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Population isolation shapes plant genetics, phenotype and germination in naturally patchy ecosystems

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Abstract

Aims
Habitat connectivity is important in conservation since isolation can diminish the potential of a population for adaptation and increase its risk of extinction. However, conservation of naturally patchy ecosystems such as peatlands has mainly focused on preserving specific sites with exceptional characteristics, neglecting the potential interconnectivity between patches. In order to better understand plant dynamics within a peatland network, we assessed the effect of population isolation on genetic distinctiveness, phenotypic variations and germination rates using the peatland-obligate white-fringed orchid (*Platanthera blephariglottis*).

Methods
Fifteen phenotypic traits were measured for 24 individuals per population (20 distinct populations, Quebec, Canada) and germination rates of nearly 20 000 seeds were assessed. Genetic distinctiveness was quantified for 26 populations using single nucleotide polymorphism markers obtained *via* a pooled genotyping-by-sequencing approach. Geographic isolation was measured as the distance to the nearest population and as the number of populations occurring in concentric buffer zones (within a radius of 2, 5 and 10 km) around the studied populations.

Important Findings
All phenotypic traits showed significant differences among populations. Genetic results also indicated a pattern of isolation-by-distance, which suggests that seed and/or pollen exchange is restricted geographically. Finally, all phenotypic traits, as well as a reduced germination rate, were correlated with either geographic isolation or genetic distance. We conclude that geographic isolation likely restricts gene flow, which in turn may affect germination. Consequently, it is imperative that conservation programs take into account the patchy nature of such ecosystems, rather than targeting a few specific sites with exceptional character for preservation.

Keywords: trait variation, population genetics, peatland, orchid, *Platanthera blepharigottis*, genotyping by sequencing GBS

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INTRODUCTION

Degradation and destruction of habitats are among the principal threats to terrestrial biodiversity around the world (Krauss *et al.* 2010; Sala *et al.* 2000). Increasing pressure from land use change often results in habitats and populations that are fragmented within a human-modified matrix (Lande 1988, Young *et al.* 1996). Human-induced isolation resulting in the loss of connectivity among populations may reduce their fitness and capacity for adaptation, eventually resulting in a

higher risk of extinction (Aguilar *et al.* 2008; Frankham 2005; Newman *et al.* 2013). As such, it is essential to document the effects of population isolation and evaluate how these affect fitness in natural systems.

The response of plant species to habitat fragmentation and subsequent isolation depends to a great extent on their life-history traits (Ewers and Didham 2006; Henle *et al.* 2004). For example, genetic drift will be more important for species with shorter generation time (Young *et al.* 1996). As well, plants that produce short-lived seeds are more susceptible to genetic loss than those that safeguard genetic diversity by producing a durable seed bank (Henle *et al.* 2004) or relying on vegetative propagation (Honnay *et al.* 2005). Obligate outcrossing plants are also more likely to experience deleterious impacts from a reduction in population size and isolation than are selfing plants (Aguilar *et al.* 2008; Honnay and Jacquemyn 2007; Leimu *et al.* 2006).

Although threatened or rare species have often been associated with higher vulnerability to fragmentation and resulting isolation (Cruzan 2001; Ellstrand and Elam 1993; Gonzales and Hamrick 2005; Leimu *et al.* 2006; Wallace 2002), a recent meta-analysis by Honnay and Jacquemyn (2007) reported that common species could be as vulnerable as rare ones. Furthermore, fragmentation and isolation could have a more severe negative effect on species that became rare as a consequence of anthropogenic habitat loss than species that have been historically rare (Aguilar *et al.* 2008). Even though some studies have been made on naturally patchy ecosystems, such as serpentine and sandstone outcrops (Gil-López *et al.* 2014; Harrison 2000; Wolf and Harrison 2001), most studies to date have mainly focused on anthropogenically fragmented ecosystems, such as forests and grasslands (Heinken and Weber 2013). In naturally patchy ecosystems, plant populations may already have evolved traits providing them the ability to survive in patchy environments (Putz *et al.* 2015) and they may respond differently to human-induced disturbance such as additional fragmentation (Wolf *et al.* 2000).

The conservation of naturally patchy ecosystems such as cedar glades, alvars, sandstone outcrops, sky islands and serpentine outcrops, has mainly focused on the exceptional character of specific sites, without considering their potential interconnectivity (Bragg *et al.* 2003; Poulin *et al.* 2006). Although peatlands may have suffered from fragmentation or habitat loss in some parts of the world, they also often represent a naturally patchy ecosystem, especially in temperate regions, for which connectivity among plant populations has seldom been investigated. In addition, the few studies that have examined how isolation influences genetic and phenotypic diversity in peatland plant populations were focused on the impact of artificial isolation resulting from anthropogenic landscape transformation. For instance, Thingsgaard (2001) observed lower genetic diversity in the peatland moss *Sphagnum affine* Ren. & Card. of undisturbed habitats compared with anthropogenically fragmented areas. In another study, peat mining was found to increase differentiation in *Polytrichum commune* Hedw. despite the fact that it possesses effective spore dispersal mechanisms (Wilson and Provan 2003) and often relies on vegetative reproduction. Finally, anthropogenic fragmentation was shown to reduce genetic diversity and plant fitness in the common fen species *Swertia perennis* L. (Lienert *et al.* 2002). Taken together, these results suggest that peatland plant species remain sensitive to anthropogenic isolation, even if they have evolved within a naturally patchy ecosystem. However, the effects of natural isolation by itself on populations of peatland plants remain unclear.

