OUTCROSSING MATING SYSTEM OF THE EARLY-DIVERGENT MOONWORT FERN (*BOTRYCHIUM LUNARIA*, OPHIOGLOSSACEAE) REVEALED IN THE EUROPEAN ALPS

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Premise of research. Vascular plants depend on sexual recombination for generating new genetic variability to meet environmental needs. Nevertheless, members of the early-divergent fern genus *Botrychium* (Ophioglossaceae) typically maintain gametophytic selfing and show strong inbreeding within populations. To explain this evolutionary anomaly, the existence of previous or current but undiscovered outcrossing, genetically rich precursors of the existing genetically depauperate taxa, has been hypothesized.

Methodology. Using allele expression at 13 independently assorting enzyme loci, we compared allelic diversity and levels of heterozygosity in 471 *B. lunaria* individuals across 16 populations in the Alps and Jura Mountains of Switzerland. We examined habitat characteristics influencing mating systems and investigated population genetic structure on the basis of a discriminant analysis of principal components (DPAC) and a graph-theoretic framework. We tested the pattern of isolation by distance and explored the biological processes favoring or limiting spore dispersal at a regional scale.

Pivotal results. We found high genetic diversity within and among populations and similar observed heterozygosity (H_O) and expected heterozygosity (H_S) across loci ($H_O = 0.020$ – 0.450 and $H_S = 0.040$ – 0.590 , respectively). Estimates of H_O (0.050 – 0.272) were lower than those of H_S (0.144 – 0.305) across individuals, indicating a weak deficit in heterozygotes. Mean values of departure (D) from expected heterozygosity were close to what would be expected with random mating ($D_L = 0.061$ and $D_P = 0.059$ across loci and populations, respectively). The mean inbreeding coefficient (F_{IS}) was low ($F_{IS} = 0.247$), and the overall genetic differentiation (F_{ST}) was moderate (global $F_{ST} = 0.083$).

Conclusions. Together, the DPAC and the population graph revealed a complex population genetic structure and weak genetic differentiation, with substantial gene flow among most populations. One population (Arosa), sampled from a site recently released from permafrost, demonstrated a probable founder effect not correlated with geographical isolation. The topology of the population graph identified three subgroups with several key populations (Bosco Gurin, Chandolin Low, Grand St. Bernard, San Bernardino, and Val Roseg) that maintained genetic connectivity of populations from distant locations.

Keywords: dispersal, gametophytic selfing, Last Glacial Maximum, mating system, sporophytic outcrossing, early vascular plants.

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Introduction

Diverse reproductive modes among vascular plants have played a key role in the transmission of their genetic diversity during their evolutionary history (Hamrick and Godt 1996; Barrett 2002). Among early-divergent vascular plants, ferns exhibit diverse and unique life cycles that have strongly sculpted

genetic variability and population genetic structure among species (Haufler et al. 2016).

Homosporous ferns have three possible sexual mating systems: sporophytic outcrossing, sporophytic selfing, and gametophytic selfing. The third mating system, which results from the fusion of genetically identical egg and sperm, is exclusive to homosporous ferns and lycophytes, most of which have the potential for producing bisexual (hermaphroditic) gametophytes (Soltis and Soltis 1987; Haufler et al. 2016; Sessa et al. 2016). Gametophytic selfing is an extreme form of inbreeding that produces completely homozygous sporophytes in a single generation (Klekowski and Lloyd 1968). Although inbreeding and

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genetic drift are often perceived as detrimental, selfing may confer key evolutionary abilities for the dispersal and colonization of homosporous lineages (Ranker and Haufler 2008; Wubs et al. 2010; Haufler et al. 2016).

The genus *Botrychium* Sw. (Ophioglossaceae) is a classic example among early-divergent homosporous ferns, with a predominance of gametophytic selfing for the majority of its species (Soltis and Soltis 1992; Hauk and Haufler 1999) and sporophytic

outcrossing for a restricted number of species (*B. campestre* var. *lineare* (W. H. Wagner) D. R. Farrar; Farrar and Gilman 2017). Bisexual gametophytes of *Botrychium* are underground (Wagner et al. 1985) and nonphotosynthetic and are colonized by arbuscular mycorrhizal fungi of Glomeromycota (Winther and Friedman 2007), which not only provide minerals and water but also provide carbohydrates and other organic products from nearby vascular plants to which the fungi are also connected (Berch and

