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Floral and genetic divergence across environmental gradients is moderated by inter-population gene flow in *Platanthera dilatata* (Orchidaceae)

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Understanding how natural selection acts on intraspecific variation to bring about phenotypic divergence is critical to understanding processes of evolutionary diversification. The orchid family is well known for pollinator-mediated selection of floral phenotypes operating among species and along environmental or geographic gradients. Its effectiveness at small spatial scales is less understood, making the geographic scale at which intraspecific floral variation is examined important to evaluating causes of phenotypic divergence. In this study, we quantified phenotypic variation in the orchid *Platanthera dilatata* across 26 populations in coastal Southeast Alaska and compared this to edaphic and genetic variation at microsatellite loci. We sought to determine (1) if flower morphological variation is structured at smaller geographic scales, (2) the extent of genetic divergence in relation to phenotypic divergence, (3) the scale at which inter-population gene flow occurs, and (4) the relative importance of geographic distance and abiotic factors on population genetic structure. Two morphological groups were found to separate based on lip and spur length and are restricted to different habitats. Small-flowered forms occur in muskeg bogs, whereas large-flowered forms occur in fens and meadows, and rarely in sub-alpine habitat. Genetic analyses were concordant with the morphological clusters, except for four small-flowered populations that were genetically indistinguishable from large-flowered populations and considered to be introgressed. In fact, most populations exhibited some admixture, indicating incomplete reproductive isolation between the flower forms. Pollinators may partition phenotypes but also facilitate gene flow because short-tongued Noctuidae moths pollinate both phenotypes, but longer-tongued hawkmoths were only observed pollinating the large-flowered phenotype, which may strengthen phenotypic divergence. Nevertheless, pollinator movement between habitats could have lasting effects on neutral genetic variation. At this small spatial scale, population genetic structure is only associated with environmental distance, likely due to extensive seed and pollinator movement. While this study corroborates previous findings of cryptic genetic lineages and phenotypic divergence in *P. dilatata*, the small scale of examination provided greater understanding of the factors that may underlie divergence.

KEYWORDS

cryptic divergence, flower variation, genetic structure, gene flow, soils, isolation by distance, isolation by environment, *Platanthera dilatata*

1. Introduction

Understanding the origin and maintenance of intraspecific variation is of central importance to evolutionary biology because they inform our understanding of diversification across space and time and illuminate the process of speciation (Pinheiro et al., 2018). Substantial intraspecific phenotypic variation may indicate the maintenance of polymorphisms over otherwise connected populations (Nobarinezhad and Wallace, 2022), or it could indicate the presence of evolutionarily divergent cryptic lineages that exhibit parallel ecological responses (Kahl et al., 2021). Whereas a polymorphic species is expected to experience spatially and temporally heterogeneous gene flow among populations, cryptic lineages exhibiting genetic divergence should be isolated from one another (Surveswaran et al., 2018). While genetic tools have been especially useful for identifying cryptic lineages, integrated approaches involving multiple data types and widespread sampling of populations provide not only the identification of cryptic lineages but also clues about their divergence and geographic spread (Surveswaran et al., 2018; Liu et al., 2022). When examined deeply, many species have been found to comprise cryptic lineages (Pinheiro et al., 2018). Linking such divergence with pollinator selection of floral traits is critical to understanding how it integrates with co-evolutionary processes in determining ecological speciation (Van der Niet et al., 2014).

The orchid genus *Platanthera* (L.) Rich. contains many phenotypically polymorphic species (e.g., Robertson and Wyatt, 1990; Wallace, 2003a; Bateman and Sexton, 2008; Bateman et al., 2013; Adhikari and Wallace, 2014) and potentially cryptic lineages (Wettewa et al., 2020). As in many orchids, this phenotypic variation is frequently attributed to pollinator-mediated selection (Hapeman and Inoue, 1997; Van der Niet et al., 2014). Such selection has been shown to operate even within species (Robertson and Wyatt, 1990). At larger geographic scales or along environmental gradients, pollinator-mediated selection is a reasonable hypothesis for morphological polymorphism if pollinators exhibit habitat preferences or have distributional limits. However, at smaller geographic scales, other factors must also be considered to explain the maintenance of phenotypic variation in *Platanthera* species. Characterizing the geographic scale of phenotypic variation within species is important for distinguishing among competing factors in the maintenance of this variation.

Platanthera dilatata (Pursh) Lind. ex L.C. Beck is distributed across the northern U.S. and Canada, reaching as far south as New Mexico and as far north as Alaska. This species has been treated as representing three varieties based on nectar spur length, which are thought to partition pollinators by corresponding proboscis lengths (Sheviak, 2002). However, as noted by Sheviak (2002), "the recognized varieties of *P. dilatata* are evidentially merely endpoints in a very complex variation pattern," leading to unanswered questions as to why polymorphism in this species exists.

In this study, we examined phenotypic and genotypic divergence among populations of *P. dilatata* in Southeast Alaska and across elevational, climatic, and edaphic gradients. In the study area, *P. dilatata* populations do not readily fit into the varieties outlined by Sheviak (2002). Thus, we sampled across an area covering many habitats and flower types to quantify variation in soil characteristics, climatic variables, flower morphological traits, and genetic variation at microsatellite loci. We used these data to address the following questions: (1) Is flower morphological variation structured at smaller geographic scales, (2) Are floral phenotypes genetically divergent, (3) Does gene flow occur across morphologically distinct populations, and (4) How do geographic distance and environmental differences influence population genetic structure? We predicted strong isolation by distance at the regional scale (i.e., encompassing all study populations) because of limitations on gene flow *via* seeds and selection on flowers by pollinators, but at a local scale (i.e., less than 50 km between populations), we predicted that environmental factors would more strongly influence genetic structure because seeds should be capable of dispersal over these distances but may differ in adaptation to habitats and pollinators.

2. Materials and methods

2.1. Study area

This study took place in Southeast Alaska, United States. which comprises an 800 km mountainous coastline and adjacent island chain along the northwest coast of North America (Figure 1). The climate of this region is primarily wet maritime, averaging over 300 cm annual precipitation. The average maximum temperature reaches 18°C in July, and the average minimum temperature reaches -4°C in January (Shulski and Wendler, 2007; Bienek et al., 2012). The predominant coastal vegetation is northern rainforest; about 17% of the area is non-forested shrubland and peatland (Kirchoff et al., 2016). This area was glaciated <10,000 years BP; as a result, climate and post-glacial migration strongly affect vegetation composition (Andersen, 1955; Mathewes, 1985), but glacial refugia present during the late Wisconsin glaciation also may have allowed persistence and recolonization of vegetation within this region (Carrara et al., 2007).

