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Research

Strong gene flow explains lack of mating system variation in the perennial herb, *Vincetoxicum hirundinaria*, in a fragmented landscape

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Fragmented landscapes may have implications for the genetic structure of populations and for the microevolution of plant species. In particular, landscape fragmentation and/or population isolation might affect the evolution of plant mating systems. Here, we study the consequences of landscape fragmentation on the genetic structure of populations of a perennial herb, *Vincetoxicum hirundinaria* with a mixed mating system. Our study area, the south-western Finnish archipelago, was formed after the glacial ice sheet started to retreat 12 000 years ago. Due to the isostatic land uplift following the glacial retreat, suitable habitats have been formed gradually, and as a consequence, populations of *V. hirundinaria* differ in age, size and their degree of isolation in the area. We hypothesized that a mixed-mating system has been selected for in these populations due to the advantage of self-fertilization in newly colonized areas and the advantage of outcrossing in adaptation to heterogeneous environments. To test this hypothesis, we collected seeds of open-pollinated flowers from 13 *V. hirundinaria* populations differing in size, age and isolation, and used 15 microsatellite markers to perform progeny-array analysis to estimate population-level outcrossing rates, population genetic indices and population structure. We found that *V. hirundinaria* is almost completely outcrossing in the study area with no signs of past self-fertilization and/or mating among relatives. The overall low inbreeding coefficients indicate that even in small populations mating among relatives is rare. High allelic richness of both maternal and offspring genotypes as well as limited genetic differentiation among the studied populations indicate strong gene flow among them. Our findings suggest that *V. hirundinaria* has successful seed and pollen dispersal among populations that has allowed colonization of new habitats in this fragmented landscape and led to a genetically well-mixed group of populations at the scale of the study.

Keywords: dispersal, Finnish archipelago, inbreeding, isolation, microsatellites, outcrossing rate, selfing

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Introduction

Fragmented landscapes, whether natural or man-made, may have implications for the population genetic structure and adaptive evolution of plant species (Hoebee et al. 2007). For instance, gene flow should diminish and among population genetic differentiation should increase with increasing population isolation (Young et al. 1996, Gómez-Fernández et al. 2016). Such developments can, on the one hand, be devastating for the adaptive potential of a species or populations existing in such an environment (Fischer and Matthies 1998, Garant et al. 2007). On the other hand, limited gene flow among populations can allow for adaptation to particular local environments (Lenormand 2002, Blanquart et al. 2013, Kalske et al. 2016). In either case, plant populations are not necessarily helpless in the face of fragmentation but can instead adapt to the prevailing population configuration, i.e. fragmentation, through traits that increase gene flow and population connectivity and/or reduce the reliance on recurrent gene flow (Dubois and Cheptou 2017).

A particularly important plant trait affecting population genetic diversity and differentiation, and therefore the evolutionary fate of the plant populations, is the mating system (Hamrick and Godt 1996). The degree of landscape fragmentation, or the isolation of populations, can also have consequences for the evolution of plant mating systems (Llorens et al. 2012, Coates et al. 2013, Gauli et al. 2014, Sampson et al. 2014, Breed et al. 2015). Most plants reproduce either through self-fertilization or cross-fertilization, but about 40% of plant species have a mixed-mating system producing a part of their seeds via self-fertilization and another part via cross-fertilisation (Goodwillie et al. 2005). While the efficiency of genetic transmission is suggested to be the main genetic force promoting an evolutionary shift to self-fertilization (Griffin and Willi 2014), reproductive assurance is an ecological force that favors self-fertilization under gamete limitation (Reinartz and Les 1994, Cheptou et al. 2002, Kalisz et al. 2004, Mable and Adam 2007, Griffin and Willi 2014). Gamete limitation might occur due to low pollinator visitation or reductions in population size (Jain 1976) both of which can be a consequence of fragmentation. While self-fertilization has been suggested to promote species invasion and establishment into novel or remote habitats (Baker 1955, Pannell 2015) it might also be advantageous in fragmented landscapes.

The downside of self-fertilisation is that it may lead to inbreeding depression, and thus reduced performance and fitness, as well as reduced herbivore resistance and/or tolerance of the offspring (Schemske and Lande 1985, Muola et al. 2011, Kalske et al. 2014). Inbreeding depression results from the reduction in heterozygosity that increases the probability of the expression of recessive, deleterious alleles (Charlesworth and Charlesworth 1987, 1999, Carr and Dudash 2003, Charlesworth and Willis 2009). Selection is likely to favor self-fertilization if inbreeding depression is less than the transmission advantage (Maynard Smith 1978, Lande and Schemske 1985), and if self-fertilization helps a species to establish in novel and remote habitats despite fitness losses. Deleterious alleles can also be purged by selection, which is likely to facilitate evolution towards selfing (Charlesworth and Charlesworth 1987, Arunkumar et al. 2015). In addition, small population size and restricted pollen and seed dispersal might lead to mating with close relatives that may eventually have similar negative consequences as self-fertilisation (Kéry et al. 2000, Kolb 2005). Furthermore, in a species with a mixed-mating system, a low outcrossing rate reduces the accumulation and maintenance of genetic variation in the population, which limits local adaptation (Arunkumar et al. 2015). Therefore, in fragmented landscapes, we would predict an increasing outcrossing rate with increasing population size and age and with decreasing isolation among populations.

Here, we study the population genetic structure of a mixed-mating perennial herb, *Vincetoxicum hirundinaria*, in a naturally fragmented landscape Fig. 1a). In addition, we test whether selfing and/or mating among relatives correlates with the size, age and isolation of populations of this species. Our study area, the south-western (SW) Finnish archipelago, is a naturally fragmented landscape that has been formed relatively recently after the ice sheet started to retreat 12 000 years ago (Lundqvist 1986). In this area, isostatic land uplift is approximately 4–5 mm per year and, thus, suitable habitats on novel islands and newly exposed coastline are gradually being formed and subsequently get colonized by terrestrial plants and animals (Ekman 1996). *Vincetoxicum hirundinaria* is relatively abundant in the SW Finnish archipelago, although it is at the NE edge of its continental Eurasian distribution range. It is estimated to occur on over 700 islands and is expanding its distribution (Mikael von Numers, pers. comm.). As a consequence of fragmentation and the ongoing colonization, populations of *V. hirundinaria* are differing in age, size and the degree of isolation. These are all factors that individually and interactively determine the opportunities for gene flow and shape the population genetic structure. Whereas population isolation should correlate negatively with gene flow and positively with population differentiation (Slatkin 1987), the predictions are unclear for population age and size.

