* 2007; Ward 2007; Tylkowski 2009; Verheyen *et al.* 2009; McCartan & Gosling 2013; Gruwez *et al.* 2014; Broome *et al.* 2017). Seeds also exhibit deep dormancy and germination remains sporadic, with unlikely germination until their second to fifth spring. No long-term persistent (>5 years) seed bank exists (Thomas *et al.* 2007).

Studied populations

We sampled ten remnant *Juniperus communis* subsp. *communis* populations in southern Belgium and two large reference populations from France and Germany for genetic analyses (Table 1, Fig. 1). Soil type, population size (number of trees) and sex ratio (when possible; male/female) were recorded for each population. As the sex ratio was close to 1:1 for most populations (Table 1), this variable was not analysed further. As an exhaustive inventory of the remaining populations was carried out in southern Belgium (L.-M. Delescaille, unpublished data), we estimated the spatial isolation level of the studied populations by calculating population density as the number of trees from surrounding populations located at 1, 2, 3 and 5 km distance of the studied populations (Table S1).

DNA analyses

Sampling

Leaves were collected from the 12 populations in autumn 2018. We collected 30 mg of leaves from each individual. When feasible, up to 20 individuals were sampled across the population, every 50 m, for a total of 228 individuals (Table 1). Leaves were sampled from both male and female individuals of varying ages. Leaves were stored at −80 °C before genetic analysis.

DNA extraction and amplification

The DNA was extracted from leaf tissue using the Qiagen DNeasy Plant Kit (Qiagen, Venlo, The Netherlands), after grinding with a Retsch MM400 mixer mill (Thermo Fisher Scientific, Waltham, MA, USA) at 30 Hz for 4 min. DNA was quantified using a ND1000 Nanodrop spectrophotometer (Thermo Fisher Scientific) and diluted to 10 ng μl^{-1} for subsequent PCR amplification.

Table 1. Population details and estimates of within-population genetic variation and of null allele frequencies per locus for 12 populations of *Juniperus communis* at four nuclear microsatellite loci.

Code	Population	Soil type	Pop. size	Male prop.	*n*	$A_{[11]}$	H_o	H_e	F_{IS}		F_{ISnull}		IIM model
1	Mont	Calcareous	>400		20	6.90	0.392	0.729	0.453	*	0.210	(0.001–0.410)	inbr
2	Dourbes	Calcareous	<50		18	7.37	0.503	0.768	0.320	*	0.087	(0.000–0.244)	no inbr
3	Buis	Calcareous	32	0.63	19	7.79	0.463	0.756	0.359	*	0.160	(0.001–0.308)	inbr
4	Vaucelles	Calcareous	11	0.70	11	6.50	0.546	0.701	0.221	*	0.080	(0.000–0.233)	no inbr
5	Furfooz	Calcareous	20–50		18	7.89	0.410	0.802	0.479	*	0.188	(0.001–0.395)	inbr
6	Prelleu	Calcareous	44	0.50	18	7.66	0.530	0.751	0.273	*	0.105	(0.000–0.243)	inbr
7	Aise	Calcareous	88	0.49	19	7.40	0.553	0.790	0.298	*	0.144	(0.000–0.309)	no inbr
8	Boton	Calcareous	166	0.52	20	5.83	0.367	0.736	0.493	*	0.318	(0.000–0.530)	inbr
9	Pairées	Calcareous	>160		20	7.93	0.488	0.821	0.406	*	0.329	(0.021–0.487)	inbr
10	Cour	Acidic	68	0.53	19	6.20	0.378	0.750	0.488	*	0.185	(0.002–0.383)	inbr
11	Crépale	Acidic	79	0.54	17	4.35	0.392	0.596	0.338	*	0.087	(0.000–0.277)	no inbr
12	Allendorf	Calcareous	>400		19	7.72	0.392	0.815	0.507	*	0.358	(0.072–0.590)	inbr
	Mean					6.96	0.451	0.751	0.386	*			
	SD					1.08	0.070	0.061	0.098				

Pop. size, population size (number of flowering trees); Male prop., proportion of male trees; *n*, sample size; $A_{[11]}$, allelic richness for a fixed sample size (11); H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, Wright's inbreeding coefficient; F_{ISnull}, inbreeding coefficient corrected for null alleles (mean value and 95% highest posterior density interval); IIM model, best fitting model in the IIM analysis, including inbreeding (inbr, *nfb*) or not (no inbr, *nb*); SD, standard deviation; ns, not significant.
*$P < 0.05$.