The white fringed orchid, *Platanthera blephariglottis var. blephariglottis* (Willd.) Lindl., is a peatland obligate species and is commonly found in ombrotrophic peatlands (bogs) (Brown and Scott 1997; Laroche *et al.* 2012). The presence and the abundance of this charismatic species have been identified as indicators of ecological integrity (Laroche *et al.* 2012) and could therefore play a role in the design of conservation networks to safeguard peatland sites of high integrity. Populations of *P. blephariglottis,* generally composed of hundreds to thousands individuals, are known to harbor notable morphological variability, which may be due to phenotypic plasticity or be genetically determined (Argue 2012; Brown and Scott 1997; Hardin 1961). The white fringed orchid is pollinated by at least 11 different species (Cole and Firmage 1984), including hawkmoths (*Hemaris sp.*) that are considered to be strong flyers with vagrant habits (Tartaglia 2013). This may facilitate gene flow among closely situated populations and consequently lead to patterns of isolation by distance (Wright 1943). *Platanthera blephariglottis* is a diploid, self-compatible but mainly outcrossing species (Cole and Firmage 1984) with $2n = 40$ (Argue 2012), which should facilitate genetic assessments. Finally, its white conspicuous flowers are visually distinguishable from a considerable distance, allowing good detectability in the landscape. Taken together, these characteristics, along with recent data on population occurrences and associated vegetation community (CDPNQ 2013; Laroche *et al.* 2012), make *P. blephariglottis* a prime candidate to investigate the effects of peatland isolation.

The primary objective of this study was to assess how population isolation among peatlands, a naturally patchy ecosystem in many parts of the world, affects plant genetics, phenotype and germination of a locally abundant plant, *P. blephariglottis*. Increasing our understanding of plant population responses to the dynamics of naturally patchy ecosystems is of crucial importance to conservation, as restricted gene flow among patches can affect the overall persistence of local populations or whole metapopulations (Bossuyt 2007; Vandewoestijne *et al.* 2008). We hypothesised that geographical distance would hamper gene flow in a linear fashion. Moreover, we predicted that population isolation would increase genetic distinctiveness, generate phenotypic differentiation and reduce germination rates. To test these predictions, germination rates and the phenotypic variability of 20 populations were measured, while a pooled genotyping-by-sequencing (GBS) approach allowed us to calculate genetic distance among 26

populations and to investigate links between phenotypic and genetic variation.

METHODS

Study area, sampling and phenotype measurement

The study area is located in southeastern Canada, within 60 km of the southern shore of the St. Lawrence River (Fig. 1). Numerous peatlands are scattered throughout the forested (45%) and agricultural (40%) landscape, with >50 peatlands larger than 40 ha. Inter-peatland distances range from a few hundred meters to >5 km. Peatlands have always been naturally patchy in this landscape while a few of them have been very recently fragmented or completely destroyed by human activities (Poulin *et al.* 2004, 2016). However, the surroundings matrix is heavily impacted by human activities due to forest harvesting and further land conversion.

Twenty-four populations of *P. blephariglottis* had been previously identified in peatlands of the study area (CDPNQ 2013; Laroche *et al.* 2012). These populations were sampled for genetic analyses from 4 July to 2 August 2013. Additionally, two distant populations 208 km north (SNA) and 132 km south (HER) of the main study area were sampled for comparative purposes, totaling 26 populations for genetics analyses. Leaf material was collected from 24 randomly chosen individuals within each population, and placed in anhydrous calcium sulphate (Drierite, 8 mesh).

Among the 24 populations from the study area (Fig. 1), 20 were selected for phenotypic analyses based on data availability (Laroche *et al.* 2012) of plant communities, i.e. relative cover of companion plant species evaluated using a pinpoints intercept method. In peatlands, plant communities reflect local abiotic conditions such as water table level and minerotrophy (Gignac *et al.* 1991, 2004; Vitt and Slack 1984). Phenotypic variability among individuals of the same species could therefore be indirectly influenced by the surrounding plant community and not be necessarily genetically determined. Consequently, the sampling sites were chosen to maximize the similarity of their plant communities. Within each site, sampling was conducted where the density of *P. blephariglottis* was maximal.