Fig. 1 Sampling sites of *Botrychium lunaria* populations in Switzerland. A, Populations sampled in the Swiss Alps and Jura Mountains for allozyme investigation. B, Morphology of *B. lunaria*, with aboveground sterile and fertile parts. C–E, Sexual development stages displayed by outcrossing *B. campestre*, with the initial first development of either female (C) or male (D) gametangia followed by a mix of both sexes (E). ARO = Arosa; BER = Bernina; BGU = Bosco Gurin; CHA = Chasseral; CHH = Chandolin High; CHL = Chandolin Low; CHO = Chasseron; GOP = Gotthard Pass; GRP = Grimsel Pass; GSB = Grand St. Bernard; LAA = Laax; SBE = San Bernardino; SIM = Simplon; VDC = Val di Campo; VRO = Val Roseg; ZER = Zermatt.

Kendrick 1982). Young stages of gametophytes initially display male or female gametangia, followed by continuous production of a mix of male and female gametangia (see fig. 1). This close proximity of mature male and female gametangia on the same gametophyte likely promotes selfing because of the low probability of fertilization by sperm swimming through soil from a different gametophyte. Several species (*B. campestre* W. H. Wagner & D. R. Farrar, *B. pallidum* W. H. Wagner, and *B. pumicola* Colville) also have vegetative reproduction via gemmae produced in the leaf axils of underground stems, which provides an alternative for overcoming the difficulties of cross-fertilization as well as selfing mating systems (Farrar and Johnson-Groh 1990).

Botrychium lunaria has a broad circumtemperate and circumboreal distribution in the Northern Hemisphere and is widespread in mountainous areas of Europe, where it occurs in alpine meadows, recently deglaciated areas, and human-disturbed soils (e.g., road shoulders and ski slopes; Farrar 2011). Previous studies investigated the genetic diversity and phylogenetic relationships among taxa of the *Lunaria* species complex on the basis of a worldwide sampling (Dauphin et al. 2014, 2017, 2018; Stensvold and Farrar 2017) that supported the monophyly of the *Lunaria* clade and seven well-supported subclades, including six named taxa. In addition, a regional study highlighted the large haplotype diversity found in the *Lunaria* clade, suggesting an ancient demographic history in the central European Alps during several glacial and interglacial periods of the Quaternary (Maccagni et al. 2017).

Two scenarios about the demographic fate of European alpine species during the Last Glacial Maximum (LGM) are often discussed (Stehlik 2000; Taberlet and Cheddadi 2002)—either the tabula rasa scenario (Chodat and Pampanini 1902), in which case no populations persisted in the Central Alps, but some survived in peripheral areas that were able to recolonize the alpine landscape afterward, or the nunatak scenario (Brockmann-Jerosch and Brockmann-Jerosch 1926), in which populations survived in restricted ice-free locations above the trimline (i.e., maximal altitude of the ice sheet) in the Central Alps and thereafter spread out to the valleys during deglaciation. A phylogeographical study of *B. lunaria* has revealed three peripheral putative refugia in the Alps that harbored alpine populations during the LGM (Maccagni et al. 2017), which supports the second scenario as the more likely—although the number of specimens analyzed was limited (two or three plants per population) for assessing genetic diversity metrics. Palynological data attest to the presence of *B. lunaria* in the Central Alps (Lake Fully, Valais, Switzerland) during at least the Holocene, with a demographic expansion 2000 yr ago characterized on that highland plateau (Finsinger and Tinner 2007). Also, it was reported that several locations in the Central Alps were ice-free at moderate elevations (2000–2600 m) during the LGM (Kelly et al. 2004), especially on the south-exposed slopes, where the trimline was generally located lower than on north-exposed flanks: 2300 m at the Gotthard Pass (GOP; Hippe et al. 2014), 2290 m at the Grimsel Pass (Wirsig et al. 2016), and 2500 m at the Simplon Pass (Dielforder and Hetzel 2014).