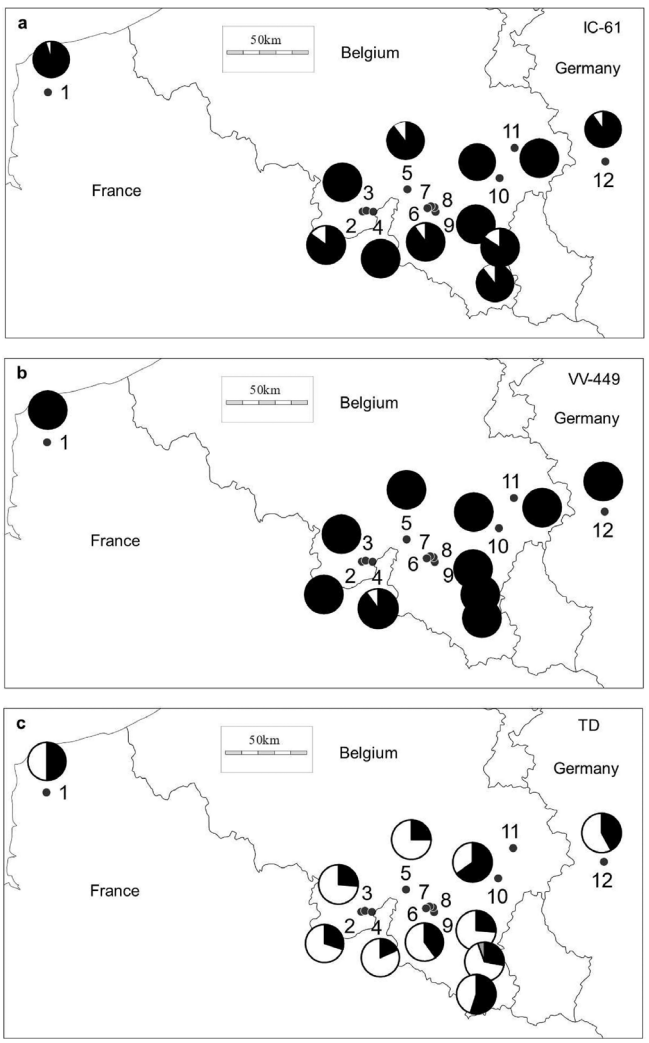

Fig. 1. Location of the 12 populations of *Juniperus communis* in Belgium, France and Germany, with the multilocus haplotype frequencies of three polymorphic SNPs (a, IC-61; b, VV-449 and c, TD) in each population (except populations 6 and 11). For population codes, see Table 1.

Nuclear microsatellite analysis

We used four nuclear microsatellites previously developed by Michalczyk *et al.* (2006). The primer JC37 did not reliably amplify a fragment (as in Provan *et al.* 2008) and was not used. Protocols were adapted from Michalczyk *et al.* (2006). PCR was carried out on a Mastercycler pro-thermal cycler (Eppendorf, Hamburg, Germany) with the Type-It Microsatellite PCR Kit (Qiagen). Each total 10-µl sample contained 3 µl DNA (10 ng μl^{-1}), 5 µl Type-It Multiplex PCR Master Mix 2x, 1.6 µl H_2O and 10 pmol (0.2 µl) of each primer. Optimal PCR parameters were as follows: initial denaturation at 94 °C for 4 min, followed by 37 denaturation cycles at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR products were diluted ten-fold (milli-Q water) before genotyping. Genotyping was performed on a Prism 3130xl capillary sequencer (Applied Biosystems, Waltham, MA, USA). Genotypes were identified using GeneMapper (Applied Biosystems).

Plastid allele-specific single nucleotide polymorphism analysis

We used five plastid (chloroplastic) allele-specific single nucleotide polymorphisms (SNP; AS-PCR: IC-61, VV-435, VV-449 and BD-616), previously developed by Provan *et al.* (2008). The AS-PCR protocol was as follows (adapted from Provan *et al.* 2008): initial denaturation at 94 °C for 3 min followed by 11 denaturation cycles at 94 °C for 60 s, annealing at 65 °C for 60 s (−0.7 °C $cycle^{-1}$) and extension at 72 °C for 60 s; followed by 24 denaturation cycles at 94 °C for 60 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. For the *trn*T-*trn*D (TD) marker, PCR parameters were as follows: initial denaturation at 94 °C for 3 min followed by 35 denaturation cycles at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR was carried out in a total volume of 10 µl, containing 100 ng genomic DNA, 10 pmol of forward primer, 10 pmol of reverse primer, 10 pmol SNRP-selective primer, 2.5 mM 1× PCR reaction buffer (7.5 mM Tris-HCl, pH 9.0; Roche, Basel, Switzerland), 200 µM dNTP mix (Promega, Madison, WI, USA) and 0.5 U *Taq* polymerase G2 (Promega). PCR products were resolved on 2% agarose gel and visualized with ethidium bromide staining. A long-range DNA ladder (VWR 100–1000 bp molecular weight scale) was used.

Genetic data analyses

Extent of clonality based on nuclear and plastid markers

A test for genotypic disequilibrium between pairs of nuclear microsatellite loci was performed with sequential Bonferroni-type correction (Rice 1989) to assess the independence of the loci using FSTAT (Goudet 2003). To estimate the extent of clonality, we used GenAlEx 6.5 (Peakall & Smouse 2012) to identify putative clones and calculate probability estimates (p_{se}) of encountering the same multilocus genotype a second time in the sampled individuals. The multiple occurrence of a multilocus genotype is likely to be generated by clonal propagation of a single genet when $p_{se} < 0.05$, and by sexual reproduction when $p_{se} > 0.05$ (Parks & Werth 1993). Individuals with missing data were excluded from these analyses. Two analyses were performed: on the four nuclear microsatellite loci (185 individuals from 12 populations) and on the four microsatellite loci together with the three plastid polymorphic SNPs (145 individuals from ten populations).