In the study area, *P. dilatata* is most abundant in open bog and fen peatlands, coastal, lakeshore, and riverine meadows, and anthropogenic-disturbed roadsides (Figure 2). Bogs, also known as muskeg, are usually ombrotrophic and develop at low to mid-elevations but grade into subalpine conditions with less organic matter. These habitats usually comprise sapric to hemic peat, and support plant species of open bogs, including *Sphagnum* L. sp., *Carex* L. sp., and Ericaceous shrubs (Neiland, 1971). Fens usually occur at low elevations along drainage ways and range from weak to moderately minerotrophic, receiving greater nutrient input than bogs (Fellman and D'Amore, 2007; Fellman et al., 2008; D'Amore et al., 2010, 2015). They comprise floating or solid mats of hemic to fibric peat and support a subset of bog and meadow vegetation. Coastal meadows, also termed uplift meadows, are developed in fine-textured glacial outwash and lacustrine deposits and are undergoing isostatic uplift following glaciation. They are dominated by broad-leaved herbs, with a minor component of graminoid species, and may zonate along tidelands (Stone, 1993). Anthropogenic roadsides have mineral soils developed from grading and gravel deposition and tend to represent a subset of meadow vegetation that tolerates disturbances such as seasonal mowing.

The mycorrhizal fungi *Ceratobasidium* sp. and *Tulasnella* sp. have been identified in *P. dilatata* root samples from the study area. Two of three *Ceratobasidium* isolates were from muskeg, while 10 of 11 *Tulasnella* samples were from fen, meadow, or anthropogenic habitat (Melton, 2020; M. McCormick, pers. comm.; L. Zettler, pers. comm.).

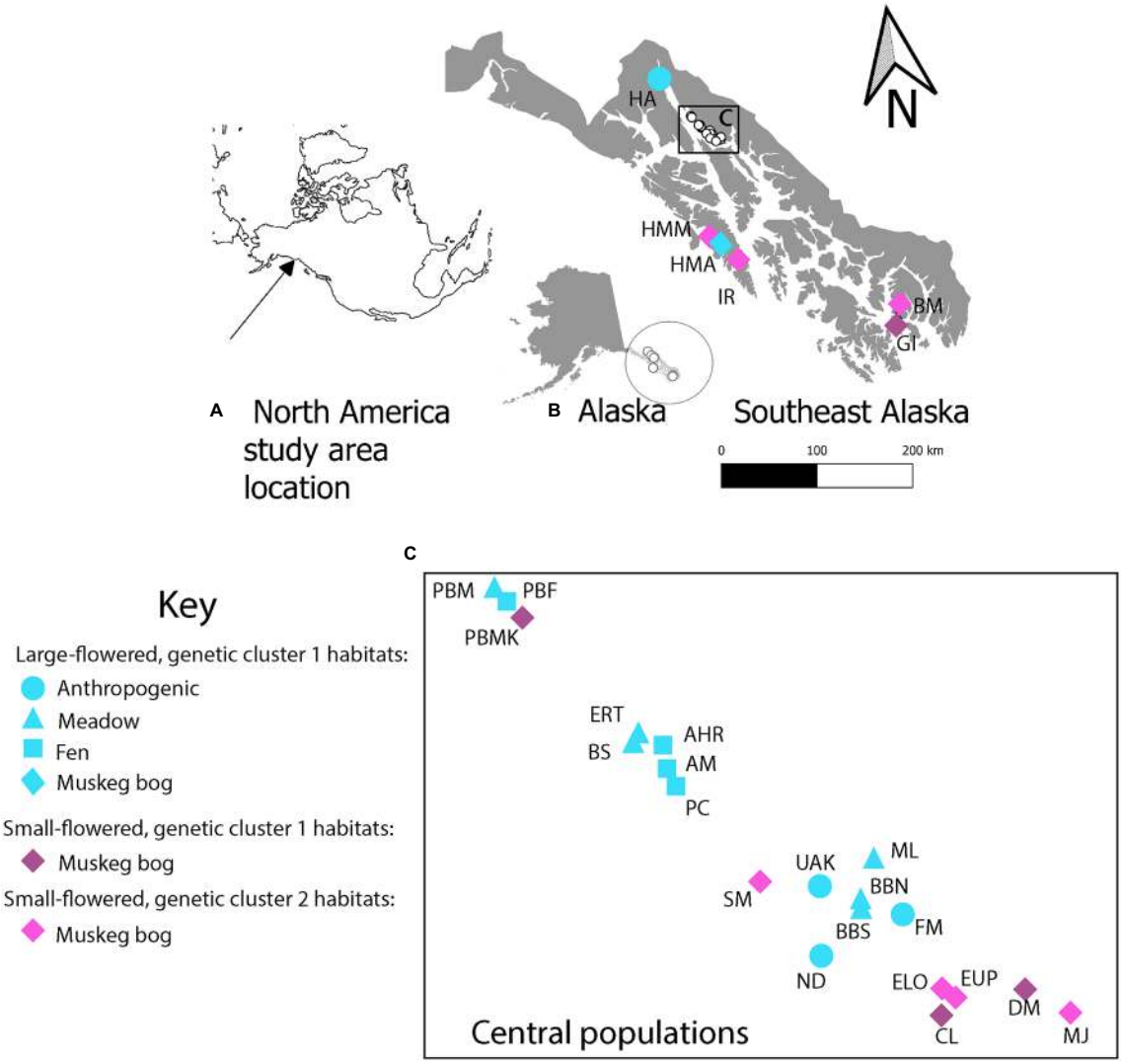


FIGURE 1
Location of **(A)** study area in North America, **(B)** Southeast Alaska collection sites of *Platanthera dilatata* sampled for morphology, genetic markers, and soil characteristics, and **(C)** distribution of populations shown in the box in panel B and referenced as central in landscape genetic analyses. In panels B and C, shapes and colors indicate the six combinations of habitat x flower size x genetic cluster observed in this study. Site names follow those in Table 1 and Supplementary Table S1.

Pollinators of *P. dilatata* include Noctuidae moths, the hawkmoth *Hyles gallii* (Rottemburg, 1775), and the butterfly *Pieris marginalis* Scudder, 1861 (Figure 3); pollinia were deposited on the proboscises of these insects (Bowles and Armstrong, 2021). Noctuidae moths appear to be primary pollinators across all habitats, but hawkmoths may be most frequent in fens and meadows.

2.2. Site selection

The 26 study sites represented 12 muskeg bogs, six meadows, four fens and four anthropogenic roadsides, spanning *ca.* 500 km from north to south (Figure 1; Table 1; Supplementary Table S1). Although fen, meadow, and anthropogenic habitats may occur in southern Southeast Alaska, study sites for these habitats were restricted to northern Southeast Alaska. Sites were selected based on accessibility, lack of anthropogenic disturbance (excluding roadsides) presence of >10 flowering plants at each site, and regional distribution to maximize sampling in morphologically diverse populations and environmentally variable sites.