In this study, we addressed key evolutionary issues for the population genetics and mating systems of early-divergent ferns. We used the broadly distributed fern *B. lunaria* as a model to infer the predominant breeding system maintained in highly heterogeneous alpine habitats. Moreover, we examined habitat

characteristics influencing mating systems and explored the potential consequences of the LGM on genetic structure and diversity in the Alps. We further investigated the intensity of gene flow between populations and the key biological processes favoring or limiting spore dispersal at a regional scale. Finally, we discuss our results for population genetic structure in the context of the postglacial recolonization, with a focus on the species' ecological features putatively enabling in situ survival during the LGM.

Material and Methods

Sampling

We sampled 16 populations of *Botrychium lunaria* in the Alps and Jura Mountains (fig. 1), covering its main biogeographical and altitudinal (1550–2700 m asl) range in Switzerland (table 1). Population sizes, approximated from the number of emergent sporophytes, varied from 80 to more than 1000 individuals. The number of plants sampled per population ranged from 24 to 40, for a total of 471 sporophyte plants collected (table 1). Fresh leaves were stored in cold conditions during transport from the field to the electrophoresis laboratory at Iowa State University. All genotyped specimens were individually numbered, and vouchers (i.e., 471 plants) were deposited at the Ada Hayden Herbarium (ISC, Iowa State University) for morphological study.

Enzyme Electrophoresis

Allozyme data were generated following the procedures of Stensvold and Farrar (2017). As soon as possible after collection, 1 cm of tissue was cut from the base of the leaf stalk and crushed in a buffer solution containing phosphate-polyvinylpyrrolidone (Cronn et al. 1997). The grindate was stored for up to 12 mo in microcentrifuge tubes at -80°C until electrophoresis was conducted. Enzyme electrophoresis was performed in horizontal 12% starch gel using the three buffer systems, 7, 9, and 11, from Soltis et al. (1983) to reveal allelic variants at 22 loci of 10 enzyme systems by differentiating gel migration patterns. Thus, loci of those enzyme systems were resolved for aspartate aminotransferase (AAT) and triose phosphate isomerase (TPI) with buffer system 7; for malate dehydrogenase (MDH), phosphoglucumutase (PGM), phosphogluconate dehydrogenase (PGD), and phospho-glucosomerase (PGI) with buffer system 9; and for aconitase, diaphorase (DIA), isocitrate dehydrogenase (IDH), and shikimate dehydrogenase (SKDH) with buffer system 11. The stain recipes used followed Soltis et al. (1983).

Allele letter codes (i.e., scoring) were assigned with reference to alleles previously determined for *Botrychium* taxa of the *B. lunaria* complex by Stensvold and Farrar (2017). The consistency of the scores was checked by multiple runs of the same specimens when necessary. Allele variants for each locus were numbered, assigning the lowest number to the fastest (most anodal) migrating allele. In analyses, we removed rare alleles (population frequencies, <0.05) to avoid artificial inflation of polymorphism estimates due to experimental error, as recommended by Hartl and Clark (1998), and excluded uninformative loci that were monomorphic across all populations. Loci with missing scores in more than 30% of samples were excluded.

Table 1
Sampling Locations and Genetic Diversity among Swiss *Botrychium lunaria* Populations

Code	Locality	Longitude ^a	Latitude ^a	Elevation (m) ^b	No. individuals	No. loci	No. polymorphic loci	Polymorphic loci (%)	No. alleles across loci
ARO	Arosa	9.63885	46.78957	2644	24	12	5	42	19
BER	Bernina	10.04977	46.41120	2066	29	13	11	85	29
BGU	Bosco Gurin	8.47011	46.31574	1911	29	13	10	77	25
CHA	Chasseral	7.04625	47.12800	1552	29	13	10	77	27
CHH	Chandolin High	7.62394	46.25086	2513	40	13	13	100	32
CHL	Chandolin Low	7.60002	46.25330	2071	37	13	11	85	29
CHO	Chasseron	6.53919	46.85266	1574	29	12	10	83	25
GOP	Gotthard Pass	8.56179	46.59290	1788	26	12	7	58	23
GRP	Grimsel Pass	8.35811	46.56677	2067	26	12	11	92	28
GSB	Grand St. Bernard	7.19378	45.90279	1905	30	13	10	77	28
LAA	Laax	9.22446	46.86409	2204	26	11	9	82	23
SBE	San Bernardino	9.17264	46.48341	1944	26	12	10	83	26
SIM	Simplon	8.03214	46.24785	2029	40	13	13	100	33
VDC	Val di Campo	8.87715	46.55818	1965	26	11	7	64	20
VRO	Val Roseg	9.86097	46.43056	2025	28	13	11	85	28
ZER	Zermatt	7.75168	45.99337	2534	26	12	9	75	26

Note. Location codes, geographic coordinates, elevation, number of samples, number of loci, number and percentage of polymorphic loci, and number of alleles per locus are given.