Genetic variation within populations based on nuclear microsatellite markers

All analyses of nuclear microsatellite data were performed using GEN-SURVEY (Vekemans & Lefèbvre 1997) or FSTAT, except when specified. The following measures of genetic diversity were calculated for each population over all loci: mean allelic richness ($A_{[N]}$) for a fixed sample size ($N = 11$), expected heterozygosity (H_e), corrected for small sample size (Nei 1978), and Wright's inbreeding coefficient (F_{IS}) corrected for small sample size. The significance of the F_{IS} values (for each locus and over all loci) estimated for each population was tested with randomization tests and a sequential Bonferroni-type correction. In case of highly positive F_{IS} values, we might suspect the presence of null alleles as previously detected in four microsatellite loci (Michalczyk *et al.* 2006). Therefore, each locus for the whole dataset and for each population was

checked for potential null alleles using INEST 2.2 (Chybicki & Burczyk 2009). We used the Bayesian approach (IIM) with 10^6 Markov Chain Monte Carlo iterations and 10^5 Burnin cycles, and tested two models: a full model (*nfb*, including null alleles, inbreeding and genotyping failures) and a model (*nb*) where inbreeding was set at 0. The lowest value of the deviation information criterion (DIC) was used to choose the best fitting model. Null allele frequencies for each locus and mean inbreeding coefficient values (F_{ISnull}) for each population and their 95% highest posterior density intervals (HPDI) were estimated after adjusting allele frequencies with INEST 2.2. To investigate whether there were relationships between genetic variables ($A_{[11]}$, H_o, H_e, F_{IS} and F_{ISnull}), population size and population density, we carried out non-parametric Spearman or Gamma correlation analyses using STATISTICA (Dell Inc 2015).

Genetic diversity within populations based on plastid SNP markers

For each plastid SNP, the proportion of each multilocus haplotype (excluding missing data) was calculated for each population and plotted in geographic space. Mean haploid genetic diversity (H) was calculated for each population over all SNPs using GenAlEx 6.5 (Peakall & Smouse 2012). Crépale and Prelleu, which had one missing SNP, were excluded from the calculations. We tested the relationships between the number of multilocus haplotype or mean haploid genetic diversity (H) and population size and population density with Gamma or Spearman correlation analyses, using STATISTICA.

Genetic structure among populations based on nuclear microsatellite markers

We used several approaches to characterize the genetic structure among populations based on the four polymorphic nuclear microsatellite loci. First, we investigated population genetic structure using Nei's total genetic diversity (H_T) and mean within-population diversity (H_S) with a correction for small sample size according to Nei & Chesser (1983), and using Weir & Cockerham's (1984) estimate of the between-population component of diversity (F_{ST}).

Second, we computed pairwise F_{ST} values (Weir & Cockerham 1984) and tested their significance with randomization tests and Bonferroni correction. We tested for isolation by distance, by regressing $F_{ST}/(1 - F_{ST})$ ratios for all pairs of populations against the logarithm of their geographic distance using SPAGeDI version 1.2 (Hardy & Vekemans 2002). The significance of the regression was tested with a 1000-permutation Mantel test. We also calculated F_{ST} values with the *ENA* correction using FreeNa (Chapuis & Estoup 2007) to take the possible presence of null alleles into account.

Third, we calculated Cavalli-Sforza & Edwards' (1967) genetic distances between all pairs of populations, both with and without the *INA* correction (considering possible null alleles) using FreeNa. With only four loci, bootstrapping could not be performed using FreeNa. To summarize the patterns of differentiation between populations, a neighbour-joining (NJ) cluster analysis was performed on the matrix of Cavalli-Sforza & Edwards' distances using the software Populations 1.2.3.1 (Olivier Langella, Montpellier, France). The cluster analysis was visualized using FigTree v.1.4.3 (Rambaut, 2016).

Fourth, we performed a Principal Coordinates Analysis (PCoA) based on a standardized distance matrix using GenAlEx 6.5. Finally, we used STRUCTURE version 2.3.4 (Pritchard *et al.* 2000), a Bayesian clustering method to identify the number of clusters (K) which our data could be divided into distinct gene pools. We performed analyses for $K = 1$ to $K = 12$ clusters (ten different runs), using an admixture ancestry model with correlated allele frequencies, run length of burn-in period of 10^6 iterations, and 2 10^6 Markov Chain Monte Carlo replications. Two models were generated per analysis: (1) a model ignoring the presence of null alleles and (2) treating null alleles as recessive and considering genotypes with missing data as homozygous for null alleles (Falush *et al.* 2007). The most likely number of K clusters was inferred as described in Evanno *et al.* (2005), using STRUCTURE HARVESTER (Earl & vonHoldt 2012). The most likely estimated membership (Q) values of the ten independent runs obtained with CLUMPP version 1.1.2 (Jakobsson & Rosenberg 2007) were visualized on plots using DISTRUCT version 1.1. (Rosenberg 2004).

Genetic structure among populations based on plastid SNP markers

For the plastid SNP data, the genetic differentiation between populations was estimated using pairwise Φ_{PT} values, tested for significance with 999 permutations and a Bonferroni correction using GenAlEx 6.5. As locus VV-449 was missing for Prelleu and locus TD was missing for Crépale, Φ_{PT} values for these populations were calculated without these SNP(s).

Seed collection and extraction

Ripe cones were harvested in autumn 2017 from the 12 populations and stored in a cold room (4 °C) before seed extraction. Cones were placed in water for at least 2 h to soften the pulp (McCartan & Gosling 2013). Seeds collected from cones were classified into two categories: aborted (empty) seeds or full (well-developed embryo) (García 2001; Tylkowski 2009; Verheyen *et al.* 2009). Cones with viable seeds were mixed for a few seconds in a blender and rinsed with demineralized water to obtain seeds for germination experiments (Pack 1921; McCartan & Gosling 2013).