2.3. Morphological data collection and analysis

Lip and spur length are the most important variables for distinguishing among varieties of *P. dilatata* (Adhikari and Wallace, 2014). These metrics were obtained from single flowers selected from 10–28 (mean = 17.4, se = 1.01) inflorescences from each study population. Flowers were collected in 2018–2019. Flower collection was stratified to represent the range of inflorescence sizes present; flowers were collected from the lower third of inflorescences to avoid nectar spurs that were not fully developed. Flowers were stored in zip-lock plastic bags at 4°C, and measured within 48 h. Each flower

TABLE 1 Genetic diversity at eight microsatellite loci across sampled locations of *Platanthera dilatata* in Southeast Alaska.

Site name	Habitat	N	Na	%P	H_O	H_E	F_{IS}
Large-flowered populations							
FM	Anthropogenic	18.4	3.0	100	0.413*	0.497	0.106
HM	Anthropogenic	20.0	3.4	100	0.500	0.496	0.031
ND	Anthropogenic	23.6	4.5	100	0.516	0.604	0.034
UAK	Anthropogenic	19.0	3.7	100	0.507	0.501	0.033
BS	Meadow	22.0	3.6	88	0.381*	0.458	0.113
BBN	Meadow	15.9	3.6	100	0.557	0.528	0.039
BBS	Meadow	22.7	3.9	100	0.460*	0.513	0.088
ERT	Meadow	15.0	3.6	100	0.467*	0.511	0.068
ML	Meadow	9.0	3.5	88	0.542	0.542	0.054
PBM	Meadow	9.0	3.0	100	0.403*	0.428	0.133
AHR	Fen	12.6	3.6	100	0.544	0.513	0.090
PC	Fen	22.6	3.7	100	0.432*	0.501	0.078
AM	Fen	24.0	3.6	100	0.458	0.462	0.043
PBF	Fen	10.0	2.9	100	0.400	0.376	0.051
HMA	Muskeg bog	10.0	1.5	50	0.288	0.226	0.025
Small-flowered populations							
DM[a]	Muskeg bog	24.0	4.0	100	0.641	0.578	0.013
PBMK[a]	Muskeg bog	20.7	3.5	100	0.459	0.491	0.040
CL[a]	Muskeg bog	23.0	4.2	100	0.565	0.600	0.035
GI[a]	Muskeg bog	16.0	2.7	100	0.539	0.470	0.033
ELO	Muskeg bog	20.9	3.2	100	0.431*	0.508	0.069
EUP	Muskeg bog	19.0	2.5	75	0.270	0.284	0.053
HMM	Muskeg bog	21.7	2.2	88	0.319	0.364	0.032
IR	Muskeg bog	23.9	2.7	100	0.350	0.385	0.031
MJ	Muskeg bog	21.0	2.7	88	0.310	0.316	0.043
BM	Muskeg bog	14.6	3.1	100	0.434	0.436	0.044
SM	Muskeg bog	19.0	2.6	100	0.395	0.451	0.071
Mean–large-flowered populations		16.9	3.4	95	0.458	0.477	0.066
Mean–small-flowered without hybrid populations[a]		20.0	2.7	93	0.358	0.392	0.049
T-test[b] *P*		--	2.486 0.022	0.369 0.72	3.074 0.006	2.206 0.039	1.228 0.233

N = mean number of individuals sampled across all loci, Na = mean number of alleles per locus, % *P* = percentage of polymorphic loci, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient.
*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).
[a]Small-flowered populations suspected of having introgression from large-flowered populations.
[b]*T*-tests were conducted without the inclusion of hybrid small populations.

was dissected to remove the lip and spur, and their lengths were measured to the nearest 0.5 mm. Most spurs were falcate, and they were flattened under a flexible sheet of transparent plastic for linear measurement.

Population means (+ se) were calculated for flower and lip length. To assess whether morphological groups could be identified, a k-means cluster analysis was performed in NCSS statistical software (Hintze, 2013). This test evaluated 1–5 clusters, using 5 random starts to produce an optimum solution in which within-cluster sum of squares is minimized. A goodness of fit comparison of the percent variation in each within-cluster group sum of squares relative to one group was used to evaluate which number of clusters had the greatest reduction in variation (Hintze, 2013). The Duda and Hart (1973) test was also used to evaluate whether single or multiple clusters better fit the data, followed by application of the Calinski and Harabasz (1974) index to further evaluate the most likely number of clusters beyond one.

We calculated P_{ST} (Brommer, 2011) to estimate the degree of differentiation in lip and spur length among populations. P_{ST} was then compared to F_{ST} estimated from the microsatellite data (see below) to evaluate the relative potential for selection and genetic drift to drive the observed differences in floral traits. P_{ST} was estimated separately for lip length and spur length using the R package Pstat (Blondaeu Da Silva and Da Silva, 2018). Data were subjected to Atchinson transformation and the value of c/h^2 was set to 1; bootstrap analysis with 1,000 replicates was used to calculate 95% confidence intervals for P_{ST} and this was compared to our estimate of F_{ST} based on microsatellite loci.

2.4. Soil data collection and analysis

Soil samples were collected from each study site in 2018–2022. Each sample comprised multiple excavations made to rooting depth with a hand trowel, which were combined into a single collection for each site. Samples were analyzed by Waypoint Analytical (Richmond, Virginia, USA) for percent organic matter (POM); parts per million (PPM) Ca, K, Mg, and P; percent base saturation (PBS) Ca, K and Mg; percent H saturation (PHS); and cation exchange capacity (CEC, meq/100 g). Analytic methods followed Horton (2011).

Soils data were analyzed with ANOVA and multivariate statistics. One-way ANOVA was used to test whether soils variables differed among muskeg, fen, meadow, and anthropogenic habitat groups, which supported different orchid phenotypes (see below). For these tests, transformations were used to approximate normality for POM and PBS K (arcsin transformation), PPM P (log transformation), PPM Ca and Ca (square root transformation). Non-metric Multidimensional Scaling (NMS) was used on PCORD (McCune and Mefford, 2011) to ordinate habitat groups using POM, pH, CEC, PPM P, PBS K, PBS Mg, PBS Ca, and PHS as metrics. A relative Euclidian distance measure with a random seed starting configuration and 100 runs with real data were used to project three axes using a Varimax rotation, for which stability was tested with a randomization test. Relationships of each metric with the first and second NMS axis were tested with correlation analysis. A Multi-Response Permutation Procedures (MRPP) test was used on PCORD to assess whether habitats differed in their multivariate distributions based on soils metrics. Because of skewed metrics in anthropogenic habitat soils, the MRPP test was repeated with this group excluded from the analysis.