Although *V. hirundinaria* exhibits a mixed mating system (Leimu 2004), it is also known to suffer from inbreeding depression expressed as reduced performance and herbivore resistance in selfed offspring (Muola et al. 2011, Kalske et al. 2014). However, the ability to self-fertilize could be an adaptation to the heterogeneous environment of the SW Finnish archipelago as well as a way to foster the colonization of new islands. The outcrossing rate is likely to increase after population establishment through the increasing availability of potential mates. Moreover, the fragmented landscape of the SW Finnish archipelago is likely to be reflected in patterns of gene flow and in the population genetic structure of *V. hirundinaria*.

Here we report on a study designed to ask the following questions: 1) is there variation in outcrossing rate among the *V. hirundinaria* populations in the SW Finnish archipelago?

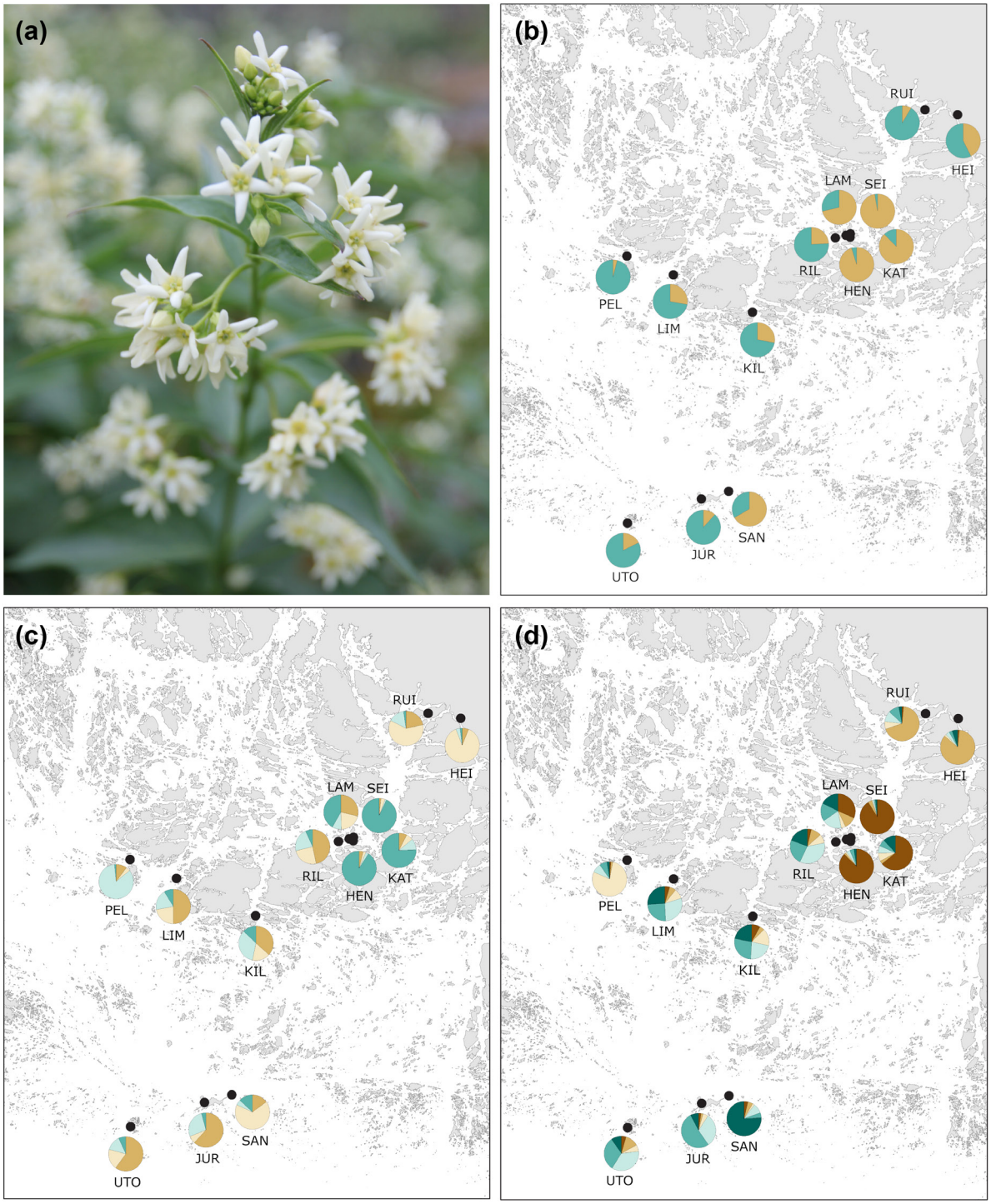

Figure 1. (a) Study species *Vincetoxicum hirundinaria* and STRUCTURE sample assignments for (b) K = 2, (c) K = 4 and (d) K = 6.

Does this variation correlate positively with the size and age, and negatively with the isolation of the populations? We tested this by estimating population-level outcrossing rates from seeds of open-pollinated flowers collected from thirteen *V. hirundinaria* populations differing in size, age and isolation, using microsatellite markers. 2) Do population-level fixation indices vary, suggesting past selfing and/or mating among relatives? Does this variation in fixation indices decline with population size and age and increase with isolation? 3) Are allelic richness and among-population genetic structure correlated with population size, age, isolation or geographical distance?

Material and methods

Study species

Vincetoxicum hirundinaria Medik. (Apocynaceae) is an insect-pollinated long-lived perennial herb. The main pollinators are large flies (Timonin and Savitskii 1997). Although it has a late-acting self-incompatibility system occurring via late-acting ovarian rejection (Wyatt and Broyles 1994, Lipow and Wyatt 2000, Leimu 2004), in our study area part of *V. hirundinaria* individuals are able to produce seeds by self-fertilisation, which leads to varying levels of realised self-fertilisation among populations (Leimu 2004, Muola et al. 2011). In Finland, flowering begins in the middle of June and lasts until the beginning of August. Fruits normally ripen at the end of August or early September. Each pod contains approximately 20 seeds with adaptations for wind dispersal (Leimu 2004).