Pre-germination treatments and germination tests

Seeds were placed in Petri dishes in batches of 30 viable seeds (three batches per tree and per treatment) on a 5-mm layer of sterilized sand, which was moistened with milliQ water. Dishes were inspected once a week. Four seed pre-treatments, considered the most effective for improving germination (Tylkowski 2009; McCartan & Gosling 2013), were performed: (1) cold stratification at 4 °C only; (2) chemical scarification in concentrated sulphuric acid (H_2SO_4 18 M) for 15 min, followed by rinsing with demineralized water, then cold stratification at 4 °C; (3) thermal scarification in boiling water for 5 min followed by soaking until water returned to room temperature, then cold stratification at 4 °C; and (4) cold stratification cycles for 3 months at 4 °C, 3 months at 20 °C, then 3 months at 4 °C. Seeds were inspected weekly for fungal pathogens; infected seeds were treated with 5% NaClO when necessary. Seed substrate moisture was also adjusted and germination was recorded. A seed was considered germinated when the exposed radicle was at least 1/3 of the length of the seed (Tylkowski, 2009). Germination rate was calculated as the percentage of germinated seedlings after 12 months. To investigate whether there were relationships between germination rate and

population size, population density and genetic variables ($A_{[11]}$, H_o, H_e, F_{IS} and F_{ISnull}), we carried out non-parametric Gamma correlation analyses using STATISTICA.

RESULTS

Extent of clonality based on nuclear and plastid markers

No linkage disequilibrium was found between the six pairs of loci ($P > 0.008$, not significant after sequential Bonferroni correction), except between JC31 and JC32. Only three multilocus genotypes occurred twice or three times. One genet was found in both Cour and Crépale populations separated by 20.8 km, whereas the multiple occurrences of the two other genets were found in Crépale, but were likely to be accounted for by sexual reproduction and not by clonal propagation ($p_{se} > 0.05$). All other individuals (with no missing data) showed distinct multilocus genotypes.

Null allele prevalence in nuclear microsatellite loci

Null alleles were found in all four microsatellite loci, with a frequency of 0.259 (95% HPD: 0.192–0.316) for JC16, 0.362 (0.312–0.424) for JC31, 0.126 (0.075–0.186) for JC32 and 0.037 (0.003–0.081) for JC35.

Genetic variation within populations based on nuclear microsatellite markers

Multiple alleles were scored in the four microsatellite loci for each population (12–27), for a total of 79 alleles. All loci were polymorphic in all populations. Allelic richness ($A_{[11]}$) ranged from 4.35 to 7.93, observed heterozygosity (H_o) from 0.367 to 0.553, expected heterozygosity (H_e) from 0.596 to 0.821, and Wright's inbreeding coefficient (F_{IS}) from 0.221 to 0.507. All populations showed significantly ($P < 0.05$) positive F_{IS} values, differing from Hardy–Weinberg expectations, with a deficit of heterozygotes (Table 1). The IIM analysis indicated that inbreeding made an important contribution to the F_{IS} values in most populations (lowest DIC for the *nfb* model), but the presence of null alleles also contributed to the observed F_{IS} values in many populations, resulting in lower F_{ISnull} values (0.080–0.358; Table 1, Fig. 2). There was a significantly positive correlation between the inbreeding coefficients (F_{IS} and F_{ISnull}) and population size (Spearman correlation coefficient $r_s = 0.581$ and 0.683, $P = 0.047$ and 0.014, respectively; Fig 2a). The other variables ($A_{[11]}$, H_o and H_e) were not correlated with population size (r_s ranging from −0.453 to 0.147, $P > 0.100$). There was a significantly positive correlation between H_o and population density at 5 km distance (Gamma correlation coefficient = 0.524, $P = 0.042$) and between H_e and population density at 1 km distance (Gamma coefficient = 0.833, $P = 0.014$; Fig. 2b). The other correlations between the genetic variables and population density (at 1, 2, 3 or 5 km distance) were not significant (Spearman or Gamma coefficients ranging from −0.596 to 0.500, $P > 0.070$).

Genetic diversity within populations based on plastid SNP markers

Three out of the five SNPs were polymorphic: IC-P61 (two haplotypes), VV-449 (two haplotypes, with one haplotype only present in Vaucelles) and TD (three haplotypes, with one haplotype only present in Boton). VV-435 and BD-616 were monomorphic. A combined total of six multilocus haplotypes were found (Table 2, Fig. 1). All populations showed at least two different multilocus haplotypes, with up to five haplotypes found in Boton, except Crépale, which was monomorphic for the two amplified SNPs (IC-P61 and VV-449). Prelleu showed four different multilocus haplotypes for the two amplified SNPs, IC-P61 and TD. Mean haploid genetic diversity (H) ranged from 0.136 to 0.261. The number of multilocus haplotypes and H were not correlated with population size (Gamma correlation coefficient = 0.034 and $r_s = 0.450$, $P > 0.100$, respectively) nor with population density (Gamma coefficients ranging from −0.500 to 0.308, $P > 0.100$).