In each sampling site, three flowers per individual were randomly sampled for 24 randomly chosen individuals per population. We measured a total of 15 traits using electronic callipers (supplementary appendix 1). Traits were categorized as 'flower traits' or as 'plant traits' and were selected based on their propensity to vary between individuals (Argue 2012) and the capacity to measure them accurately. Measurements were performed from 21 to 31 July 2013. This brief period should not have induced bias, since *P. blephariglottis* inflorescences can bloom for up to 46 days and each flower remains receptive for up to 10 days (Cole and Firmage 1984; Smith and Snow 1976).

Germination trials

In each of the 20 populations studied for phenotypic traits, we collected four mature but closed capsules from 10 random individuals. *Sphagnum* mosses were collected from each site and then thoroughly mixed together to serve as a germination substrate (Diez 2007). Seeds from the same individual were pooled, and *ex situ* germination trials were conducted. See Lemay *et al.* (2015) for a detailed description of the

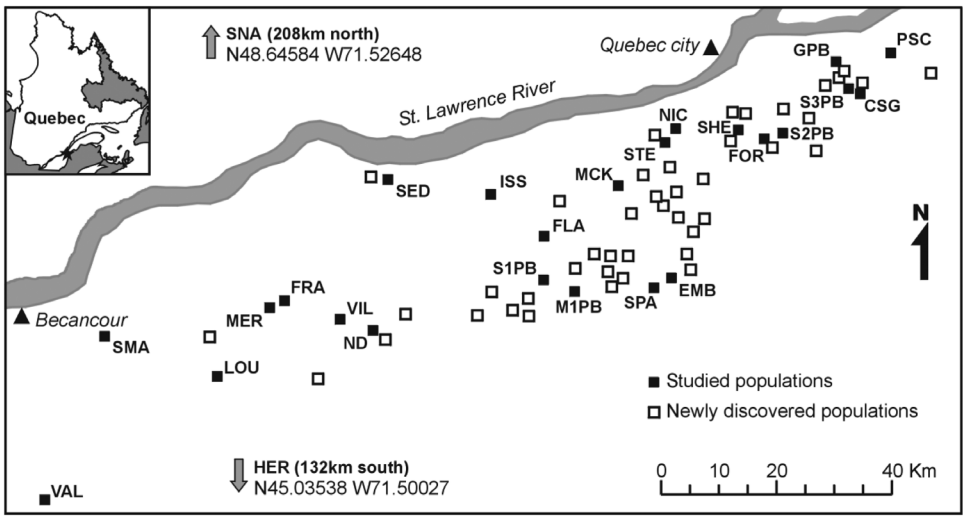

Figure 1: study area. Studied populations are indicated by filled squares (n = 24 on the map), excluding populations SNA and HER located 208 km north and 132 km south of the study area, respectively. Coordinates of these distant populations are given on the map. The 20 populations used for phenotypic analysis are each labeled with a three-letter code. These codes are used in Fig. 2 and in Tables A1, A2 and A3 (supplementary appendix 2). Four populations have a four-letter code and were only used for genetic analyses. To get accurate estimates of population isolation, we checked for presence of *Platanthera blephariglottis* in surrounding peatlands and identified 46 unarchived populations (open squares). Quebec and Becancour cities are represented by gray triangles.

protocol. About 100 seeds per individual were assessed for a total of 19 050 seeds. Population averages were calculated from individual germination rates.

Population isolation and environmental factors

To quantify geographic isolation among our 24 populations, we searched for the presence of undetected populations by visiting all surrounding peatlands with at least 10 ha of suitable *P. blephariglottis* habitat (following Poulin *et al.* (2002) and Laroche *et al.* (2012)). Fifty-seven additional peatlands met the habitat criterion and field visits revealed that *P. blephariglottis* was present in 46 of these peatlands. While this approach may have missed some populations in peatlands smaller than 10 ha, our extensive knowledge of these sites (Laroche *et al.* 2012; Poulin *et al.* 2002) has shown that such peatlands seldom contain orchids due to a lack of proper habitat. The degree of isolation of each studied population was estimated as (i) the linear distance to the nearest population, hereafter referred to as the nearest neighbor (NN), and (ii) the number of populations within each buffer zones of 2, 5 and 10 km around each studied population. (supplementary appendix 2: Table A1). For each population, the peatland area was calculated. We characterized plant communities using the same method as Laroche *et al.* (2012). These communities were considered to reflect environmental factors potentially affecting phenotype and were characterized to confirm that sampled populations of *P. blephariglottis* had similar environments.

Statistical analyses on phenotypic variation, germination rates and plant community composition

To summarize phenotypic traits, we conducted two principal component analyses (PCA): one on flower traits and a second on plant traits (Table 1). Missing values for the length of the longest leaf were imputed after confirming with a chi square test and Markov chains that missing data due to grazing (28%) were randomly distributed throughout the data set ($P = 0.239$). To test for differences in phenotype and germination among populations, an ANOVA was performed on the mean PCA scores of each population and on germination results using the SAS MIXED procedure, which accounts for data heterogeneity using the group option of the repeated statement (SAS Institute, Inc. 2011). To assess the influence of landscape parameters (buffers, NN, area) on phenotype and germination, a regression model with populations as random factor was conducted in the SAS MIXED procedure.