Study populations

We selected thirteen *V. hirundinaria* populations that are located on separate islands in the SW archipelago of Finland. We estimated three population characteristics: size, age and level of isolation (Table 1). The size of the study populations varied from small (50 individuals) to large (10 000 individuals). In small populations (under 100 individuals) we counted all mature *V. hirundinaria* individuals. In populations with more than 100 individuals, two or three researchers estimated population sizes independently by counting the number of *V. hirundinaria* individuals along a transect and then extrapolating it to the total area. The age of the study populations was assessed based on the elevation of the midpoint of each population above sea level from topographical maps provided by the National Land Survey of Finland (<www.maanmittauslaitos.fi/en/e-services/mapsite>). Since the populations usually colonize the shores, the current elevation can, via the rate of land uplift in this area, be used as an estimate of the maximum age of the population. Population age estimates varied between 200 and almost 5000 years. The distances among populations varied from 550 m to over 80 km, and their isolation in terms of proportion of sea around the populations ranged over one magnitude at each of three spatial scales (circles of 2, 5 and 10 km radii from the population).

Sampling

We collected seeds of open-pollinated flowers from 20 maternal plants from 12 study populations in September 2007. The 13th population (PEL) was sampled in September 2012. Seed production in both years of sampling was generally high, but due to extensive spatio–temporal variation in seed quality as well as in pre-dispersal seed predation by *Euphranta connexa* the number of seed families used in our study varied from 9 to 18 among populations (Table 2). Although in the majority of populations, sampled plants represented a random selection of all reproductive plants irrespective of their size, we sampled every single reproductive plant in the small population HEN. Seeds from different pods of the same maternal plant were pooled and dried for three weeks at room temperature (+22°C) and then stored at +4°C. We germinated the seeds in the greenhouse (Ruissalo Botanical Garden, Univ. of Turku) during the summer following the collections (2008 or 2013). In 2008, we germinated up to 100 randomly chosen seeds per maternal plant. If a maternal plant had less than 100 seeds we germinated all the seeds. In 2013, we followed the same procedure for seeds from PEL except that we did not count sown seeds. Following germination, seedlings were allowed to grow for four weeks after which we sampled leaf material of around eight randomly selected seedlings per maternal plant (mean ± SD = 8.25 ± 1.28; range 5–16). The samples were flash frozen in liquid nitrogen in 2008 and put in vials containing air-dried silica gel in 2013, and subsequently stored at −80°C. Altogether we had 1369 samples from 163 maternal seed families.

Microsatellite development and genotyping

We developed microsatellites for *V. hirundinaria* in collaboration with Genoscreen (Lille, France) and the Center of Evolutionary Applications (CEA) at the Univ. of Turku,

Table 1. Characteristics of 13 sampled populations of *Vincetoxicum hirundinaria* in the SW Finnish archipelago.

Population		Coordinates (N; E)	Size	Age (years)	Isolation 2	Isolation 5	Isolation 10
Heikkilä	HEI	60°42'53.25'', 22°25'33.06''	50	4974	0.64	0.38	0.28
Henrysaari	HEN	60°23'15.00'', 21°95'85.98''	50	519	0.87	0.72	0.58
Jurmo	JUR	59°82'54.08'', 21°57'91.50''	1800	1002	0.92	0.99	0.97
Katava	KAT	60°23'33.67'', 21°95'43.92''	380	1014	0.87	0.73	0.58
Killingholm	KIL	60°10'86.83'', 21°68'48.50''	1000	2994	0.87	0.68	0.74
Lammasluoto	LAM	60°23'36.92'', 21°94'63.53''	5261	2994	0.86	0.74	0.58
Limskär	LIM	60°15'47.69'', 21°44'01.64''	4800	1509	0.96	0.74	0.75
Pellonkare	PEL	60°17'64.49'', 21°29'81.49''	50	219	0.94	0.79	0.83
Rilot	RIL	60°22'88.17'', 21°91'48.96''	150	3984	0.90	0.74	0.60
Ruissalo	RUI	60°42'90.72'', 22°15'37.25''	100	1014	0.74	0.49	0.22
Sanden	SAN	59°84'00.91'', 21°65'79.05''	500	513	0.99	0.97	0.96
Seili	SEI	60°23'65.19'', 21°95'84.98''	83	2004	0.88	0.74	0.57
Utö	UTO	59°78'03.39'', 21°36'61.19''	10 000	1980	0.95	0.99	0.99

Isolation 2, 5 and 10 indicate the proportion of water within radii of 2, 5 and 10 km around the centre of the population, respectively.

Table 2. Genetic indices of 13 sampled populations of *Vincetoxicum hirundinaria* in the SW Finnish archipelago.

	Maternal seed families	A_m	A_o	ΔA	H_{S_m}	H_{S_o}	H_{O_m}	H_{O_o}	F_{IS_m}	F_{IS_o}	t_m	t_s	t_m–t_s	Selfing maternal genotypes	F/M
HEI	7	43.0	46.0	3.0	NA	0.568 (0.549–0.587)	0.512	0.569 (0.560–0.578)	0.007	−0.018 (−0.055–0.019)	1.000 (0.000)	0.976 (0.017)	0.024 (0.017)	0	2.71
HEN	9	35.5	46.1	10.6	0.518	0.574 (0.566–0.582)	0.557	0.543 (0.532–0.554)	−0.071	0.051 (0.031–0.072	1.000 (0.000)	0.978 (0.008)	0.022 (0.008)	0	3.11
JUR	18	53.6	55.3	1.7	0.650	0.647 (0.640–0.653)	0.623	0.623 (0.617–0.629)	0.000	0.035 (0.023–0.046)	1.000 (0.000)	0.971 (0.007)	0.029 (0.007)	0	4.28
KAT	15	47.1	50.0	2.9	0.669	0.564 (0.557–0.571)	0.565	0.504 (0.498–0.511)	0.017	0.103 (0.089–0.117)	0.989 (0.013)	0.978 (0.012)	0.011 (0.014)	1	3.87
KIL	8	49.4	52.7	3.3	0.576	0.639 (0.626–0.653)	0.597	0.609 (0.600–0.617)	−0.035	0.038 (0.014–0.063)	1.000 (0.000)	0.985 (0.007)	0.015 (0.007)	0	4.63
LAM	9	51.4	54.6	3.2	0.638	0.654 (0.647–0.662)	0.698	0.611 (0.602–0.621)	−0.122	0.065 (0.053–0.077)	1.000 (0.000)	0.953 (0.025)	0.047 (0.025)	0	3.89
LIM	13	53.6	56.8	3.2	0.664	0.663 (0.656–0.670)	0.619	0.590 (0.582–0.599)	−0.013	0.108 (0.097–0.120)	0.991 (0.009)	0.929 (0.024)	0.061 (0.020)	0	4.08
PEL	15	50.6	51.5	0.9	0.643	0.667 (0.660–0.674)	0.676	0.652 (0.645–0.660)	0.015	0.021 (0.008–0.033)	1.000 (0.000)	0.947 (0.020)	0.053 (0.020)	0	2.08
RIL	11	48.1	50.0	1.9	0.634	0.600 (0.592–0.608)	0.654	0.573 (0.566–0.581)	−0.018	0.041 (0.027–0.056)	1.000 (0.000)	0.969 (0.014)	0.031 (0.014)	0	3.81
RUI	13	51.5	52.4	0.9	0.567	0.621 (0.612–0.630)	0.600	0.568 (0.562–0.574)	−0.023	0.082 (0.067–0.096)	0.991 (0.010)	0.943 (0.019)	0.047 (0.019)	0	2.31
SAN	11	51.6	55.0	3.4	0.638	0.651 (0.638–0.664)	0.663	0.594 (0.586–0.602)	−0.057	0.079 (0.061–0.098)	1.000 (0.000)	0.968 (0.019)	0.032 (0.019)	0	3.64
SEI	8	39.0	48.5	9.5	0.552	0.635 (0.627–0.644)	0.646	0.555 (0.547–0.564)	−0.197	0.123 (0.109–0.138)	1.000 (0.000)	0.981 (0.006)	0.019 (0.006)	1	4.63
UTO	16	53.2	57.1	3.9	0.635	0.679 (0.671–0.687)	0.666	0.629 (0.623–0.636)	−0.071	0.072 (0.060–0.083)	0.985 (0.009)	0.964 (0.013)	0.021 (0.011)	0	3.75
Across populations	153	48.3	52.0	3.7	0.615 (0.584–0.646)	0.627 (0.604–0.652)	0.621 (0.589–0.654)	0.586 (0.562–0.610)	0.010 (−0.025–0.043)	0.073 (0.029–0.119)	–	–	–	–	3.60