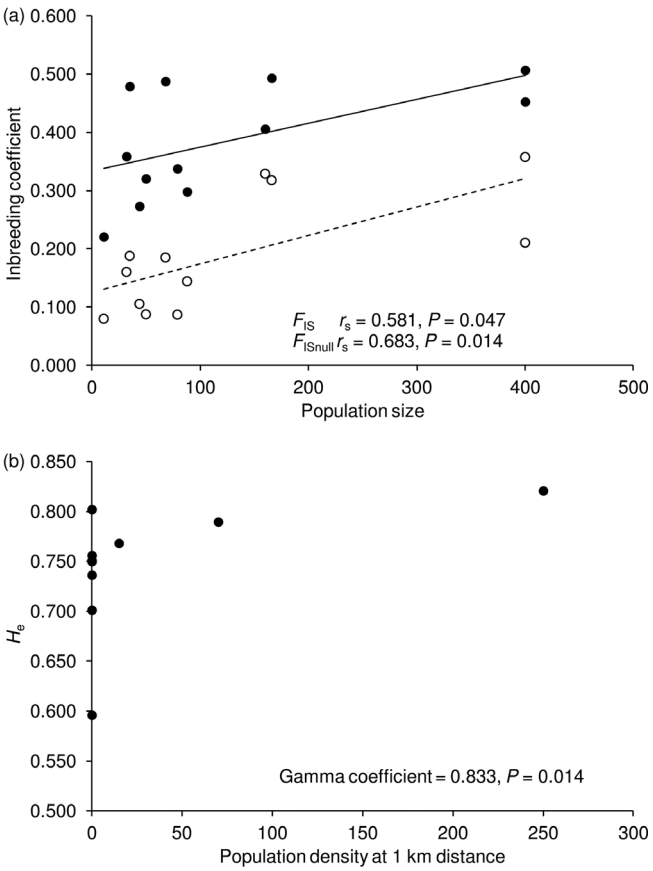

Fig. 2. Relationship between (a) population size and inbreeding coefficient (black circles: F_{IS} and white circles: F_{ISnull}) and (b) population density at 1 km distance and H_e for the studied populations of *Juniperus communis*.

Genetic structure among populations based on nuclear microsatellite markers

The total (mean over loci) genetic diversity (H_T) for all populations was 0.786 (range: 0.588–0.905) and the mean (over populations and loci) within-population genetic diversity (H_S) was 0.761 (0.567–0.882). The mean (over loci) between-population component of diversity (F_{ST}) was 0.031 and F_{ST} values ranged from 0.012 (JC35) to 0.050 (JC31). The Crépale population was significantly differentiated from the other populations (mean F_{ST} values = 0.084–0.133, $P < 0.05$). F_{ST} values were low

Table 2. Frequency of multilocus haplotypes and mean haploid genetic diversity (*H*) for ten populations of *Juniperus communis* based on five plastid SNP markers.

		Haplotype						
Code	Population	1	2	3	4	5	6	*H*
1	Mont	0.421	0.526	–	0.053	–	–	0.209
2	Dourbes	0.150	0.700	–	0.150	–	–	0.237
3	Buis	0.211	0.737	–	0.053	–	–	0.170
4	Vaucelles	0.100	0.800	–	–	–	0.100	0.176
5	Furfooz	0.211	0.684	–	0.053	0.053		0.198
6	Prelleu	–	–	–	–	–	–	–
7	Aise	0.278	0.722	–	–	–	–	0.136
8	Boton	0.188	0.625	0.063	0.063	0.063	–	0.261
9	Pairées	0.474	0.421	–	0.105	–	–	0.240
10	Cour	0.650	0.350	–	–	–	–	0.160
11	Crépale	–	–	–	–	–	–	–
12	Allendorf	0.389	0.556	–	0.056	–	–	0.235

(mean $F_{ST} < 0.050$) and not significant ($P > 0.05$ after Bonferroni correction) for most other population pairs (Table S2). No significant association was found between $F_{ST}/(1-F_{ST})$ and geographic distance matrices ($R^2 = 0.023$, $P = 0.208$; Fig. 3), but the correlation was significant at geographic distances <100 km ($R^2 = 0.326$, $P = 0.004$). When applying the *ENA* correction, the pairwise F_{ST} values were slightly lower (mean $F_{ST} \leq 0.102$) than without *ENA* correction (Table S2, Fig. 3), but the two measures were highly correlated ($r_s = 0.923$, $P < 0.001$).

The overall pattern of between-population differentiation based on Cavalli-Sforza and Edwards' genetic distances with the *INA* correction for null alleles is summarized in the tree in Fig. 4. Several clusters, however with short branch lengths, were distinguished, but not related to the geographic location of the populations. The same clustering was found when calculating the Cavalli-Sforza & Edwards' genetic distances without the *INA* correction for null alleles (results not shown). No clear genetic structure among populations could be detected based on the PCoA in relation to the geographic, soil or sex origin of the individuals (Fig. 5). Similarly, no genetic structure was detected in the STRUCTURE analysis, even at the most likely inferred number of *K* clusters, *i.e.* $K = 3$ (or $K = 5$ when considering the null alleles as recessive) (Fig. 4).

Genetic structure among populations based on plastid SNP markers

Pairwise Φ_{PT} values based on SNPs ranged from 0.000 to 0.274 and were not significant ($P > 0.05$ after Bonferroni correction), except between Cour and Furfooz (Table S3). No geographic pattern was found for the SNP haplotypes (Fig. 1).