Even though sampling sites were chosen to minimize variability in plant communities and therefore ensure similar environments between sampled populations, we assessed whether orchid phenotypic variability could be due to among-site variation of *in situ* plant communities. Species cover data were Hellinger-transformed to account for double absences (Legendre and Gallagher 2001). A PCA was conducted on plant species composition (species cover) and we conserved enough principal components to reach a minimum of 50% cumulative proportion of variance explained. The influence of plant species composition on population traits (mean of the PCA vectors) and on germination was assessed by a multiple linear regression model in R (R Development Core Team 2014). Since all sites had at least hundreds (usually thousands) of individuals (supplementary appendix 2: Table A1), and because we investigated genetic distinctiveness and distances rather than genetic diversity, population size was not included in statistical analyses.

DNA extraction, sequencing and sequences processing

Individual DNA extractions were conducted using the Qiagen (Venlo, NL) DNeasy 96 plant kit (cat. no. 69181) using 15 mg of dried material. DNA was quantified using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and DNA quality was checked on 1% agarose gels.

Table 1: list of phenotypic traits of studied populations and results of the PCA on flowers and plants traits

	Component			Component	
	1	2		1	2
Flower traits	Size	Shape	Plant traits	Size	Foliage
Variability explained (%)	40.77	15.07	Variability explained (%)	51.23	17.27
Central sepal length	0.45	−0.16	Inflorescence height	0.39	−0.17
Central sepal width	0.41	0.23	Inflorescence size	0.47	−0.12
Lateral petal length	0.42	−0.23	Number of flowers	0.49	0.03
Lateral petal width	0.26	0.60	Number of leaves	0.01	0.68
Column width	0.34	0.19	Longest leaf length	0.18	−0.59
Labellum length	0.39	−0.33	Longest leaf width	0.39	0.32
Labellum width	0.22	0.44	Stem diameter	0.45	0.19
Spur length	0.27	−0.42			

Data show the degree of correlation of studied traits with the component, with 1 and −1 being the highest possible correlations and 0 the lowest correlation. The PCA components were associated with a size and a shape effect for the flower traits, and a size and foliage effect for the plant traits.

For the 26 studied populations, population pools were prepared at a concentration of 20 ng/µl, by mixing equal amounts of DNA extracted from each of the 24 sampled plants. Pool replicates were made from the same individual DNA extracts for four populations to test the accuracy of the method. A *Pst*I/*Msp*I enzyme combination was used to prepare a 30-plex GBS library for a genomic analysis platform (Institut de Biologie Intégrative et des Systèmes (IBIS), Laval University, Quebec City, QC, Canada) according to Elshire *et al.* (2011) and Poland *et al.* (2012). The resulting GBS library was sequenced on a single lane of an Illumina HiSeq2000 (San Diego, CA) at the McGill University-Génome Québec Innovation Center (Montreal, QC, Canada). Finally, allelic frequencies were estimated as the fractions of reads supporting one allele versus another.

To validate our DNA-pooling approach, we individually genotyped 15 plants from two populations as well as a DNA pool of the same individuals for these two populations. These 32 samples were prepared as described above and sequenced as part of a 93-plex library with samples from an unrelated study. This allowed verifying that the allelic frequency estimates obtained with the DNA-pooling approach were in agreement with those obtained by individual level genotyping.

Given that no reference genome for *P. blephariglottis* was available, we used the UNEAK pipeline (Lu *et al.* 2013) from TASSEL v3.0, (Bradbury *et al.* 2007) to discover and call variable sites (single nucleotide polymorphisms, SNPs). We used a minimum minor allele frequency (mnMAF) of 0.05 and minimum call rate (mnC) of 0.80. Using Microsoft Excel (2011 for Mac v14.4.5), we then performed pairwise comparisons of allelic frequencies between genotypes from the four pools and their replicate at different minimum coverage thresholds to identify a filtering strategy that maximized the reliability of the DNA pooling approach. Based on these comparisons, we performed additional filtering in Excel to remove sites that had a mean coverage depth of <48 reads, as well as every SNP that had a score of 127 for both alleles in one or more pools (because the signal would be saturated since the UNEAK pipeline is capped at 127 reads/allele).

Genetic analyses and isolation-by-distance

To evaluate the genetic distinctiveness of populations, allelic frequencies were used to generate a distance matrix between populations based on Nei's genetic distance (Nei 1972) using the R package 'ade4' (Dray and Dufour 2007). A UPGMA phenogram was then built from the resulting distance matrix using the 'cluster' R package (Maechler *et al.* 2014). We tested for the optimal number of clusters by comparing the original distance matrix to binary matrices computed from the phenogram cut at various levels in order to choose the level where the matrix (Mantel) correlation was the highest (Borcard *et al.* 2011). In addition, we tested for an isolation by distance pattern by means of redundancy analysis (RDA) of the Cartesian coordinates on the Hellinger-transformed allelic frequencies. The Hellinger transformation of allelic frequencies was used to avoid giving small genetic distances between populations based on the shared absence of alleles (Legendre and Gallagher 2001). Statistical significance was evaluated using 10 000 permutations. Distant populations (SNA and HER) were excluded from the isolation by distance analysis since we were mainly interested by the genetic structure within the studied area.