A – allelic richness; ΔA – difference in allelic richness between maternal and offspring genotypes; H_S – genetic diversity; H_O – observed heterozygosity; F_{IS} – fixation index; $_m$ – based on maternal genotypes; $_o$ – based on offspring genotypes. t_m – multi-locus outcrossing rate; t_s – single-locus outcrossing rate; t_m–t_s – bi-parental inbreeding coefficient; F/M – average number of fathers siring a mother plant. 95% confidence intervals are given for offspring-based genetic diversity, observed heterozygosity and the fixation index, whereas t-values are accompanied by their standard deviation.

Finland. The CEA performed all subsequent genotyping. More detailed description of microsatellite development can be found in the Supporting information.

For DNA extraction, we first homogenized a 1–2 cm^2 piece of leaf with a TissueLyser and then subjected it to a silica-based purification method modified from Elphinstone et al. (2003). We diluted DNA 1:16 and genotyped samples with 15 microsatellite markers (Vince-02, Vince-04, Vince-06, Vince-08, Vince-11, Vince-14, Vince-15, Vince-23, Vince-26, Vince-31, Vince-32, Vince-39, Vinc-101, Vinc-102 and Vinc-118; Tada et al. 2009, Supporting information). To improve the microsatellite peak profiles, we added a GTTT-tail to the 5′ end of each non-labelled primer (Brownstein et al. 1996).

We carried out PCR amplification in two 8 µl multiplexed reactions using QIAGEN Multiplex PCR Kit with an annealing temperature of 58°C, the primer concentration varying from 0.1 to 0.4 µM and using 1 µl of diluted DNA. PCR profile was according to the manufacturer's standard protocol for microsatellites. We performed amplifications on PTC-100 (MJ Research), AB 2720 (Applied Biosystems) and S1000 (Biorad) thermal cyclers. For electrophoresis we pooled the PCR products by combining 1.0 µl of each multiplexed PCR and diluted these with 100 µl of sterile water. We combined 2 µl of the pooled and diluted PCR product with GS600LIZ size standard (Applied Biosystems) and HiDi-formamide (Applied Biosystems). We denatured the samples at 98°C for three minutes and determined the size of the fragments by capillary electrophoresis on an ABI PrismTM 3130xl genetic analysis instrument.

We scored the genotypes using GeneMarker ver. 1.96 and, following visual inspection, exported them to a spreadsheet program for downstream analyses. We assessed the genotyping error by repeating the PCR amplification of 60 individuals, analysing these samples blindly and comparing with the original genotypes. Direct count genotyping error rate per allele per locus varied 0–0.9%, with a mean over all loci of 0.17%.

Twenty-five samples failed to yield proper genotypic data and we therefore omitted these, leaving 1344 samples for the analyses. Analysis with the program INEST (Chybicki and Burczyk 2009) indicated that null alleles were likely present in two loci, Vince-6 and Vince-15, across populations and we therefore omitted these loci from further analyses.

Mating system analysis

We used progeny-array analyses in the multi-locus maximum-likelihood estimation program MLTR ver. 3.4 (Ritland 2002) with the expectation–maximization method in order to determine outcrossing rates for mating system analysis and maternal genotypes for analysis of population genetic structure. We first checked the quality of the data by assessing non-compatibility of offspring with maternal genotypes (i.e. when it was not possible to determine the maternal genotype based on offspring genotypes) in MLTR. Such cases were probably mostly due to null alleles rather than to genotyping errors, since in all cases, the conflict was due to three or more

different 'homozygotes' rather than to high allelic richness. We omitted the whole seed family when non-compatibility with maternal genotypes occurred for more than two loci (ten seed families). If non-compatibility occurred for a certain locus in more than two seed families in a population, the complete locus was removed (two populations). If one or two loci or seed families were non-compatible we removed the loci only for those seed families (28 occurrences). This pruned data set was used to determine outcrossing rates and maternal genotypes (153 mother plants; Table 2).

We assessed the multi-locus outcrossing rate (t_m), the averaged single-locus outcrossing rate (t_s) and the difference between t_m and t_s, which is an estimate of the bi-parental inbreeding coefficient (i.e. mating among relatives), at the population level. We used 1000 bootstraps using families as sampling units to calculate standard deviations of our estimated mean variables. We estimated pollen and ovule allele frequencies separately. We also analysed the multi-locus and single-locus outcrossing rate per family within each population with the same method as described above to investigate within-population variability in outcrossing rate. This will shed light on the assumption in MLTR that outcrossing rates are equal among all maternal plants within each population. Other assumptions include that 1) all mating events are due to either selfing or random outcrossing; that 2) pollen pools are homogeneous and there is no assortative mating, and that 3) there is neither mutation nor any kind of selection between fertilisation and sampling. Finally, we calculated the most likely average number of fathers siring mother plants.