Germination

Seed viability varied from 0 to 30%, depending on the tree and the population. The first germination was recorded after 15 weeks of cold stratification. Seedlings were only observed in the cold stratification (4 °C) treatment, and in the chemical scarification followed by cold stratification treatment. One exception was two seedlings from the hot water pre-treatment, followed by cold stratification, which came from a single tree

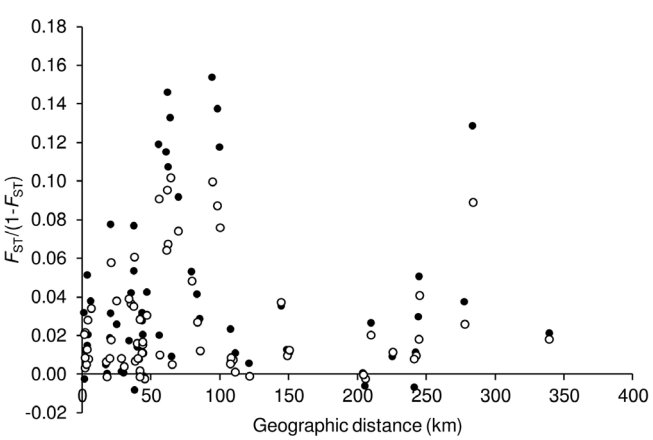

Fig. 3. Relationship between pairwise geographic distances and $F_{ST}/(1-F_{ST})$ ratios for 12 populations of *Juniperus communis* without (black circles) and with (white circles) *ENA* correction.

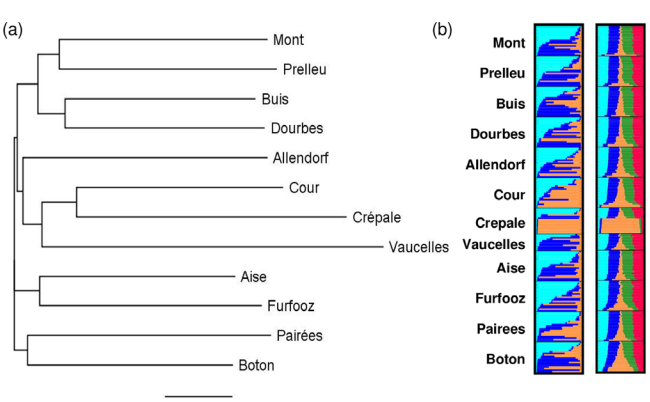

Fig. 4. (a) Neighbour-joining tree based on Cavalli-Sforza and Edwards' genetic distances with the *INA* correction for null alleles and (b) results of Bayesian clustering (modal $K = 3$ and 5) for 12 populations of *Juniperus communis*. In the barplot, each individual is represented by a horizontal bar, showing the probability of membership to each of the 3 or 5 modal clusters.

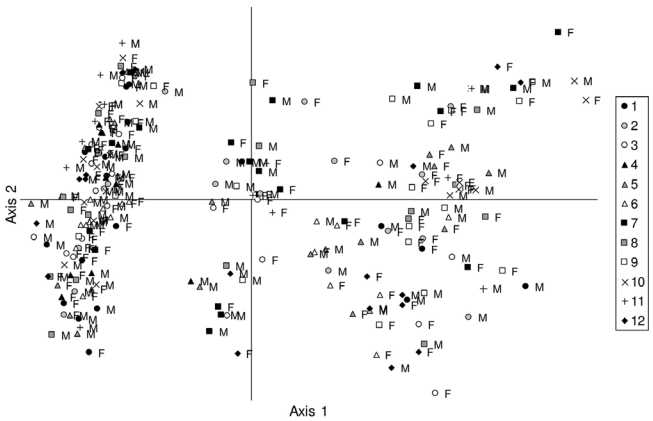

Fig. 5. Principal Coordinates Analysis (PCoA) plot for 12 populations of *Juniperus communis* (M = male, F = female). Axes 1 and 2 explained 14.1% and 10.3% of the total variation, respectively. For population codes, see Table 1.

from the Aise population. The highest germination rate (8.9%) was observed for seeds from Aise with the cold stratification treatment (Table 3). A low number of seedlings were obtained from Boton, Prelleu and Pairées populations (Table 3), while no germination was observed from the other populations. Germinated seedlings were recorded for populations surrounded by the highest population densities at 2, 3 and 5 km distance (Gamma correlation coefficient = 0.933–0.724, $P \leq 0.016$; Table S1). Germination rate was not correlated with population size or any estimates of genetic variation or inbreeding (Gamma coefficient = −0.211–0.400, $P \geq 0.222$).

DISCUSSION

Low germination rates and limited clonal propagation

Small, ageing remnant populations of juniper usually have suppressed regeneration capability. This is likely due to a number of reasons. First, germination rates are very low, around 1%, and seeds often germinate in natural populations only after two to five winter seasons (Verheyen *et al.* 2005; Thomas *et al.* 2007). This low germination was not related to population size or to the genetic diversity or inbreeding levels. This rather suggests that endogenous and exogenous primary dormancy, as well as secondary dormancy, are responsible for low germination (Thomas *et al.* 2007; Tylkowski 2009; McCartan & Gosling 2013).

Table 3. Germination rate after 12 months for two germination treatments (cold and chemical stratification) and total number of seedlings obtained for four populations of *Juniperus communis*.