2.5. Genetic data collection and analysis

Leaf samples used in genetic analyzes were collected in 2018–2019. A 5 cm length of fresh leaf tissue was removed from one leaf

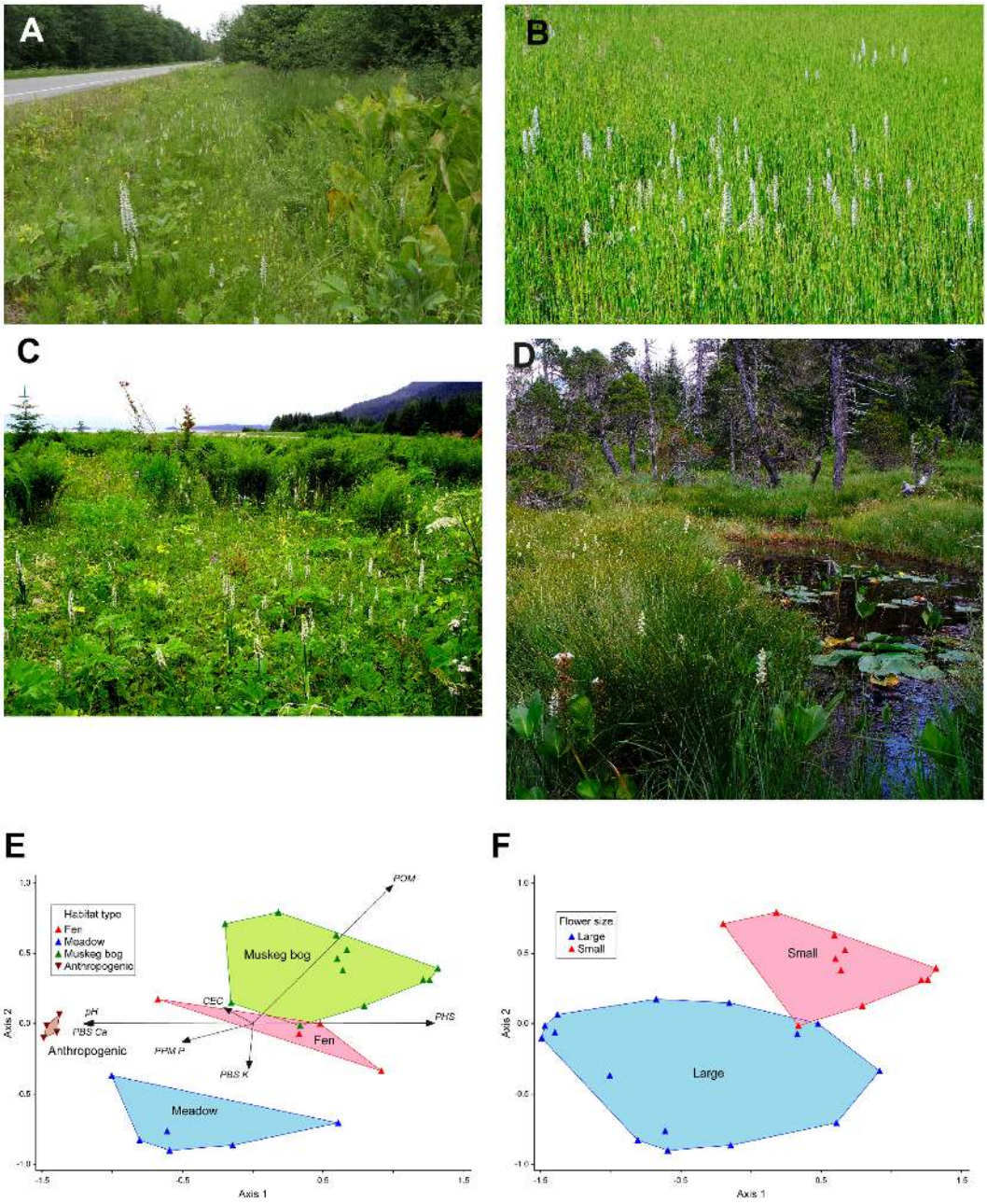


FIGURE 2
Variation in habitats and soils occupied by *Platanthera dilatata*. **(A)** anthropogenic roadside, **(B)** fen, **(C)** uplift meadow, **(D)** and muskeg bog. Panels **(E,F)** show the NMS ordination of habitat vegetation types in relation to soil characteristics and flower group as indicated by the K-means clustering analysis of floral traits **(F)**. Ordination final stress=3.4891, final instability=0.0; probability of final stress obtained by chance (Axis 1 p=0.002, Axis 2 p=0.044). Cumulative correlations between ordination distances and distances in the original n-dimensional space: Axis 1 r^2=0.828, Axis 2 r^2=0.990. MRPP: all habitats (A=0.41613688, p<0.0001); anthropogenic habitats excluded (A=0.26071168, p<0.0001). See Supplemental Table S4 for soils variables axis correlation statistics.

from 9–26 (mean = 18.8 se = 1.3) plants from each study site. Leaf samples were stored in zip-lock plastic bags at 4°C. These samples were dried within 24 h. by placing them in folded aluminum foil containing silica gel crystals and then sealed within double zip-lock plastic bags. DNA was extracted from dried leaves using the SYNERGY 2.0 Plant DNA extraction kit (OPS Diagnostics, Lebanon, New Jersey, USA) and stored in 1X TE buffer. DNA samples were standardized to 10 ng/μl for use in PCR. Each sampled plant was genotyped at nine microsatellite loci that were developed from a transcriptome library of *P. dilatata* (Wallace, unpublished data). Primer sequences are provided in Supplementary Table S2. The nine loci were amplified using a multiplex PCR with the Kapa 2G Fast multiplex PCR kit (Roche Sequencing and Life Science, Wilmington, Massachusetts, USA) and fluorescent labeled primers following protocols in Culley et al. (2013). Each fluorescently labeled primer contained a sequence that matched a tag sequence located on the 5′