Population structure

MLTR analysis yielded one most likely maternal genotype for each of the 153 seed families, and these 153 maternal genotypes were subsequently used for the analysis of population genetic structure. We also constructed 100 datasets with 153 randomly drawn offspring genotypes (using equal numbers of offspring per population as mother plants sampled, each random offspring from its mother plant). All subsequent genetic analyses run on the mother genotypes were likewise run on these 100 offspring datasets and subsequently averaged across offspring datasets. We used the package *hierfstat* (Goudet 2005) in R (<www.r-project.org>) to determine the following per population genetic diversity indices: allelic richness (A) summed over loci and rarefied down to seven individuals to allow population comparisons, observed heterozygosity (H_O), gene diversity (H_S), fixation index (F_{IS}). We also calculated the overall and pairwise F_{ST}. *STRUCTURE* 2.3.4 (Hubisz et al. 2009) was used to assign individuals to genetic clusters. We used *Structure Harvester* (Earl and von Holdt 2012) to visualise diagnostic results and *CLUMPP* (Jakobsson and Rosenberg 2007) to average results across replicate runs.

Correlations between genetic diversity indices and population characteristics

To test whether population size, age and isolation correlate with population genetic properties, we performed Pearson correlations between these population characteristics and the genetic diversity indices A, H_O, H_S and F_{IS} based on mother and offspring genotypes. Assuming that allelic richness (A) reflects gene flow, then allelic richness of maternal genotypes is a measure of past gene flow, whereas allelic richness of offspring is a measure of both past and the current year's gene flow. Due to absence of variability in the outcrossing rate, we did not perform any correlations between this variable and population characteristics.

F_{ST} based on offspring is expected to be lower than F_{ST} based on maternal genotypes due to gene flow among populations neutralising among-population differentiation. We applied Pearson correlations to pairwise F_{ST} of offspring and maternal genotypes with geographic distance, and Pearson correlations of the average pairwise F_{ST} per population with population size, age and isolation. Here, F_{ST} was transformed to the index $F_{ST}/(1 - F_{ST})$ (Rousset 1997).

Results

Outcrossing rate

The outcrossing rate was almost completely invariable, with only two maternal plants from different populations (KAT and SEI) showing potentially selfed offspring in the year of sampling, 2008 (Table 2). The amount of mating among relatives was low and ranged from 1.1 to 6.1%. The number of fathers per mother siring the tested offspring ranged from 2.08 to 4.63 (average 3.60).

Genetic diversity indices

For maternal plants, the cross-population cross-locus allelic richness was 48.3 and ranged from 35.5 to 53.6 among the 13 populations (Table 2). For offspring, the average allelic richness was 52.0 and ranged from 46.0 to 57.1 with each population having higher values for offspring than for maternal plants. Population level allelic richness was, thus, on average 3.7 higher in offspring compared to maternal plants (range 0.9–10.6), which constituted a significant difference (paired t-test, $t = 3.7$, $p < 0.001$). Population level allelic richness of maternal plants and offspring were strongly correlated ($r = 0.89$, $p < 0.0001$). For maternal and offspring plants, the mean genetic diversity (H_S) were 0.615 and 0.627, respectively, their confidence intervals overlapped (Table 2), and a pairwise t-test (omitting population HEI) showed no difference ($t = -1.19$, $p = 0.258$). The observed heterozygosity (H_O) across populations based on maternal and offspring plants were 0.621 and 0.586, respectively, and their confidence intervals likewise overlapped (Table 2), but a pairwise t-test showed that observed heterozygosity was lower in offspring compared to mother plants ($t = 2.93$, $p = 0.013$).

Inbreeding

The maternal plant-based overall fixation index, F_{IS}, was 0.010 and was not significantly different from zero (Table 2).

Overall F_{IS} of offspring was 0.073 which suggests very low level of inbreeding. Thus, overall, populations were likely not or only little inbred and had no heterozygosity excess. Although F_{IS} of mothers and offspring differed (pairwise t-test, t = 4.4, p = 0.001), they did not correlate with each other (Pearson correlation, r = −0.483, p = 0.09).

Genetic differentiation

Mean pairwise genetic differentiation (95% CI) across the 13 populations based on maternal genotypes was 0.100 (0.083–0.116) (Supporting information), which indicates limited genetic differentiation among populations. Averaged F_{ST} across the 13 populations based on randomly drawn sets of offspring genotypes was 0.098 (0.072–0.127), i.e. similar to the maternal genotype-based value. All pairwise F_{ST} values were significantly different from zero except for the LIM–RIL pair based on maternal plants. Pairwise F_{ST} values based on maternal plants were correlated with pairwise F_{ST} values based on offspring plants (Mantel test, r = 0.54, p = 0.001) and all pairs had overlapping confidence intervals between maternal plants and offspring (data not shown).

Population genetic structure

In STRUCTURE the likely number of genetic clusters can be inferred by different methods. Using Evanno's method (Evanno et al. 2005), our results indicate that a division of maternal genotypes into two clusters (K = 2) was the most likely genetic structure in our data, showing the highest ΔK = 35.0 (Supporting information). Another method bases the likely number of genetic clusters on the average ln(P)d, which was highest for K = 6 (ΔK = 4.5) (Supporting information). We therefore investigated the genetic structure results using 2, 4 and 6 clusters. At the most coarse level (K = 2), the investigated *Vincetoxicum hirundinaria* populations are broadly separated into two genetically differentiated groups (Fig. 1b). When assigning populations to groups based on average majority affinity, one group was composed of the populations HEN, KAT, LAM, SAN and SEI, which are all located close together except for SAN, which is one of the outer populations sampled. For K = 4 (Fig. 1c), SAN split off from this group and became more closely associated with RUI and HEI, two of the oldest populations on, or close to, the mainland which split off from the other large group for K = 2. Furthermore, PEL, the smallest and youngest population, formed a group weakly associated with KIL. For K = 6 (Fig. 1d), SAN again split off, this time without any associations to other populations. JUR tended to break off from LIM, RIL and UTO and associated with KIL. PEL became a group on its own.

Correlations between genetic diversity indices and population characteristics

Given the near absence of variation in outcrossing rate (Table 2), we did not perform correlations of outcrossing rate with population characteristics. We did find, however, that the average number of fathers siring a mother plant was positively correlated with population size (r = 0.55, p = 0.05; Table 3), though not with age or isolation.

The correlation of allelic richness of both maternal and offspring genotypes with population size was significant and positive (Table 3, Fig. 2). The correlation of allelic richness with population age was not significant for neither the maternal genotypes nor for the offspring genotypes (Table 3). The correlation of allelic richness with isolation (for all three radii) was not significant for maternal genotypes whereas it was significant for offspring genotypes (Table 3, Fig. 3). Similarly, genetic diversity (H_S) based on offspring, but not maternal, genotypes correlated positively with isolation (for all three radii; Table 3).