	Germination rate (%) (number of seeds tested)		
Population	Cold stratification	Chemical scarification	Total number of seedlings
Aise	8.7 (1050)	1.1 (450)	98
Boton	1.1 (450)	0.6 (180)	6
Prelleu	2.4 (900)	0.0	22
Pairées	0.2 (900)	0.9 (450)	6

Despite pre-germination treatments for breaking dormancy tested in this study, germination rates remained low across populations. Germination was only recorded in the area of high population density, suggesting that isolation related to habitat fragmentation reducing population density might also negatively affect seed fitness, *e.g.* through lower pollen diversity (Breed *et al.* 2012). Second, the age of individuals affects their seed viability, with trees over 75 years old producing only 4% viable seeds, compared to 70% viability for 10-year-old individuals (McCartan & Gosling 2013). Finally, early survival of seedlings is suppressed due to predation or over-shading (Pack 1921; Thomas *et al.* 2007). Low germination rates not only represent a constraint for *in situ* conservation, but also for *ex situ* conservation based on seed storage in seed bank facilities.

We did not detect any clonality in the populations we sampled, despite the small number of markers used. As a result, recruitment is likely to have occurred through sexual reproduction in these populations. With limited clonal reproduction and low establishment rates, regeneration of these juniper populations is expected to be associated with very long recruitment time lags. In the meantime, small populations can be very sensitive to demographic and environmental stochasticity (Verheyen *et al.* 2005), and translocation of young trees should be considered as a buffer against such risk (Frankham *et al.* 2004).

Genetic status of the remnant populations

The populations of juniper we studied exhibited high levels of genetic variation for both nuclear and plastid markers, despite their small size (usually fewer than 100 male and female trees together). Values of genetic variation are similar or even higher than the levels reported in Germany (Reim *et al.* 2016) and Ireland (Provan *et al.* 2008) based on the same markers. Hence, we conclude that these small populations are not genetically depauperate compared to large populations. Other studies of *J. communis* or *J. brevifolia* in Western Europe also reported high genetic diversity levels within small populations (Bettencourt *et al.* 2015; Reim *et al.* 2016). This is not surprising for a long-lived species, as small population remnants may hold old individuals which reflect pre-fragmentation genetic variation (Van Geert *et al.* 2015; Reim *et al.* 2016; Van Rossum & Raspé 2018). Post-fragmentation changes are often seen in the newly established individuals, which may be inbred and show lower genetic diversity (Van Geert *et al.* 2008; Van Rossum 2008; Breed *et al.* 2012). These small populations have high conservation value, especially when they are still connected by gene flow or when connectivity has been restored, *e.g.* through biological corridors, which are needed to avoid possible inbreeding in the recruits. They can also be used as seed sources for translocations if they are mixed (Kramer *et al.* 2008; Van Rossum & Triest 2012; Van Geert *et al.* 2015).

In our study, only the Crépale population displayed relatively low genetic diversity, with several individuals sharing the same genotype. This might reflect the geographic isolation of this population or be an artefact of planting from cuttings, a practice probably not rare in the past. For instance, the Cour population was planted in the 19th century (Ph. Frankard, personal comunication). It is possible that other populations were also maintained by landowners or pharmacists during the 19th–20th centuries (such as Crépale population).

All populations exhibited significantly positive F_{IS} and F_{ISnull} inbreeding coefficients, indicating a deficit in heterozygotes. This pattern has also been reported in previous studies of *J. communis* (Oostermeijer & De Knegt 2004; Reim *et al.* 2016) or other members of the *Cupressaceae* in general (Ritland *et al.* 2001; Bettencourt *et al.* 2015). The presence of null alleles affects the F_{IS} values in our studied populations, but not the overall genetic structure. This indicates that other factors likely contribute to the observed pattern in inbreeding coefficients. These inbreeding coefficients (F_{IS} and F_{ISnull}) have been found to be even higher in large populations, which might reflect a genetic substructuring of the populations (Wahlund effect) related to a patchy spatial distribution of the individuals (Somme *et al.* 2014). These patterns may result from specific germination ecological requirements (microniches) combined with spatially heterogeneous environmental conditions. Restricted seed dispersal due to preferential deposition of seeds by birds near the maternal trees may reinforce this process (García 2001). A non-random distribution of male trees in the populations and Allee effects may also favour biparental inbreeding (Oostermeijer & De Knegt 2004).

There was low to moderate genetic differentiation among juniper populations for both nuclear and plastid markers in southern Belgium, with no clear geographic structure or relationship with soil type. No isolation by distance was detected between distant populations, but there was isolation by distance detected for nearby populations. These patterns are consistent with other studies of populations in continental Europe (Oostermeijer & De Knegt 2004; Michalczyk *et al.* 2006; Vanden Broeck *et al.* 2011; Reim *et al.* 2016). By contrast, Irish and English insular populations exhibit geographic structuring, which was explained by different post-glacial migration routes from multiple sources (Van der Merwe *et al.* 2000; Provan *et al.* 2008). Continental populations are believed to have been derived from historically large and interconnected populations, characterized by high genetic diversity (Van der Merwe *et al.* 2000; Provan *et al.* 2008; Vanden Broeck *et al.* 2011; Reim *et al.* 2016). Studies of other juniper species (Bettencourt *et al.* 2015) and other gymnosperms (Vendramin *et al.* 1998; Richardson *et al.* 2002; Naydenov *et al.* 2005; Petit *et al.* 2005) have generally shown little differentiation between populations (<10%) due to low isolation by distance (Bettencourt *et al.* 2015).

The patterns in genetic differentiation observed in this study may be related to the pollen and seed dispersal abilities of juniper. In wind-pollinated conifers, pollen dispersal can range from 10 to 100 km (Bettencourt *et al.* 2015). Nevertheless, it is assumed that most pollen is locally deposited for *J. communis*. Michalczyk *et al.* (2006) found only 1% of *J. communis* pollen more than 100 m from the male plant. The prevalence of short-distance pollen dispersal in *J. communis* is also supported by palynological studies (20–50 m; L.-M. Delescaille, unpublished data). However, spatially isolated female trees often produce cones with full seeds, indicating that pollen flow events over longer distances can occur.