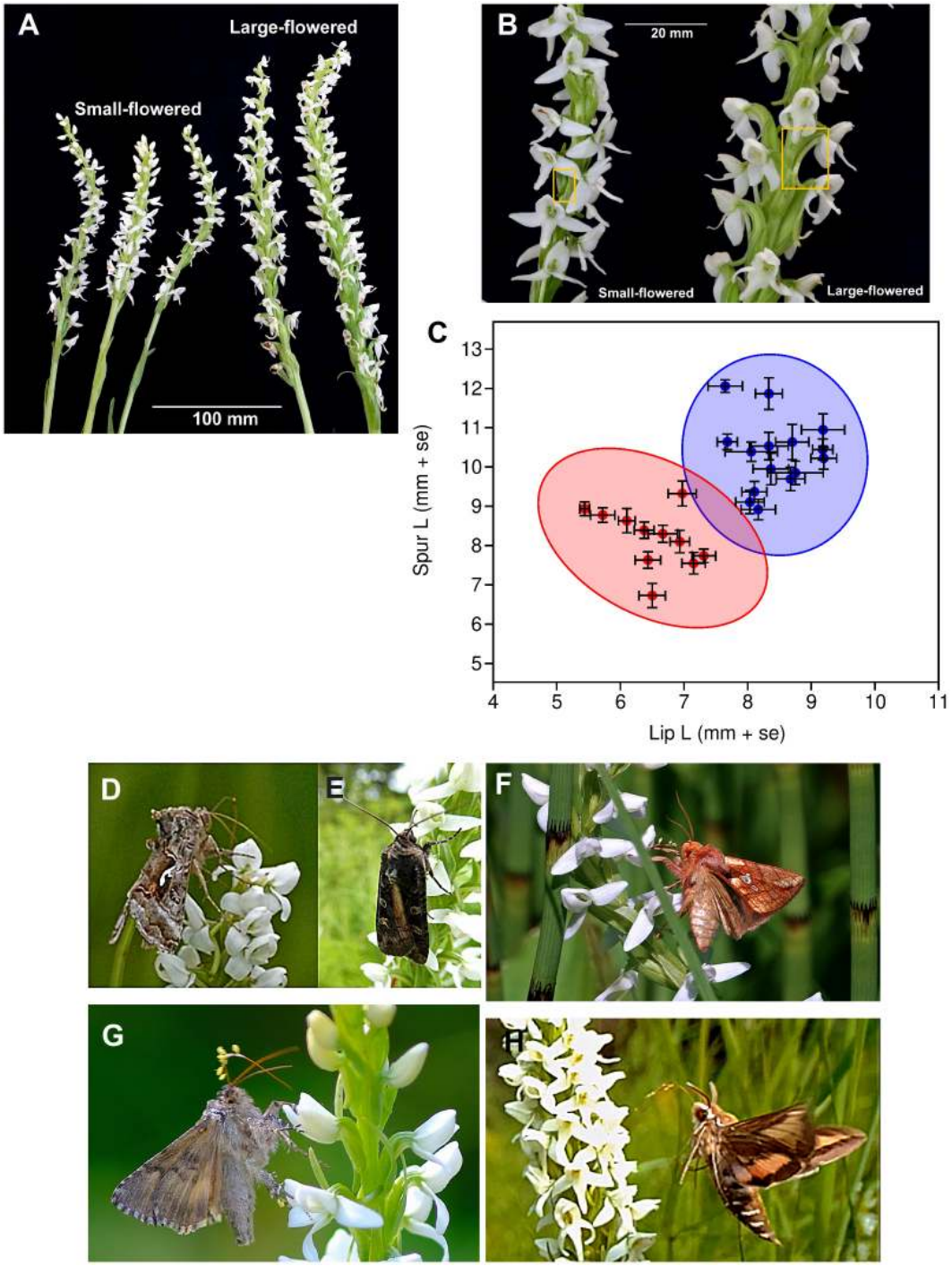


FIGURE 3
Variation in flower traits and pollinators of *Platanthera dilatata* in the study area of Southeast Alaska. Differences in **(A)** inflorescence size and **(B)** the lengths of spurs (in boxes) between plants grouped by flower size and **(C)** mean (+ se) lip and spur length of flowers, with the ellipses indicating 95% concentrations of populations within large and small-flowered groups identified by k-means cluster analysis. Pollinators observed on *P. dilatata* flowers in the study populations include **(D)** *Autographa corusca* Strecker, 1885 on small-flowered phenotype (photo: R. H. Armstrong), **(E)** *Actebia fennica* (Tauscher, 1806) on small-flowered phenotype (photo: G. Bayluss), **(F)** *Plusia* sp. Ochsenheimer 1816 on the large-flowered phenotype (photo: R. H. Armstrong), **(G)** *Autographa corusca* Strecker, 1885 on large-flowered phenotype, and **(H)**, *Hyles gallii* (Rottemburg, 1775) on large-flowered phenotype (photo: R. H. Armstrong); D-G are Noctuidae species, and H is a Sphingidae species. Large-flowered group: lip mean=8.43 (se=0.13), spur mean=10.31 (se=0.23), *t*-test of lip and spur lengths: $t=-7.089$, $p<0,001$; small-flowered group: lip mean=6.49 (se=0.173), spur mean=8.11 (se=0.25),), *t*-test of lip and spur lengths: $t=-5.385$, $p<0.001$. One-way ANOVA between morphological groups: Lip $F_{1,24}=85.45$, $p<0.0001$, Spur $F_{1,24}=40.50$, $p<0.0001$. Lip-spur correlations: among groups ($r=0.6409$, $p=0.0004$); small-flowered group ($r=-0.3877$, $p=0.237$); large-flowered group ($r=-0.0581$, $p=0.8435$).

end of the locus-specific forward primer. For each sample, each multiplex reaction was performed in a final volume of 10 µl in the presence of 10 ng of template DNA, 100 µmole of each of the reverse and tagged fluorescently labeled primers and 10 µmole of tagged forward primer using KAPA 2G Fast Multiplex PCR mix. The thermal cycler program used to amplify loci included 3 min at 95°C, 30 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, and a final extension step of 1 min at 72°C. Amplified products were genotyped at the Institute of Biotechnology at Cornell University with LIZ 500 size standard, and individual alleles were sized using GeneMarker (SoftGenetics, State College, Pennsylvania, United States).

The presence of null alleles in each locus and population was checked using the program FreeNA (Chapuis and Estoup, 2007). Null allele frequencies <0.2 are not expected to greatly influence the results of population genetic analyzes (Dakin and Avise, 2004; Carlsson, 2008). Thus, we considered further only loci exhibiting a null allele frequency > 0.2, which occurred at three loci, 72267, 99945, and 107223, in eight, three, and three populations, respectively. Locus 72267 was removed from the dataset because of the extensive occurrence of potential null alleles. We further investigated inbreeding as a potential cause of null alleles for the other two loci. Heterozygote deficiency, which is a potential sign of null allele presence, has often been reported in association with significant F_{IS} in other orchids (Chung et al., 2004; Alcantara et al., 2006; Andriamihaja et al., 2021). For each of the five populations suspected of having null alleles, we compared a model based on the inclusion of null alleles, inbreeding, and genotyping errors (i.e., nfb) with one lacking inbreeding (i.e., nb) using the software INEST v. 2.2 (Chybicki and Burczyk, 2009). These analyzes were implemented using a Bayesian approach with 1 million MCMC cycles, keeping every 100th result, and a burn-in of 10,000 prior to summarizing the results. DIC values were compared between the two models to evaluate the impact of inbreeding on observed diversity. For population PC, the full model had a substantially lower DIC than the model without inbreeding. For the other four populations (i.e., HMM, IR, SM, and ND), the difference in DIC between the two models was less than 1.5. As these results suggest that inbreeding may account for the lack of heterozygous individuals in these populations at the suspected loci, we chose to retain data for these locus-population combinations for further analysis of genetic diversity and structure.