Average pairwise F_{ST} per population of mothers correlated negatively with population size, but not with population age or isolation (Table 3). Average pairwise F_{ST} per population of offspring did neither correlate with population size, nor with age or isolation. A Mantel test of pairwise F_{ST} with pairwise geographic distance was neither significant for mother plants (Mantel's r = 0.04, p = 0.39) nor for offspring (Mantel's r = 0.003, p = 0.49). Neither F_{IS} of mothers nor of offspring nor the degree of mating among relatives correlated with population size, age or isolation (data not shown).

Table 3. Correlations between genetic diversity indices and population characteristics. Pearson correlation coefficients and their significance are shown in the table. p-values < 0.05 are indicated in bold.

	log(size)		Age		Isolation 2 km		Isolation 5 km		Isolation 10 km	
	r	p	*R*	p	*r*	p	*r*	p	*r*	p
A_m	0.68	**0.01**	−0.12	0.69	0.37	0.21	0.39	0.19	0.45	0.12
A_o	0.86	**0.0002**	−0.27	0.37	0.60	**0.03**	0.63	**0.02**	0.64	**0.02**
Hs_m	0.54	0.07	0.03	0.93	0.53	0.08	0.46	0.13	0.48	0.12
Hs_o	0.60	**0.03**	−0.27	0.37	0.62	**0.03**	0.59	**0.03**	0.65	**0.02**
Ho_m	0.43	0.14	−0.21	0.49	0.68	**0.01**	0.63	**0.02**	0.55	**0.05**
Ho_o	0.40	0.17	−0.05	0.87	0.38	0.19	0.42	0.14	0.58	**0.04**
F_{STm}	−0.80	**0.001**	0.03	0.93	−0.47	0.10	−0.45	0.12	−0.49	0.09
F_{STo}	−0.52	0.07	0.31	0.31	−0.32	0.28	−0.40	0.17	−0.41	0.17
F/M	0.55	**0.05**	0.18	0.55	0.41	0.17	0.42	0.15	0.39	0.19

n = 13 for all indices. We used population level mean pairwise values for F_{ST_m} and F_{ST_o}. F/M is the average number of fathers siring a mother plant. $_m$ – based on maternal genotypes; $_o$ – based on offspring genotypes.

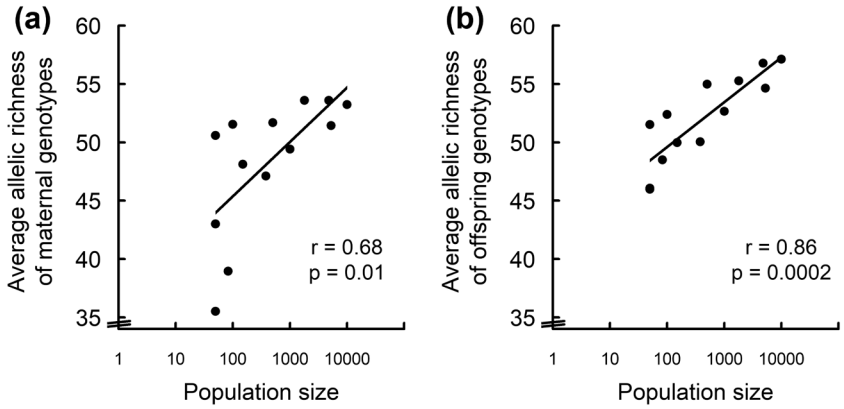

Figure 2. Positive relationship between allelic richness of (a) maternal genotypes and (b) offspring genotypes with population size (on a logarithmic scale).

Discussion

We found that *Vincetoxicum hirundinaria* is almost completely outcrossing in the SW archipelago of Finland, which is contrary to our expectation of an increasing amount of selfing in smaller, younger and more isolated populations. Only two of the sampled mother plants, each on a different island, produced selfed offspring. Furthermore, we did not detect signs of past selfing and/or mating among relatives. This, together with the overall low fixation indices and estimates of bi-parental inbreeding coefficients, indicates that mating among relatives is rare even in small populations. High allelic richness and limited F_{ST} values observed in both maternal and offspring genotypes indicate strong gene flow among populations. This is further supported by the lack of isolation by distance and by the STRUCTURE results that show two major genetic clusters among the 13 populations and clustering of distant populations when K was increased. Our findings thus suggest that *V. hirundinaria* has successful seed and pollen dispersal among populations which has allowed colonization of new sites and persistence of genetically viable populations in this fragmented landscape, and has led to genetically rather well-mixed groups of primarily outcrossing populations.

Selfing and mating among relatives

Despite the relatively low fixation indices (F_{IS}) and the predominantly outcrossing mating system, it is conceivable that the first *V. hirundinaria* individuals colonizing new islands in the study area reproduced uniparentally (Pannell 2015). Plant mating systems are known to be plastic (Pannell 2015) and our earlier observations and experiments confirm that, despite the late-acting self-incompatibility system (Wyatt and Broyles 1994, Lipow and Wyatt 2000, Leimu 2004), *V. hirundinaria* is indeed capable of producing seeds via autonomous self-fertilization (Leimu 2004, Muola et al. 2011). Occasional self-fertilization may assure reproduction in colonizing populations of *V. hirundinaria*. Yet, our results clearly show that the ability of *V. hirundinaria* to self-fertilize is not generally realized in natural populations, at least not during the two years of sampling. Strong inbreeding depression, which *V. hirundinaria* exhibits both in performance and in herbivore resistance of selfed offspring (Muola et al. 2011, Kalske et al. 2014), may explain the high outcrossing rates due to strong selection against frequent selfing (Lande and Schemske 1985). The very low rate of mating among relatives in our study may likewise relate to inbreeding

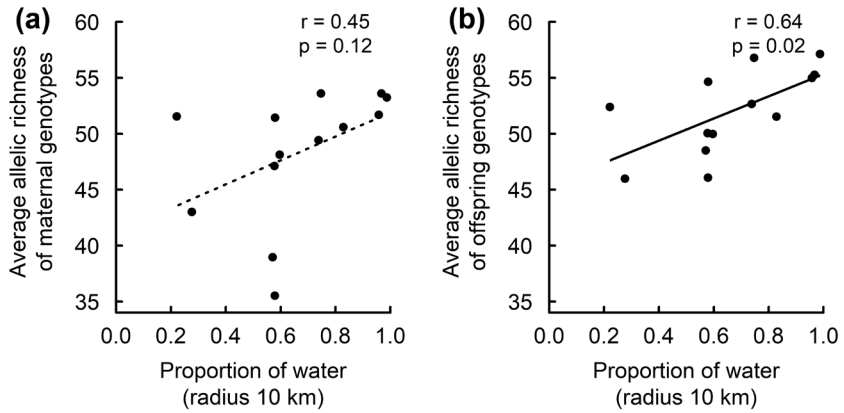

Figure 3. Relationship between allelic richness of (a) maternal genotypes and (b) offspring genotypes with the proportion of water to total area within a radius of 10 km as a measure of population isolation. Similar results were obtained for radii of 2 km and 5 km.