Juniper seeds are predominantly dispersed by thrushes and blackbirds (*Turdus* spp.), which tend to contribute to dispersal over short distances, but also over long distances during bird migration (García 2001; Vanden Broeck *et al.* 2011). For instance, in southeastern Spain, migrating thrushes spend time foraging on juniper cones, which induces an accumulation of seeds at the base of maternal trees. However, long-distance dispersal events occur when these fruit-eating birds further migrate (García 2001). Vanden Broeck *et al.* (2011) estimated that 3−14% of individuals in juniper populations had migrant genotypes.

The decline of juniper populations in Europe dates back to the beginning of the 20th century (Thomas *et al.* 2007; Vanden Broeck *et al.* 2011; Reim *et al.* 2016) when spatial isolation caused by reforestation may have begun to affect pollen flow (Oostermeijer & De Knegt 2004). These small fragmented populations may also have become less attractive to birds due to low cone availability or suffer low seed viability due to ageing and predation, suppressing seed dispersal between nearby populations (García *et al.* 1999; Provan *et al.* 2008). The patterns in genetic diversity we observed are therefore consistent with historically high levels of pollen- and seed-mediated gene flow moderated by population fragmentation at a local scale. However, even low levels of gene flow might also explain the diversity of young individuals compared to older trees (Vanden Broeck *et al.* 2011).

Implications for population restoration

Poor natural regeneration is recognized as the main threat to the long-term preservation of juniper populations across species range (Oostermeijer & De Knegt 2004; Thomas *et al.* 2007; Vanden Broeck *et al.* 2011). Even if small, isolated, populations of juniper maintain high genetic diversity, inbreeding levels are already high. In a fragmentation context possibly restricting gene flow, inbreeding might increase in the newly arising generations as well as genetic erosion due to demographic stochasticity and genetic drift (Van Geert *et al.* 2008; Vranckx *et al.* 2012, 2014). Plant translocations are, therefore, clearly necessary for successful long-term rehabilitation of juniper populations. Self-reinforcement of populations through cuttings is a short-term solution, but is not a sufficient long-term solution. Cuttings represent clones of the same age, and adding the same genotypes does not increase the effective population size. Even if genetic differentiation between populations remains low, introducing cuttings from several source populations in each site is a viable option to increase genotype diversity. Nevertheless, further investigation is needed to characterize the levels of local adaptation (*e.g.* dry *versus* wet and/or acidic *versus* calcareous soil types) to avoid potential outbreeding depression effects.

Recruitment from seeds is important for the long-term sustainability and rejuvenation of plant populations (Menges 2008; Weeks *et al.* 2011). Seedling establishment can be negatively impacted by overgrazing (Ward 1982), but also by a lack of grazing or mowing (Verheyen *et al.* 2005; Delescaille 2015). Management actions such as creating openings in old closed shrub canopies, reducing competition from other species or protection against herbivores should be considered to optimize seed germination and the survival of the saplings (Verheyen *et al.* 2005; Delescaille 2015).

In years of high seed set, collection of seeds for *ex situ* germination may be realized to generate sufficient seedlings for translocation. Transplanting a large number of individuals (at least 50 genotypes) is recommended to provide a sufficient number of mates and limit biparental inbreeding (Godefroid *et al.* 2011; Maschinski & Albrecht 2017). Saplings should be translocated into populations after 1–2 years of cultivation in

nurseries to avoid predation at an early stage of development. Sufficient densities and interspersion of male and female plants can also avoid Allee effects (Colas *et al.* 2008; Basey *et al.* 2015; Bowles *et al.* 2015). In the long term, we also recommend conservation measures based on both *ex situ* storage in seed bank facilities and *in situ* nurseries at managed natural sites to avoid divergent selection pressures (Reim *et al.* 2016; Broome *et al.* 2017; Euroforgen network).

Finally, given the geographic isolation of the remnant populations, connectivity between populations might be restored by creating so-called 'stepping stone' populations in favourable open areas, such as the edges of fields or under power supply lines (Frankham *et al.* 2004; Van Rossum & Triest 2012; Reim *et al.* 2016; Broome *et al.* 2017). Even isolated individuals could act as stepping stones for bridging large distances between groups of trees or populations.

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AUTHOR CONTRIBUTIONS

LMD initiated the study, obtained funding and supervised the project. LMD, CB and ALJ collected the plant material. CB performed germination tests and DNA amplification. FVR performed statistical analyses. ALJ, FVR and LMD wrote the paper. All authors discussed the results and contributed to the writing.

SUPPORTING INFORMATION

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

Table S1. Population density as the number of trees from surrounding populations located at 1, 2, 3 and 5 km distance of the studied populations of *Juniperus communis* in southern Belgium.

Table S2. Pairwise genetic differentiation between populations (F_{ST} values) for 12 populations of *Juniperus communis* (upper half of the matrix; the significant values, i.e. $P < 0.05$ after Bonferroni correction, are in italics) and with the *ENA* correction (lower half of the matrix).

Table S3. Pairwise genetic differentiation between populations based on plastid SNPs (Φ_{PT} values) for 12 populations of *Juniperus communis*.

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