Within each population, we tested for significant departures from Hardy–Weinberg expectations using a global test of heterozygote deficiency in GENEPOP version 3.2 (Raymond and Rousset, 1995; Rousset, 2008). Genotypic linkage disequilibrium was measured for each pair of loci in each population and tested through Fisher's exact test using GENEPOP version 3.2 (Raymond and Rousset, 1995; Rousset, 2008) and applying a Bonferroni correction (Holm, 1979). Genetic diversity within populations was assessed as number of alleles per locus (N_a), observed heterozygosity (H_O), expected heterozygosity (H_E), and percent of polymorphic loci (% P) using GenAlEx version 6.503 (Peakall and Smouse, 2012). Inbreeding coefficients were calculated in INEST (Chybicki and Burczyk, 2009) as described above. To determine if genetic diversity varies between large and small-flowered populations, as identified in the morphological K-means clustering, we compared mean values of N_a, H_O, H_E, and F_{IS} using *t*-tests in SPSS v. 27 (IBM Corp, 2020). $p < 0.05$ was used to identify significant differences in genetic diversity between the flower groups.

We evaluated population genetic structure according to the groups identified by morphological analyzes and NMS of the soil variables, that is, between large-flowered and small-flowered groups and separately among the four habitat types (Table 1) using analysis of molecular variance (AMOVA) (Excoffier et al., 1992), conducted in GenAlEx version 6.503 (Peakall and Smouse, 2012). Statistical significance of AMOVA was assessed by 9,999 permutations. We used the Bayesian clustering approach implemented in STRUCTURE v. 2.3.4 (Pritchard et al., 2000) to test for admixture and assignment of individuals to distinct genetic clusters. These analyzes were conducted using an admixture model with correlated allele frequencies, a 'burn-in' period of 50,000 MCMC replicates, sampling 100,000 replicates, and eight iterations of each K value, from one to 13. This range of K values was used in the final run because an initial analysis of four iterations each for K values from 1 to 25 under similar run parameters indicated low probability of a K value greater than five. For the final analysis multiple posterior probability values (log likelihood (lnL) values) for the eight iterations of each K were generated, and the most likely number of clusters was determined using STRUCTURE HARVESTER (Earl and vonHoldt, 2012) and Delta K- (Evanno et al., 2005). CLUMPP (Jakobsson and Rosenberg, 2007) was used to aggregate individual assignment probabilities from the eight iterations for the selected K. STRUCTURE PLOT (Ramasamy et al., 2014) was used to generate plots of individual assignment from the CLUMPP output file.

We estimated the potential for admixture in populations using several methods. Identify scores (Q-matrix scores) from the STRUCTURE analysis were used to infer if individuals were of pure ancestry or contained an admixed background. An identity score < 0.9 in a single cluster was used to assign an individual as admixed. NewHybrids v1.1 (Anderson and Thompson, 2002) was used to assign each individual to one of six genotypic classes (i.e., pure large-flowered, pure small-flowered, F1, F2, backcross with large-flowered, or backcross with small- flowered). This analysis was conducted without specifying individuals to a particular class, and all individuals were analyzed. We ran the analysis using a Jeffreys prior, 10,000 burn-in replicates, and 1 million sweeps before assignment probabilities were determined. No individual was assigned to any of the hybrid classes with probably >0.7, so we only considered a hybrid group, rather than F1, F2, or backcross generations. Furthermore, we used a cut-off probability of >0.9 to assign individuals into one of the pure parental groups, rather than the hybrid group.

BayesAss (Wilson and Rannala, 2003) and Geneclass 2 (Piry et al., 2004) were used to estimate the proportion of immigrants and non-immigrants. Whereas BayesAss (Wilson and Rannala, 2003) is better able to detect older instances of movement, Geneclass (Piry et al., 2004) more aptly identifies first generation immigrants. For these analyzes we assigned populations to one of three groups, large-flowered populations, small-flowered populations, and hybrid populations, after considering the morphological groupings and genetic assignments suggested by STRUCTURE and NewHybrids (see results, Supplementary Table S4; Figures 3C, 4A). Hybrid populations were identified by their conflicting placement into groups based on morphological and genetic variation (i.e., small-flowered plants that were genetically similar to large-flowered plants). BayesAss analysis was conducted using 50 million iterations, a burn-in of 1 million, and sampling every 5,000 generations. The Geneclass analysis was conducted to identify first generation