We investigated whether phenotypic differences between populations were linked to genetic differences while controlling for geography. To do so, a PCA was conducted on the Hellinger-transformed allelic frequencies (Legendre and Gallagher 2001) and enough principal components kept to explain >90% of the variance of genetics data. The resulting PCs that best explained the variability of the phenotypic data were then selected by backward selection using the 'ordistep' function of the 'vegan' package in R. This function selects variables to create the model with the highest possible adjusted coefficient of determination. Partial RDAs were then conducted, using phenotypic variables as the response matrix, the selected genetic PCs as the constraining matrix and the Cartesian coordinates as the conditioning matrix. These partial RDAs allowed us to calculate the proportion of the phenotypic variance explained by the genetic PCs, after removing the proportion of variance explained by Cartesian coordinates. This procedure (selection of genetic PCs, followed by partial RDA) was performed on the whole phenotypic dataset, but also on each phenotypic variable individually (a partial RDA with one response variable is in fact a partial regression), i.e. on the mean phenotypic PCA scores of each population and on germinations means (reduced and centered). Analyses of variance (ANOVA, 10 000 permutations) were conducted to assess the global significance of these partial RDAs.

Evolutionary distinctiveness (ED; Kembel *et al.* 2010) values were also estimated for each population (excluding the two distant populations HER and SNA). The ED metric provides an estimate of a species' or population's uniqueness based on the branch lengths in an evolutionary tree. We also used SplitsTree4 vers. 4.13.1 (Huson and Bryant 2006) to build a phylogenetic network using the Neighbor-Net method and calculate a Shapley metric value (Volkmann *et al.* 2014). The SH metric is an independently derived approach based on Game Theory (Haake *et al.* 2008) that explicitly considers the species' (or population's) individual contribution to future diversity. While similar to the ED, its value is based on phylogenetic networks, which likely provide more accurate estimates of the population's genetic distinctiveness than ED values, which are based on a dichotomous tree (Volkmann *et al.* 2014). To test whether population isolation was linked to its genetic distinctiveness, a relation between both ED and SH values and geographic parameters (buffers, NN, area) was investigated by looking for significant correlations using the SAS CORR procedure.

RESULTS

Phenotypic variation, germination rates and community composition

A total of 56% and 68% of flower and plant phenotypic variability respectively, could be represented by the first two

axes of the PCAs on flower traits and plant traits. For flowers, all traits were positively correlated along the first eigenvector (Table 1). This can be attributed to a size effect that implies that populations with a higher value for this vector have larger flowers. Along the second eigenvector, all length traits were correlated together and opposed to all width traits (Table 1), representing a shape effect. Consequently, populations with a higher value for this vector had shorter and broader flowers, while populations with a lower value had elongated flowers. For plant traits, the first eigenvector also revealed a size effect (Table 1): populations with a higher value had larger plants with more flowers than those having a lower value. Along the second eigenvector, the number of leaves was opposed to the length of the longest leaf, which for *P. blephariglottis* is always at the base of the plant, with leaves decreasing upwards along the stem. Consequently, this vector indicates a foliage effect: populations with a higher value had bushier plants with many short leaves, whereas populations with a lower value were comprised of individuals with fewer but longer leaves. For all four selected eigenvectors, populations differed significantly in terms of flower size ($F_{19,505}$ = 12.20, $P < 0.0001$), flower shape ($F_{19,505}$ = 13.36, $P < 0.0001$), plant size ($F_{19,418}$ = 5.30, $P < 0.0001$) and plant foliage ($F_{19,460}$ = 9.95, $P < 0.0001$). Germination ranged from 8% to 98% for individuals and from 37% to 83% for populations (supplementary appendix 2: Table A2), with significant differences among populations ($F_{19,50.1}$ = 3.64, $P < 0.0001$).

Differences among populations in terms of phenotypic traits and germination were not linked to peatland area (Mixed Procedure, all $P > 0.05$, Table 2). Additionally, for all phenotypic and germination variables, there was no significant effect of local plant communities (multiple linear regression results of four principal components representing 56% of the variance in plant communities, 15 df: flower size r^2_{adj} = −0.03, P = 0.50; flower shape r^2_{adj} = −0.12, P = 0.73; plant size r^2_{adj} = −0.11, P = 0.72; plant foliage r^2_{adj} = −0.09, P = 0.66; germination r^2_{adj} = 0.19, P = 0.92), supporting our initial assumptions.