^a Geographic coordinates are given in decimal degree, WGS84.

^b Variables derived from a digital elevation model at 25 m of spatial resolution are available at <http://swisstopo.admin.ch/>.

Genetic Diversity and Mating System Characterization

To investigate genetic diversity and the predominant mating system maintained within populations, we calculated summary statistics of genetic diversity within populations using the R packages *adegenet* version 2.1.2 and *hierfstat* version 0.4.22 (Goudet 2005; Jombart 2008; R Core Team 2020): genotype frequencies, percentage of polymorphic loci, observed heterozygosity (H_O) and expected heterozygosity (H_S), overall gene diversity (H_T), amount of gene diversity among samples ($D_{ST} = H_T - H_S$), gene differentiation ($F_{ST} = D_{ST}/H_T$), and inbreeding coefficient ($F_{IS} = 1 - (H_O/H_S)$). We tested for each locus's departure from the Hardy-Weinberg equilibrium on the basis of the expected genotype frequencies calculated from allele frequencies using the χ^2 test implemented in the R package *pegas* version 0.12 (Paradis 2010).

Population Genetic Structure

We characterized population genetic structure using measures of pairwise F_{ST} differentiation and multivariate analyses that assume no a priori assumptions of a demographic model. We used the *genet.dist* function of *hierfstat* to calculate genetic differentiation between populations based on Weir and Cockerham's (1984) equation. To explore genotypic variation across all individuals, we performed a principal component analysis (PCA; Hotelling 1933) to summarize the overall variance along the three most informative axes using the *dudi.pca* function of the R package *ade4* version 1.7.13 (centered, not scaled). Then, in view of the low population differentiation, we performed a discriminant analysis of principal components (DAPC), which transforms the uncorrelated PCA variables and generates synthetic discriminant axes that maximize between-population

variation while minimizing within-population variation (Jombart et al. 2010). We conducted the DAPC with the population of each individual as prior information and used the *optim.a.score* function to define the optimal number of principal components (PCs) to retain on the basis of the α -score statistics.

Gene Flow and Pattern of Isolation by Distance (IBD)

Gene flow among populations was investigated within a graph-theoretic framework based on genetic covariance (Dyer and Nason 2004). This approach was developed to analyze patterns of population genetic structure and connectivity by integrating into a graph nodes as sampled populations and edges as multivariate genetic covariance measures between populations. Population graphs assume no a priori assumptions of a demographic model and therefore are relevant in study cases focusing on complex mating systems (e.g., mixed breeding behavior). In short, connectivity (i.e., genetic relationships among populations) is based on complex topologies that capture the structure of genetic covariance among populations; that is, an edge length is calculated as inversely proportional to the genetic covariance between a given pair of populations (Dyer 2015). Hence, the smallest number of edges that properly explains the genetic covariance structure is retained, leaving all other poorly informative edges to the overall covariance structure. We investigated the graph topology using the R package *GStudio* version 1.5.2, *igraph* version 1.2.4.2, and *popgraph* version 1.5.1 (Dyer 2016, 2017). We used the α threshold by default ($\alpha = 0.05$) and checked with a binomial test whether the number of edges was significantly less than the total number of possible edges in the graph topology (Xuerb et al. 2018). To interpret how genetic covariance is spatially structured in the context of the Alps, we mapped

the nodes and edges onto real space using the R package raster version 3.0.12 (Hijmans and van Etten 2016).

We tested for patterns of IBD using transformed geographic ($\ln$) and genetic distances ($F_{ST}/(1 - F_{ST})$; Rousset 1997) and 999 permutations in a Mantel test with the R package vegan version 2.5.6 (Oksanen et al. 2011). Geographic distances were assessed from latitude and longitude data of populations with the *distm* function of the R package *geosphere* version 1.5.10 (Hijmans et al. 2017), and genetic distances were calculated based on Nei's (1972) distance with the *dist.genpop* function of *ade4*.