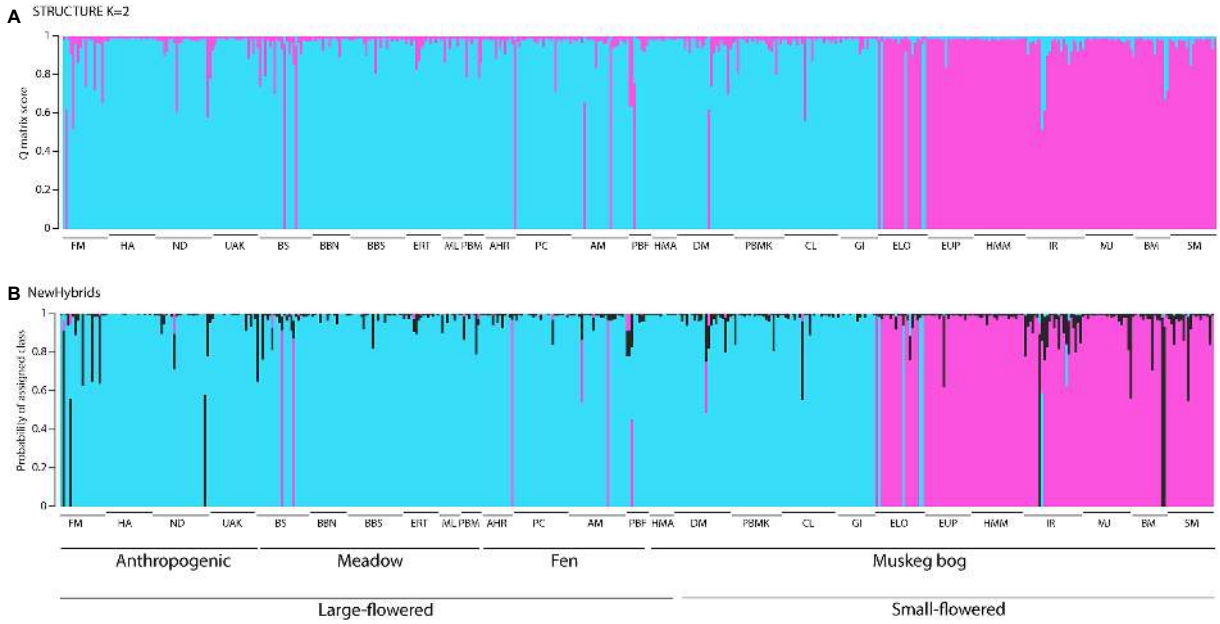


FIGURE 4
Admixture proportions for all samples of *Platanthera dilatata* from Southeast Alaska based on analysis with **(A)** STRUCTURE and **(B)** NewHybrids. For a, the Q-matrix scores for each individual according to the solution $K=2$ (blue and pink clusters) are indicated. For **(B)**, the estimated probability that an individual is from one of the pure parental groups (blue or pink) or a hybrid (black) is shown. Populations are arranged in the order shown in Table 1. See Figure 2E for alignment of habitat groups along soil gradients.

immigrants only using the criterion of Rannala and Mountain (1997) with 10,000 simulated individuals under the simulation algorithm of Paetkau et al. (2004). A $p<0.05$ was used to identify significant immigration events.

Past studies (e.g., Balkenhol et al., 2009; Kierepka and Latch, 2015) have found that different methods for assessing the role of geography, environment, or other factors on population genetic structure show only moderate agreement and recommend choosing multiple statistical approaches when testing for isolation by distance (IBD), isolation by environment (IBE), or other factors. Thus, we used multiple matrix regression (MMR) (Wang, 2013) and distance-based RDA (dbRDA) (Legendre and Anderson, 1999) to test for IBD and IBE. MMR compares pairwise population genetic distance against distance matrices based on explanatory variables using regression, whereas dbRDA assesses selected explanatory variables directly as predictors of population genetic distance. We conducted these analyzes at a regional scale, i.e., on all populations, as well as a local scale, i.e., the centrally located populations near Juneau (Figure 1B) to evaluate whether environmental factors are more predictive of genetic structure at the local scale and geographic distance at the regional scale, given that orchid seeds may be capable of dispersing over several hundred kilometers (Arditti and Ghani, 2000; Phillips et al., 2012).

For all analyzes, pairwise population genetic distances were generated using the distance metric of Cavalli-Sforza and Edwards (1967), with correction by the INA method implemented in FreeNA and described in Chapuis and Estoup (2007). For MMR, distance matrixes reflecting geography and environmental features were created in the following manner. The pairwise population geographic distance matrix was created using GPS coordinates and the distGeo() function in the geosphere package v. 1.5 (Hijmans et al., 2021) in R (R Core Team, 2022). These geographic distances were log-transformed to reduce the impact of the largest distances. For the environmental dataset we considered elevation, four soil factors that were important in the NMS ordination (i.e., POM, pH, Pppm, and PBSK), and five uncorrelated ($r^2<0.70$) climate variables (i.e., precipitation in the warmest quarter, precipitation seasonality, mean temperature of the coldest quarter, mean temperature of the driest quarter, and mean temperature of the wettest quarter) sampled from 30-s layers of the WorldClim data set (Fick and Hijmans, 2017) for all locations considered in this study. The environmental dataset was subjected to principal component analysis (PCA) using R (R Core Team, 2022). Values for each site along the first two axes, which accounted for 49% of the observed variation, were used to construct an environmental distance matrix based on Euclidean distances in Passage 2 (Rosenberg and Anderson, 2011). Multivariate MMR was conducted using the MMRR script of Wang (2013) in R (R Core Team, 2022) using the explanatory matrixes of geographic distance and environmental distance and the response matrix of genetic distances. Significance was tested using 9,999 permutations.

For dbRDA, the geographic distance matrix was used in a principal coordinates of neighbor matrices (PCNM; Borcard and Legendre, 2002; Borcard et al., 2004) in R (R Core Team, 2022) with default threshold values to generate a set of independent variables reflecting spatial relationships among the populations. The positive PCNM axes were retained and tested as predictors of genetic distance in dbRDA. To test for IBE, we used the first two axes from a principal component analysis (PCA) of the environmental variables, as described above, as predictors of pairwise genetic distances. The explanatory variables were assessed independently in marginal tests and conditioned on geographic distance to account for potential correlation between environmental factors and geography. The

dbRDA analyzes were conducted using the capscale () function of the Vegan package (Oksanen et al., 2020) in R (R Core Team, 2022). An analysis of variance was used to evaluate significance of each model. The varpart() function in R (R Core Team, 2022) was used to assess the contribution of each environmental and geographic variables to genetic distances.

3. Results

3.1. Flower morphology

In the k-means cluster analysis, a two-group solution reduced percent variation of within-sum of squares to 29.6%. Subsequent clusters further reduced variation by <10%. The Duda-Hart test indicated that the optimal clustering solution contained more than one group ($DH_K = 0.3555$, alpha = 0.99), and the Calinski-Harabasz index also was greater for two clusters. Thus, two clusters were selected as the optimal solution.

The two-cluster analysis separated populations with significantly different lip and spur lengths, which were correlated among, but not within, groups (Figure 3C). In both groups, spurs were significantly longer (by >20%) than lips, however, one group had 20% longer lips and spurs than did the other group, and thus larger flowers (Figures 3A,B). A comparison of inflorescence size among three populations of small-flowered plants and four populations of large-flowered plants (among three habitats) found that small-flowered plants also had smaller inflorescences ($N = 37$, mean = 26.95, se = 1.60) than did large-flowered populations ($N = 52$, mean = 38.85, se = 2.25); nested ANOVA of ln-transformed data: $F_{2,82} = 28.77$, $p = 0.033$. The correspondence between habitats and flower morphology is shown in Figure 2F. The group with smaller flowers comprised populations occurring only in muskeg bog habitat (Figure 2D). The group with larger flowers included all fen, meadow, and anthropogenic habitats, as well as a single muskeg site that occurred at high elevation and is a transition to alpine habitat (Figures 2A–D).

Populations showed strong differentiation in spur length and lip length as P_{ST} values were 0.94 (95% CI: 0.930–0.959) for spur length and 0.97 (95% CI: 0.969–0.980) for lip length. These estimates were robust to variation in our selection of the value for c/h^2. The critical value of P_{ST}, whereby quantitative traits are more strongly reflective of selection than genetic drift, occurred at $c/h^2 < 0.5$ (Supplementary Figure S1).