Impact of population isolation on phenotypes and germination rates

Germination rate and foliage traits were negatively correlated with distance to the NN, meaning that the shorter the distance between a peatland and its closest neighbor, the higher the germination rate and the bushier the plants. The shape of flowers, size of plants and rate of germination were all significantly influenced by the number of populations within the landscape (Table 2). More precisely, the shape of flowers was positively correlated to the number of neighbor populations in the landscape, regardless of the scale considered (2-, 5- or 10-km buffer zone). The germination rate was positively correlated with the number of neighbor populations in the landscape only at the two larger scales (5-km buffer, r = 0.3, P = 0.0039; 10-km buffer, r = 0.32, P = 0.0016) and plant size was negatively correlated with the number of neighbor populations in the landscape at the two smaller scales (5-km buffer, r = −0.26, P = 0.0052; 10-km buffer, r = −0.22, P = 0.0211).

Genetic analyses and isolation by distance

Genetic details such as the number of reads, SNPs and coverage are described in supplementary appendix 3. Similarity of allelic frequencies among replicates for the four populations tested was very high ($r \geq 0.96$) and was greater than any similarity among populations (supplementary appendices 3, 4 and 5). Moreover, the allelic frequencies calculated by the individual genotyping of 15 plants were highly correlated with allelic frequencies obtained with the DNA pooling approach for the two populations investigated (r = 0.97 and r = 0.95, supplementary appendices 3 and 6). Thus, we conclude that population DNA pooling combined with a GBS approach provides reliable allelic frequency estimates.

The cluster analysis did not reveal any significant grouping within the study area. The optimal number of clusters was two, with HER distinct from all other populations (Fig. 2A). Removing distant populations (SNA, HER) did not reveal further structure (supplementary appendix 7). The pattern obtained by the phylogenetic network approach was similar to that of the phenogram approach (Fig. 2). Again, the two distant populations SNA and HER were located on long branches, external to the network and the three most geographically isolated populations (SMA, VAL and SED) remained more distinct from the others according to both clustering approaches. We obtained a significant relationship between genetic distances and geography (ANOVA following RDA: r^2_{adj} = 0.102, $P < 0.0001$), suggesting a pattern of isolation by distance. In addition, all phenotypic variables were linked to genetic distances while controlling for geography (supplementary appendix 8), including the global model containing all phenotypic variables (ANOVA following partial RDA: r^2_{adj} = 0.275, P = 0.002).

The ED calculated from UPGMA clustering was significantly and positively correlated to the distance from the NN (r = 0.46; P = 0.04, Table 2). Similarly, ED was negatively correlated to the number of populations within the 2 and 5-km buffers: populations with more neighbors in a 2- and 5-km radius were more similar genetically compared with all other studied populations. SH values resulted in correlations very similar to ED values (r = 0.94), with P-values nearing significance for the 5 km buffer zone (Table 2).

Discussion

We investigated the phenotypic variation, germination rate and genetic distinctiveness of *P. blephariglottis*, an indicator species of peatlands in southeastern Canada. Although this species is locally abundant with populations generally larger than 1000 reproductive individuals, we found that differences in phenotypes among populations and a decrease in germination rates were significantly linked to population isolation. We also found that isolated populations were more genetically

Table 2: results of the MIXED procedure (phenotype) and CORR procedure (genetic) linking differences to isolation parameters and to peatland area

		Area		NN		2-km Buffer		5-km Buffer		10-km Buffer	
	n	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
Flower size	1440	−0.12	0.160	0.10	0.225	−0.01	0.877	−0.02	0.8295	0.11	0.1979
Flower shape	1440	0.03	0.727	−0.16	0.0594	0.24	0.0006*	0.28	<.0001*	0.19	0.0146*
Plant size	480	−0.01	0.934	0.15	0.1375	−0.26	0.0052*	−0.22	0.0211*	−0.11	0.2867
Plant foliage	480	−0.08	0.532	−0.36	0.0001*	0.20	0.0883	0.19	0.1185	0.21	0.0664
Germination rate	200	−0.06	0.645	−0.25	0.0279*	0.22	0.0606	0.30	0.0039*	0.32	0.0016*
ED	24	−0.05	0.845	0.46	0.0425*	−0.46	0.0424*	−0.52	0.0179*	−0.40	0.084
SH	24	0.06	0.808	0.37	0.1053	−0.31	0.1768	−0.44	0.0552	−0.39	0.0922

Area refers to the peatland area. NN stands for distance to the NN and X-km buffer refers to the number of populations of *Platanthera blepharigllottis* within a radius of X km. ED is the *Evolutionary Distinctiveness* and SH is *Shapley* value.
*$P < 0.05$.