Results

Enzyme Electrophoresis

After removal of inconsistently staining loci and invariant loci, we retained 13 independently assorting loci yielding results informative for breeding analyses: three loci from MDH; two loci each from AAT and PGM; and one locus each from DIA, IDH, PGD, PGI, SKDH, and TPI. Within these variable loci, a total of 35 alleles were detected at frequencies above 5% in at least one population. Allele occurrence by population is summarized in table 1. Genotype data by locus and population are available in table S1 (tables S1–S5 are available online). The percentage of missing data across loci and individuals was 9.0%. The number of expressed loci varied from 11 to 13 across populations (table 1).

Genetic Diversity and Mating System Characterization

We found high genetic diversity within and among Swiss *Botrychium lunaria* populations. The number of alleles per population varied from 19 to 33, and the percentage of polymorphic

loci ranged from 42% to 100% among populations (tables 1, S1). Values of H_O and H_S were similar across loci, with H_O ranging from 0.020 to 0.450 and H_S from 0.040 to 0.590 (fig. 2A; table S2). Estimates of H_O (0.050–0.272) were lower than those for H_S (0.144–0.305; table S3) across individuals, showing a weak deficit in heterozygotes (fig. 2B). Mean values of departure from expected heterozygosity were close to what would be expected under random mating (mean departure of 0.061 and 0.059 across loci and populations, respectively; fig. 2; tables S2, S3), but all loci significantly deviated from Hardy-Weinberg equilibrium ($P < 0.001$; table S4), suggesting an unbalanced excess and/or deficit of some heterozygotes. The mean inbreeding coefficient was low ($F_{IS} = 0.247$; table S3), with the highest value for the Arosa (ARO) population ($F_{IS} = 0.666$).

Population Genetic Structure

Overall F_{ST} was moderate (global $F_{ST} = 0.083$; $F_{ST} = 0.049$ without ARO), with ARO as the most divergent population (table S5). The first axes of the PCA summarized 9.7% and 7.8% for PC1 and PC2, respectively (fig. S1; figs. S1, S2 are available online). In total, the first three axes integrated 24.3% of the overall variance. Consistent with estimates of F_{ST} , the ARO population was segregated along the two first axes. For the DAPC, we retained eight PCs (53.6% of variance) on the basis of the α -score statistics and kept five discriminant functions (DFs) for clustering analysis. From the two first DFs, DF1 and DF2 summarized 45.5% and 17.3% of the variance, respectively (fig. 3A). The first discriminant axis (DF1) segregated the ARO population and, to a lesser extent, the GOP and Val di Campo (VDC) populations (fig. 3B), which are one northeastern and two central populations in the Alps (fig. 1). In addition, the second discriminant axis (DF2) separated the San Bernardino (SBE)

Fig. 2 Investigation of the mating system in the Swiss *Botrychium lunaria* populations. Comparison of levels of heterozygosity are given across 13 loci (A; circles) and among populations (B; squares).

Fig. 3 Population genetic structure of the Swiss *Botrychium lunaria* populations. A, Discriminant analysis of principal components (DAPC), showing genetic clusters identified with a priori sampling location information and density plots. B and C show the first and the second discriminant axes of the DAPC, respectively. ARO = Arosa; BER = Bernina; BGU = Bosco Gurin; CHA = Chasseral; CHH = Chandolin High; CHL = Chandolin Low; CHO = Chasseron; GOP = Gotthard Pass; GRP = Grimsel Pass; GSB = Grand St. Bernard; LAA = Laax; SBE = San Bernardino; SIM = Simplon; VDC = Val di Campo; VRO = Val Roseg; ZER = Zermatt; PCA = principal component analysis; DA = discriminant analysis.

population from the rest of the overall structure (fig. 3C). No more structure was detectable on additional axes.

Gene Flow and Pattern of IBD

The topology of the population graph consisted of 16 nodes and 32 edges with significant conditional genetic covariance (fig. 4A). The number of informative edges (32) was significantly lower ($P < 0.001$, 95% confidence interval = 0.19–0.36) than the maximum number of edges (120) in the given graph ($n \times (n - 1)/2$, n being the number of populations), which supports the existence of genetic structure at the metapopulation level. Three subgraphs (blue, orange, and red) were depicted in the population graph without clear geographic clustering (fig. 4B), indicating complex demographic history after glaciations. Although the Chasseron (CHO) population was geographically isolated (figs. 1, 4B), this node (purple) appeared central in how populations were connecting the three subgraphs. Two other populations, SBE and Bosco Gurin (BGU), which are relatively geographically close to each other, were central to maintaining the integrity and genetic connectivity of the metapopulation.