depression in the offspring (Fischer and Matthies 1997). Taken together, we conclude that this species expresses an outcrossing strategy rather than mixed mating, but that the ability to self-fertilize is retained as it benefits the occasional colonization (Wubs et al. 2010, Pannell 2015). In fact, *V. hirundinaria* seems to be expanding its distribution. During the past 80–90 years it has colonized at least 76 new islands and gone extinct on only 11 islands within the same surveyed area (Mikael von Numers, pers. comm.). Therefore, the ability to self-fertilize may be important for its current expansion in the SW Finnish archipelago. Similar observations of rarely self-fertilizing plants with a mixed-mating system, were made for the fern *Asplenium scolopendrium* (Wubs et al. 2010) and the herb *Campanulastrum americanum* (Kruszewski and Galloway 2006), which for the latter species was explained by cryptic self-incompatibility, i.e. a preference of the mother plant for cross versus self pollen. Although we observed that multiple fathers are involved in siring a single mother plant, reflecting successful outcrossing, it remains unknown whether this is the result of abundant outcrossing opportunities, cryptic self-incompatibility or another mechanism.

Gene flow, genetic diversity and population genetic structure

Gene flow depends on the landscape structure, mating system, pollination vectors and the adaptations of seeds for dispersal (Schmidt et al. 2009, Buehler et al. 2012, Sork et al. 1999, Cruzan and Hendrickson 2020). The naturally fragmented structure of our study area, where large bodies of water separate islands only some of which have suitable habitats for *V. hirundinaria*, suggests that there are obstacles for successful gene flow. Contrastingly, *V. hirundinaria* is mainly pollinated by large flies (Timonin and Savitskii 1997) that are likely to be able to fly long distances, even between islands in the fragmented archipelago landscape of SW Finland. Furthermore, the seeds of *V. hirundinaria* are adapted to wind dispersal (pappus; Solbreck and Knape 2017) and they are known to stay viable at least for two weeks in brackish water (Leimu unpubl.). Several related species with comose seeds are known to disperse relatively long distances (up to 80 m) even with light winds (up to 15 km h^{-1}) (Platt and Weis 1977, DiTommaso et al. 2018). However, in SW Finnish archipelago moderate and strong winds (up to 50 km h^{-1}) are relatively common when *V. hirundinaria* seed are maturing (Finnish Meteorological Inst., <www.ilmatieteenlaitos.fi/tuulitilastot>) suggesting good conditions even for long distance seed dispersal. In line with this, our findings suggest effective and successful pollen and/or seed dispersal among populations that has led to genetically rather well-mixed populations in a landscape that is geographically strongly fragmented.

The high gene flow in *V. hirundinaria* across the archipelago, as suggested by high allelic richness, limited pairwise F_{ST} values, lack of isolation by distance and our STRUCTURE results, has important implications for mating system evolution: successful dispersal renders species less dependent on selfing for reproductive assurance and, therefore, more likely to outcross (Pannell and Barrett 1998, Auld and Rubio de Casas 2013). Beyond the generally high gene flow observed in our system, which may have driven the evolution towards almost complete outcrossing, we found that specific population characteristics influenced the proxies of gene flow.

Allelic richness of mothers and offspring correlated positively with population size, which fits the expectation that larger populations harbour more genetic variation (Fischer and Matthies 1998). Surprisingly, allelic richness of offspring also correlated positively with all three radii of population isolation (as did genetic diversity of offspring), suggesting an increasing gene flow with increasing isolation of the population. Since population size and isolation were not correlated ($p > 0.12$ for all three isolation levels), association between these two factors cannot explain the significant correlations with allelic richness. The relationship between isolation and allelic richness was unexpected; one would expect decreasing gene flow with increasing isolation, i.e. more isolated populations in the archipelago should be less frequently visited by pollinators and receive fewer hydrochorous and anemochorous diaspores. An explanation for this unexpected relationship could be that, whenever more isolated populations receive pollinators, these pollinators come from a wider range of other populations compared to better connected populations, thereby introducing a broader range of alleles. Moreover, the isolated populations may even act as attractors to pollinators, making up for their larger distance to other populations. Many studies have shown that population isolation does not necessarily lead to decreased gene flow or reproductive success (Groom 1998, Young et al. 1999, Meekers and Honnay 2011), and a positive effect of population isolation on reproductive output was likewise found in a study on *Aquilegia canadensis* (Mavraganis and Eckert 2001). Interestingly, this relationship appeared only in the offspring and not in the mother plants, which may reflect selection against novel alleles to promote local adaptation in *V. hirundinaria* (Kalske et al. 2012). Since population age and isolation were not correlated ($p > 0.09$ for all three isolation levels), this disproves the possibility that patterns of allelic richness are due to longer build-up of mutation load. We also discredit the possibility that prevailing winds (SW in this region) caused a net movement of alleles towards populations with the highest allelic richness, because most of these populations (UTO, LIM, JUR, SAN) are located on the SSW edge of our study region and on the southern edge of the archipelago.

In general, the allelic richness of maternal genotypes reflects past gene flow and allelic richness of offspring reflects both past and current gene flow. The overall higher allelic richness (and genetic diversity) in offspring compared to mother genotypes, in particular in small populations (such as HEN and SEI, Table 1), is therefore another indication for strong gene flow among populations. Interestingly, since we sampled all maternal plants present in the small HEN population, the increase in allelic richness on this island

must result from gene flow from other populations. The fact that we found such a clear difference between mother and offspring allelic richness suggests that much of this influx of novel alleles might not be locally adapted and, thus, is selected against, for instance during the establishment of offspring (i.e. seedling stage). It remains unclear why the opposite pattern of lower heterozygosity in offspring compared to mothers was found, since an increase in allelic richness from the parent to the offspring generation would rather lead to an increase in heterozygosity.