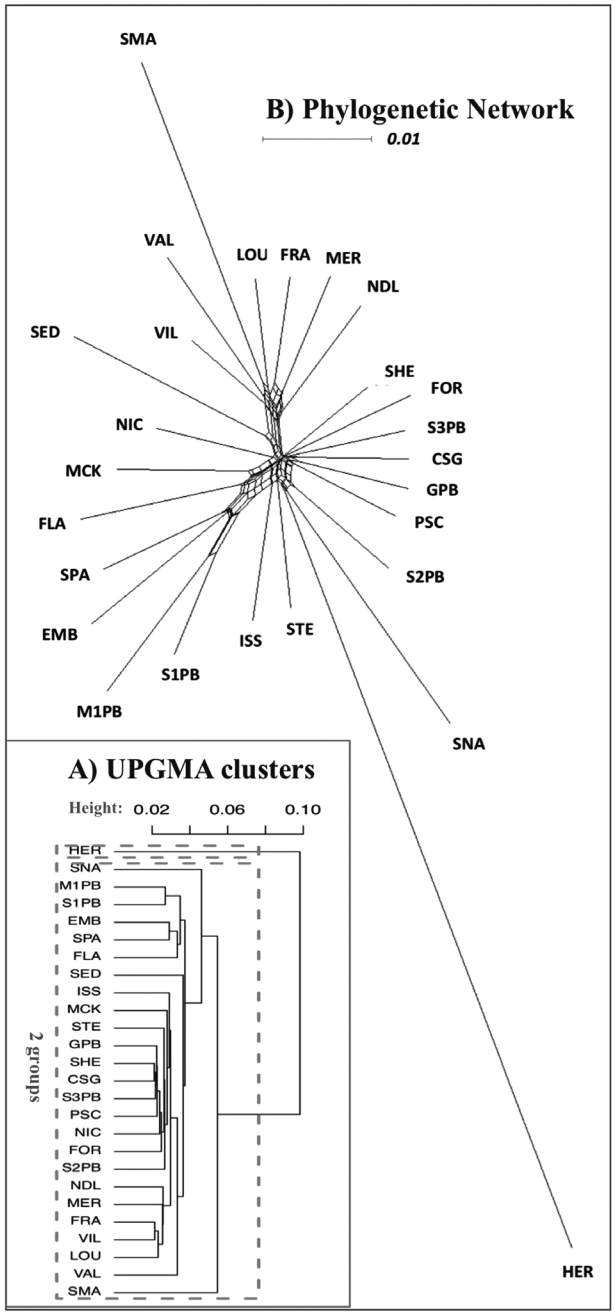

Figure 2: (**A**) Phenogram of populations built using UPGMA algorithm from Nei's genetic distance. The optimal number of clusters (dashed lines) was determined to be two. (**B**) Phylogenetic network estimated with the Neighbor-Net method. SNA and HER stand for the two distant populations (SNA: 208 km north and HER: 132 km south, see Fig. 1).

distinct and that genetic distance was linked to geography, supporting a pattern of isolation by distance. Consequently, isolation was found to have an important impact on populations. At the same time, these results imply that, even for a naturally fragmented ecosystem, gene flow among patches is important and likely keeps populations connected, as

evidenced by the pattern of isolation by distance. This gene flow seems to influence germination rate, which is a component of fitness. This has important consequences from a conservation perspective, as preserving a few isolated populations, even the ones in peatlands of high integrity, may not be an optimal strategy to ensure long-term survival of populations. While this is known for recently fragmented ecosystems like forests, our study emphasizes that even naturally patchy ecosystems are sensitive to isolation.

Impact of population isolation on phenotypes and germination rate

Population isolation had a significant impact on phenotypes and germination rates (Table 2). Differences can generally be expected in studies that extend over great distances, such as the investigation by Hardin (1961) that identified a gradient in *P. blephariglottis* populations, from a small to a large form, in a southerly direction from Maine to North Carolina (USA). Here, we observed many differences in plant size, foliage and flower shape over a much smaller area, with merely 122 km between the two most distant populations. Our study thus shows that the phenotype of *P. blephariglottis* can vary not only across its distribution range but also regionally over relatively short distances.

Population isolation is likely the main factor explaining local differences among populations. Laroche *et al.* (2012) found very low environmental variation in high-density populations of *P. blephariglottis* in our study area. This is supported by our results on the absence of effect of vegetation communities, which are known to reflect local conditions such as water table level and minerotrophy (Gignac *et al.* 1991, 2004; Vitt and Slack 1984), on the phenotypic variations observed. Smith and Snow (1976) also found no difference in the relation between capsule set and vegetation surroundings, suggesting that pollinators were able to detect flowers of *P. blephariglottis* regardless of the immediate surrounding habitat. Finally, the surface area of the peatlands we studied was not linked to any phenotypic differences in our analysis. Plasticity could be implied for some phenotypic differences but it would require significant differences in environmental characteristics, which are unlikely to occur among the studied populations based on the absence of an effect of vegetation communities. While a more extensive analysis of environmental parameters might identify such environmental differences, we believe, based on the available data, that phenotypic differentiation among populations is probably not linked significantly to differences in local environmental conditions, but is more likely due to a population isolation effect.