IBD was weak and not significant (Mantel, $r = 0.085$, $P = 0.284$; fig. S2), showing a relatively high genetic drift in the ARO population and substantial gene flow among all other populations. This lack of significance was of interest since the sampling was composed of distant populations from different biogeographical areas separated by heterogeneous habitats and major mountain massifs ranging up to 4000 m in elevation (fig. 1). Spore dispersal at both short and long geographic distances seemed to homogenize allele frequencies among populations, except for in ARO.

Discussion

Significance of Gametophytic Selfing in Homosporous Plants

Vascular plants depend on sexual reproduction for generating new combinations of genetic variability to meet environmental needs. They also depend on heterozygous shielding of initially deleterious mutations that may become advantageous in changing conditions. In homosporous vascular plants, the value of these processes is negated when sexual outcrossing is replaced by reiterative gametophytic selfing in populations founded by single spores. Allelic diversity is reduced, heterozygous shielding is lost, and the possibility of regaining allelic diversity through mutation is curtailed by selective forces imposed on homozygotes (Charlesworth and Charlesworth 1995; Goodwillie et al. 2005). Yet a prevalence of allozyme data indicates that this is the condition maintained by most taxa of the fern genus *Botrychium* and related genera of the Ophioglossaceae (McCauley et al. 1985; Soltis and Soltis 1986, 1988; Watano and Sahashi 1992; Hauk and Haufler 1999; Chung et al. 2013; Stensvold and Farrar 2017). To resolve this conundrum, previous studies have hypothesized the existence of previous or current but undiscovered outcrossing, genetically rich precursors of the existing genetically depauperate taxa.

Outcrossing Is Predominant in Swiss Alps

In our large sampling of *B. lunaria* in the Alps and Jura Mountains in Switzerland, we found high allelic variation and high levels of heterozygosity—similar to that reported in outcrossing

Fig. 4 Gene flow among the Swiss *Botrychium lunaria* populations. A, Population graph representing the genetic relationships and the barriers to gene flow. The size of the nodes represents differences in genetic variance within populations, and the edges between nodes show significant genetic covariance between populations. Colors (blue, orange, and red) represent the three main subgraphs characterized. B, Spatial population graph mapped onto real space with the Swiss digital elevation model (<http://swisstopo.ch>). ARO = Arosa; BER = Bernina; BGU = Bosco Gurin; CHA = Chasseral; CHH = Chandolin High; CHL = Chandolin Low; CHO = Chasseron; GOP = Gotthard Pass; GRP = Grimsel Pass; GSB = Grand St. Bernard; LAA = Laax; SBE = San Bernardino; SIM = Simplan; VDC = Val di Campo; VRO = Val Roseg; ZER = Zermatt.

seed plants (Gottlieb 1981; Crawford 1983, 1985)—within and among Swiss *B. lunaria* populations, contrasting starkly with reported low allelic diversity and extremely low heterozygosity among Ophioglossaceae genera and related taxa of *Botrychium* (table 2). We also determined low estimates for the inbreeding coefficient of Swiss *B. lunaria* ($F_{IS} = 0.247$; table S3) and a weak deficit of heterozygotes (fig. 2B). This finding of high genetic variation and recombination supports sporophytic outcrossing as an important element in the evolution of *Botrychium* (Haufler et al. 2016). Interestingly, in both the Swiss Alps and Rocky Mountains (for *B. campestre* var. *lineare*; Farrar and Gilman 2017), outcrossing populations have been found to occur on high-elevation slopes, where seepage from snowmelt could reliably assist sperm dispersal among gametophytes in a population, whereas populations of the same or closely related taxa occurring on lower, flatter topography exhibit genetic depauperacy indicative of inbreeding. Still, it must be asked why new spore migrants from genetically rich populations to genetically depauperate populations would not enrich the latter.