We also observed that the average pairwise F_{ST} per population of mothers correlated negatively with population size. Larger populations are less differentiated from other populations in the archipelago, perhaps because they produce a disproportionately large number of seeds which therefore also have a higher chance to disperse to other populations. On the other hand, gene flow from small populations into other populations is likely to be limited. The absence of a correlation between average pairwise F_{ST} of offspring with population size suggests that pollen is well mixed among populations and that selection against unfit offspring plays a role in shaping the genetic differentiation of populations of established individuals (Sambatti and Rice 2007). Average pairwise F_{ST} per population of neither mother plants nor offspring correlated with population age, suggesting that any genetic signatures deriving from founder events have long been erased through extensive among-population gene flow. Interestingly, a study on *Silene dioica* with similar population characteristics (Skeppsvik archipelago, Sweden) reported higher genetic differentiation in newly colonized and in very old populations compared to populations of intermediate age (Giles and Goudet 1997), i.e. a non-linear pattern. No such non-linear pattern was apparent in our data when regressing the average pairwise F_{ST} in population age and (population age)^2 (results not shown). The absence of correlations with population isolation point to the same direction of extensive gene flow irrespective of geography.

Patterns of within-population gene flow also vary among populations. The increase of the average number of fathers siring a mother plant with increasing population size is likely reflecting efficient outcrossing within larger populations, made possible through an increasing availability of pollen donors and perhaps also pollinators in larger populations. This is in agreement with plenty of studies showing an increase in pollinator visits per plant and in reproductive success with increasing population size (Mustajärvi et al. 2001, Jacquemyn et al. 2002, Brys et al. 2004), including a study on *V. hirundinaria* (Leimu and Syrjänen 2002). Thus mother plants from larger populations received pollen from a higher number of fathers and this corresponds well with our result that allelic richness increases with increasing population size. As larger populations have more potential pollen donors, this alone could explain the increase in allelic richness on the studied mother plants, but the example of HEN, where all existing plants were assessed, proves that pollen can also come from other populations.

Spatial population genetic structure

From a spatially explicit perspective, we found no evidence for isolation by distance. This is also visible in the structure analysis, which did not show clear geographic clusters of genetically similar populations. In contrast, many populations showed relationships across wide geographic distances. The most stable cluster across K=2, K=4 and K=6 is composed of HEN, KAT, LAM, SAN and SEI, of which SAN is a geographic outlier only joining this cluster for K=2. The other cluster in K=2 breaks down in K=4 and in K=6 into smaller clusters of populations with varying geographic distances and sometimes populations from other clusters in between (e.g. PEL-KIL for K=4; JUR-KIL and LIM-RIL-UTO for K=6), suggesting long-distance gene flow among populations from these clusters. Alternatively, clusters formed through historic gene flow may have been disrupted by more recent gene flow, thereby disrupting geographic clusters. This suggests long-distance dispersal of novel genetic material into established clusters.

Methodological issues

Interestingly, in an earlier study Leimu and Mutikainen (2005) found that *V. hirundinaria* populations had relatively high F_{IS} values (average $F_{IS}=0.460$) in SW archipelago of Finland and therefore suggested that *V. hirundinaria* harbours a mixed mating system with regular occurrences of selfing in this area. This obvious discrepancy between the results of the current study and those of Leimu and Mutikainen's (2005) might have resulted from different sampling designs and marker types in these studies. In our study, the leaf material used for genetic analysis originated from the germinated seeds sampled from open-pollinated flowers while Leimu and Mutikainen (2005) collected leaf material from adult plants. Our analysis is thus confined to offspring from reproducing plants whereas the study by Leimu and Mutikainen may have included plants with overall lower seed production such as individuals suffering from inbreeding depression. Given the strong inbreeding depression in this system it might be that the potentially inbred plant individuals do not produce seeds or not as many seeds as outbred individuals, and hence, would not have been sampled in the current study. Furthermore, if selfing functions as reproductive assurance, there might be annual variation in the expression of selfing. Another explanation, though, is the different marker types employed. While we used 13 microsatellite markers Leimu and Mutikainen (2005) used five allozyme loci and the total number of alleles was much lower (13) in their study, possibly increasing the chance to find allelic similarities among individuals, which could increase estimates of F_{IS}.

Conclusion

Despite the ability to self-fertilize, *Vincetoxicum hirundinaria* exhibits a predominantly outcrossing mating system. Selfing

may only occur under exceptional circumstances, which may be related to climate or weather, pollinator or pollen donor availability. Our study presents several lines of evidence suggesting a high pollen dispersal capacity in our study area. A remarkable finding is that more isolated populations show increased allelic richness in the offspring, which we explain with a higher attractivity of isolated populations for pollinators from a wider geographic range compared to better connected populations. However, the majority of novel alleles is likely selected against during the establishment phase of these offspring. Nevertheless, the availability of novel genetic material originating from a wide range of populations, already captured in offspring genotypes, may allow this species to adapt to local conditions, now and in the future. Our findings are not in line with the widely acknowledged negative association between habitat fragmentation and genetic diversity. This is likely because the fragmented nature of our study area is not anthropogenic but rather reflects the natural distribution pattern of our study species inhabiting the islands of SW Finnish archipelago. The results suggest that *V. hirundinaria* is in fact well adapted to such a landscape as it is able to maintain genetically diverse, well connected and viable populations even when the spatial configuration of populations appears challenging.

Data availability statement

Data are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.sj3tx963w> (Muola et al. 2020).

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Author contributions

A. Muola and J. F. Scheepens share first authorship. **Anne Muola**: Conceptualization (equal); Data curation (lead); Formal analysis (supporting); Investigation (lead); Methodology (equal); Project administration (lead); Resources (equal); Software (supporting); Writing – original draft (lead); Writing – review and editing (equal). **J. F. Scheepens**: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (supporting); Resources (equal); Software (lead); Visualization (lead); Writing – original draft (equal); Writing – review and editing (equal). **Liisa Laukkanen**: Conceptualization (supporting); Data curation (supporting); Funding acquisition (lead); Investigation (supporting); Resources (lead); Writing – review and editing (supporting). **Aino Kalske**: Conceptualization (supporting); Data curation (supporting); Investigation (supporting); Writing – review and editing (equal). **Pia Mutikainen**: Conceptualization (equal); Formal analysis (supporting); Funding acquisition (supporting); Methodology (supporting); Resources (supporting); Supervision (equal); Writing – review and editing (supporting). **Roosa Leimu**: Conceptualization (lead); Formal analysis (supporting); Funding acquisition (lead); Investigation (supporting); Methodology (equal); Resources (lead); Supervision (equal); Writing – review and editing (supporting).

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