Flower shape, plant size, plant foliage and germination were all linked to at least one parameter of isolation (Table 2). Additionally, the apparent link between phenotypic differences and genetic distances may imply that some populations are sufficiently isolated and gene flow sufficiently reduced for local differentiation to occur. Drift over a long period of time and differential selective pressures are two non-mutually exclusive explanations for phenotypic differentiation in isolated populations (Willi *et al.* 2007). Because of its stochastic nature, drift may result in phenotypes that have no ecological explanation. On the other hand, unstudied environmental parameters linked to isolation such as a reduction in the abundance and diversity of pollinators could drive selective pressure. Consequently, we cannot determine at the present time whether differences found in our study are due to drift or adaptive selection. This would require further detailed analyses, including common garden experiments (Hooftman *et al.* 2003), to decipher the adaptive role of trait variation.

Germination rates of *P. blephariglottis* dropped significantly with increasing geographic isolation, suggesting that even for a species occurring in a naturally patchy ecosystem, isolation may lead to inbreeding depression, perhaps through the accumulation of recessive deleterious alleles (Lynch *et al.* 1995). Germination trials may be more valuable than phenotypic trait measurements to guide conservation practices, as they represent a component of fitness. Our findings are in line with observations for another peatland specialist, *S. perennis*, whose isolated populations were shown to suffer from a reduction in fitness (Lienert *et al.* 2002). Our results also concur with studies linking geographic isolation with reductions in genetic diversity and fitness at the population or metapopulation level (Aguilar *et al.* 2006, 2008; Bossuyt 2007; Vandewoestijne *et al.* 2008; Young *et al.* 1996).

Genetic evidence of isolation

A significant pattern of isolation-by-distance (IBD) was found, indicating that gene flow occurs among geographically close populations, yet diminishes as distance increases. Connectivity among sites was also confirmed by the fact that values of ED increased as the number of populations in the surrounding landscape declined and as the distance to the nearest neighbor rose. In the studied populations, there is thus a clear tendency for genetic distinctiveness to increase with isolation.

Gene flow is likely limited by pollen dispersal and corresponds to flight estimates for common pollinators of the white fringed orchid that belong to the genus *Hemaris* (*Sphingidae* family). These sphinx moths are considered strong fliers, yet travel no further than 10 km between food sources (Janzen 1984). Moreover, even though *P. blephariglottis* produces dust seeds, that can putatively travel long distances (Eriksson and Kainulainen 2011), similarly to what has been shown also for *Sphagnum* spores (Sundberg 2013), studies that investigated dispersal range of orchid dust seeds showed that they mostly fall within a few meters of the mother plant (Jacquemyn *et al.* 2007; Jersáková and Malinová 2007; Machon *et al.* 2003). In addition to the lack of evidence for sustained long-distance gene flow, a pattern of isolation by distance was observed for plants growing in naturally patchy ecosystems, such as alpine landscapes (Gaudeul *et al.* 2000; Kuss *et al.* 2008; Stöcklin *et al.* 2009) and sandstone outcrops (Albrecht *et al.* 2014). Consequently, our results tend to support earlier

studies, demonstrating that seed dispersal over several kilometers, while theoretically possible, does not occur frequently enough to avoid patterns of IBD.

Estimation of genetic distances in this study greatly contributed to our understanding of the structure and functioning of the system. It highlighted a pattern of isolation by distance between peatlands, suggested that the presence of neighboring populations reduces the genetic distinctiveness of populations and allowed the investigation of links between phenotypic and genetic variation. Even though sequencing costs are constantly diminishing, genotyping of dozens of individuals per population for several populations remains expensive. In this study, we demonstrated that GBS and DNA pooling per population represents an accurate approach at a fraction of the cost of individual genotyping, supporting previous findings based on simulations (Ferretti *et al.* 2013) and experimental designs (Gautier *et al.* 2013). As next-generation-sequencing continues to improve at a rate exceeding Moore's law in computer science (Hayden 2014), we expect that genetic approaches such as GBS will be increasingly adopted in the context of ecological studies and conservation biology.

Further considerations

Our study illustrates the importance of considering naturally patchy ecosystems, such as peatlands, as interconnected networks. Further fragmentation of these systems could lead to negative impacts from isolation, as is the case with recently fragmented ecosystems, since long-distance dispersal events will likely be too rare to avoid genetic differentiation and inbreeding depression. Since geographical isolation can have such a major impact on genetic and phenotypic differences as well as germination rates in *P. blephariglottis*, it is conceivable that other plant species, even when occurring in populations of hundreds of individuals, could be impacted by population isolation. More worrisome, plant species that rely on few pollinators or produce seeds with little dispersal potential are likely to be even more strongly affected by further habitat loss (Kolb and Diekmann 2005). Thus, it is imperative that future conservation programs consider the interconnected nature of patchy ecosystems, rather than focusing on preserving a few specific sites with exceptional character.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology* online.

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