Restriction to Spore Dispersal Does Exist

Spore dispersal by wind, rather than vicariance events, is known to be an important feature driving fern species demography (Wolf et al. 2001), and several cases of long-range dispersal at intercontinental scales are documented for the worldwide *B. lunaria* species complex (Dauphin et al. 2017; Farrar and Stensvold 2017). Nevertheless, our analysis of gene flow among Swiss *B. lunaria* populations indicates that spore dispersal is not without limitations, even at relatively short distances. In particular, the ARO population (fig. 1), occurring on a site only recently free of permafrost (i.e., from several decades to a century; <http://map.geo.admin.ch/>), although exhibiting levels of heterozygosity similar to those of other populations at its polymorphic loci, has remarkably fewer polymorphic loci (42%; table 1). In addition, the parallel ice sheet model of the entire last glacial cycle (120,000 yr to date) in the Alps has dated the release of this site to about 11,000 yr (Seguinot et al. 2018), confirming the relatively recent retreat of the ice sheet from the ARO region. Thus, the allelic diversity

of the overall Swiss *B. lunaria* has not yet been incorporated into the ARO population because of either restriction to spore dispersal or restriction in genotype suitability to this habitat. Hence, this recently established small population (i.e., fewer than 50 sporophytic individuals) provides an empirical example of population differentiation consistent with the founder effect and provides insight into our understanding of the potential consequences of limited spore dispersal in taxon diversification.

Population Genetic Structure and Its Implications for Postglacial Recolonization

Our population genetic analyses revealed a complex population genetic structure and weak genetic differentiation, with substantial gene flow among populations (figs. 3, 4, S2; table S5). On the basis of pairwise F_{ST} values and the DAPCs, four populations (ARO, GOP, VDC, and SBE) showed a signal of differentiation not well correlated with their geographic position across the species' range (figs. 1, 3). The population graph, which does not rely on a priori assumptions of a demographic model, identified three subgroups (i.e., subgraphs) without clear geographic clustering (fig. 4B), which supports a complex spatial process of gene flow (i.e., genetic connectivity) among populations. Several key populations (BGU, Chandolin Low, Grand St. Bernard, SBE, and Val Roseg), with the notable case of CHO, maintained genetic integrity by connecting the subgroups from distant locations (fig. 4). These findings corroborate previous studies regarding the potential role of long-range spore dispersal in shaping genetic diversity (Schneller et al. 1998; Jiménez et al. 2010; De Groot et al. 2012) and support episodic long dispersal events but with a limited ability for species to maintain intense migration between populations over distances of several dozens of kilometers.

Quaternary glaciations have deeply affected the demographic history of mountain plants (Hewitt 2004; Schönschetter et al. 2005), with populations that occur in unglaciated peripheral areas of the European Alps harboring a large portion of their conspecific genetic diversity during the LGM (Petit et al. 2003). A phylogeographical study of *B. lunaria* showed such a pattern, with at least three putative peripheral refugia located in southern

Table 2

Comparison of Levels of Genetic Diversity and Heterozygosity among Seed Plants, Homosporous Ferns, Ophioglossaceae Genera, *Botrychium lunaria* Complex, and the Swiss *B. lunaria* Analyzed in This Study

Taxon group	Polymorphic loci (%)		No. alleles per locus		H_S		Reference
	Mean	Range	Mean	Range	Mean	Range	
Seed plants	58.9	...	2.29	...	...	...	Hamrick and Godt 1996
Homosporous ferns	36.0	.0–80.0	1.65	1.00–2.80	.132	.000–.330	Li and Haufler 1999
Ophioglossaceae:							
<i>Botrychium</i> (outcrossing):							
Swiss <i>B. lunaria</i>	79.0	41.7–100.0	2.03	1.38–3.75	.246	.144–.305	This study
<i>B. campestre</i> var. <i>lineare</i>	29.6	11.1–59.1	1.35	1.11–1.77	...	...	Farrar and Gilman 2017
<i>Botrychium</i> (inbreeding):							
<i>B. lunaria</i> complex	20.0	5.0–35.0	1.20	1.05–1.45	...	...	Stensvold and Farrar 2017
<i>B. campestre</i> var. <i>campestre</i>	16.7	13.6–18.2	1.21	1.18–1.23	...	...	Farrar and Gilman 2017
Nine diploid species	7.4	.0–16.7	1.10	1.00–1.20	...	...	Farrar 1998
Five diploid species	7.3	.0–21.4	1.07	1.00–1.21	.035	.000–.086	Hauk and Haufler 1999

Note. Classification is based on Schuettpelz et al. (2016). H_S = expected heterozygosity.

regions of the Alps (Monte Generoso, Mont Ventoux, and the Styria region; Maccagni et al. 2017). It also suggested two putative high-alpine refugia (e.g., Zermatt [ZER]) that could have sheltered this species during the LGM (Stehlik 2000). Several south-facing and thus insolation-rich zones along the arêtes of the main valleys (between 2300 and 3000 m; Kelly et al. 2004; Hippe et al. 2014; Wirsig et al. 2016) may have hosted *B. lunaria* by providing a habitat free of permanent snow and a soil suitable for maintenance of its vital arbuscular mycorrhizal fungi. As further evidence, the ZER locality is a well-known hot spot of intraspecific genetic diversity where some genetically rich populations might have persisted during the LGM (Taberlet et al. 2012). Previously reported for flowering plants sharing a similar habitat at high elevations (Bettin et al. 2007; Parisod and Besnard 2007), glacial refugia in the Central Alps have likely sheltered *B. lunaria* and allowed in situ survival from which the ancestral variable populations have recolonized current populations in their vicinity, leading to the current weak population differentiation. The shallow spatial structure in our results is compatible with substantial gene flow during the last interglacial period(s) that has homogenized allele frequencies through outcrossing.

Biological Features of B. lunaria for In Situ Survival during the LGM

Climatic conditions were particularly cold and dry during the LGM. The survival of *B. lunaria* in such central refugia implies key physiological and life cycle characteristics that maintained viable populations in favorable south-facing flanks of the ice-free high-alpine crests (i.e., arêtes) for many thousands of years. As a key phenological feature reported in high-elevation alpine flora (Oehl and Körner 2014), *B. lunaria* is supported by arbuscular mycorrhizal fungi that provide carbohydrate nutrition as well as water and minerals and therefore allow the maintenance of populations in thin, rocky, and poor soils (Sandoz et al. 2020). When the season is adverse, for example, under unusually dry conditions (Johnson-Groh and Lee 2002), water stress may induce dormancy of the sporophyte, in which the annual leaf remains belowground in the bud stage. Depending on the year of growth and the habitat characteristics, species of *Botrychium* have an important flexibility in the emergence period of sporophytes—up to 7 wk of fluctuation for *B. mormo*. Another phenological feature contributing to the stability of *B. lunaria* populations is the long generation time (i.e., several decades, as determined in the Swiss Alps from leaf scars along the underground stems; B. Dauphin, unpublished data), which includes a few years for the development of the underground perennial gametophytes (fig. 1C–1E; Farrar et al. 2008). On the basis of these biological features, *B. lunaria* may have been well suited for survival in glacial refugia during the LGM as well as during previous and even larger glaciations of the Quaternary.

Conclusion

This study establishes that populations of the moonwort fern, *Botrychium lunaria*, in the Alps and Jura Mountains of Switzerland maintain high allelic diversity and demonstrate strongly outcrossing breeding behavior. This observation contrasts sharply with reported selfing for other circumboreal populations of *B. lunaria* as well as other taxa of *Botrychium*. Ours and a similar finding for high mountain populations of *B. campestre* in the western mountains of North America suggest that mass flow of water on high, steep slopes may facilitate sperm transfer among underground sexual gametophyte plants of *Botrychium*, as suggested by Wagner et al. (1985). These studies indicate that two species of *Botrychium* (i.e., *B. campestre* and *B. lunaria*) maintain outcrossing populations from which an inbreeding state, as reported for most of their closely related taxa, could have been derived. A high degree of gene flow among most Swiss populations implies the effectiveness of spore dispersal over moderate distances, but the existence of one population occupying a site more recently free of permafrost and exhibiting strongly reduced allelic diversity indicates that spore dispersal may not quickly reach saturation. Dispersal over longer distances into habitats less suitable for underground sperm dispersal could account for the observed inbreeding in most *Botrychium* populations.

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Data Availability

Genotype data have been deposited in the Dryad Digital Repository (<https://dx.doi.org/10.5061/dryad.np5hqbzqc>; Dauphin et al. 2020) as part of publication of this article.

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