3.2. Soil chemistry and fertility

Most soils variables differed significantly among habitat groups (Supplementary Table S3). Percent organic matter had the strongest differentiation. It was significantly greater in muskeg (64%) intermediate in fen (37%), and lower (< 10%) in meadow and anthropogenic habitats. Other significant variables (pH, CEC, ppm Ca, and PBS Ca) were greater in anthropogenic habitat and not different among other habitats. Ca was about 400% higher in anthropogenic habitat, where it reached 1783.5 PPM. Though it did not differ significantly ($p = 0.115$), PBS K tended to be higher in meadow habitat, where it reached 4.9%.

Non-metric Multidimensional Scaling reached a stable solution for ordination of two axes after 88 iterations (Figure 2E). Only CEC and PBS Mg were not significantly correlated with either ordination axis (Supplementary Table S4). On Axis 1, muskeg bog habitat was strongly associated with positive axis scores and highly correlated with increasing POM and PHS, while anthropogenic roadside habitat was strongly associated with negative axis scores and highly correlated with increasing pH and PBS *Ca*. Meadow habitat was most strongly associated with Axis 2 and greater PBS K, but also tended to be associated with increasing PPM P along Axis 1. Fen habitat was centrally located and intermediate with respect to soil chemistry and nutrients. With MRPP, all habitats had significantly different multivariate distributions, which remained different with anthropogenic habitat excluded from the model (Figure 2E).

3.3. Genetic variation and population structure

Among the 728 inter-locus comparisons, there were six instances of significant genetic disequilibrium identified in three populations. No locus pairs exhibited significant disequilibrium in multiple populations, but one population (i.e., ELO) did have four loci out of equilibrium. Overall, these results suggest that the loci are genetically independent, and that instances of linkage disequilibrium are likely due to demographic factors unique to the affected populations. Seven populations had a deficiency of heterozygotes consistent with deviation from Hardy–Weinberg equilibrium (Table 1). Mean genetic diversity was significantly higher in populations assigned to the large-flowered group than those assigned to the small-flowered group (excluding the four hybrid populations) when considering N_a, H_O, and H_E but not for % P or the inbreeding coefficient (Table 1).

AMOVA assigned most of the observed genetic structure within populations (79–80%), then among populations (15–16%), and between the small and large-flowered groups (6%) or among the habitats (5%; Table 2). All F-statistics were significant ($p < 0.01$). The optimal number of groups, based on Bayesian analysis in STRUCTURE of the genetic variation, was two clusters (Figure 4A). The two clusters primarily align with large and small-flowered populations, although samples from four small-flowered populations (i.e., PBMK, CL, BM, and DM) were placed in the cluster with large-flowered populations. Most individuals (80%) were assigned to a single cluster with Q-matrix values >0.95.

Our analyzes indicated admixture between the phenotypic groups. This was most extensive for the four small-flowered populations that are genetically-like large-flowered populations as all but seven samples from these populations were assigned to this cluster with Q-matrix scores >0.9 by STRUCTURE, but other instances of admixture were also noted for most populations (Figure 4A). In fact, based on a cut-off of 0.9 in Q-matrix scores from STRUCTURE, all but four populations (i.e., HA, HMA, HMM, and ML) contained at least one admixed individual, resulting in *ca.* 10% of all individuals assigned as admixed. NewHybrids produced nearly identical results to those from STRUCTURE (Figure 4B), although seven populations were not predicted to contain admixed individuals by this analysis. NewHybrids also estimated more extensive hybridization in the small-flowered IR population compared to

TABLE 2 Results from an analysis of molecular variance based on allelic diversity among populations of *Platanthera dilatata* from Southeast Alaska.

Source	df	SS	MS	Percent of variance
Flower groups				
Among groups	1	92.542	92.542	6
Among populations	24	383.237	15.968	15
Within populations	936	1830.080	1.955	79
Total	961	2305.860	2.494	100
$F_{RT} = 0.063$, $P < 0.001$; $F_{SR} = 0.163$, $P < 0.001$; $F_{ST} = 0.216$, $p < 0.001$				
Habitat				
Among groups	3	122.659	40.886	5
Among populations	22	353.120	16.051	16
Within populations	936	1830.080	1.955	80
Total	961	2305.860		100
$F_{RT} = 0.045$, $P < 0.01$; $F_{SR} = 0.165$, $p < 0.01$; $F_{ST} = 0.202$, $p < 0.01$				

Groups were designated by (a) flower size and (b) habitat.

STRUCTURE, which identified admixed genotypes in only four individuals in this population.

Further support of two genetically divergent groups was found in the estimated immigration rates, which were extremely low between large-flowered and small-flowered populations. Both BayesAss and Geneclass suggested that at least 97% of the individuals in each of these groups originated within their assigned group (Supplementary Table S5). BayesAss, but not Geneclass, indicated strongly unidirectional immigration from the large-flowered group into the hybrid populations. Only a low level of immigration was detected into the hybrid group from the small-flowered group, even though these populations share a common habitat type and are morphologically similar. Additionally, low levels of recent immigration were detected between large-flowered and small-flowered groups and from the hybrid group by GeneClass.

Landscape genetic analyzes indicated that at the regional level, both geographic distance and environmental factors are predictive of genetic structure. In MMR analysis at this scale, the multivariate model placed geographic distance as the strongest factor, with environmental distance slightly less important but still significant. The overall r-square for this model is 0.27, but it is significant ($p = 0.0002$). Comparable results were obtained with dbRDA, with geographic distance explaining slightly more variation than environmental factors when considered independently (Table 3). Despite a correlation between geographic distance and environmental distance, environmental factors do remain significant in the dbRDA conditioned on geographic distance. At a smaller local scale, geographic distance was not a significant factor explaining genetic structure among populations, but environmental factors were in both MMRR and dbRDA (Table 3; Supplementary Figure S3).

TABLE 3 Results from distance-based Redundancy Analyzes (dbRDA) testing the effects of geographic distance (Geo) and environmental factors (Env) on genetic distance among the populations of *Platanthera dilatata* surveyed in Southeast Alaska.

	Marginal test			Conditional test		
Variable	*F*	*P*	% Variation	*F*	*P*	% Variation
Full						
Geo	1.904	0.01	26.62			
Env[a]	2.83	0.001	19.75	2.13	0.017	18.30
Central						
Geo	1.341	0.141	20.09			
Env[a]	3.126	0.003	26.89	2.334	0.026	25.00

[a]For conditional tests, the contribution of environmental factors was considered after removing the covariate effects of geographic distance. The analyzes were run on the full set of populations (Full) and the centrally located populations only (Central).

4. Discussion

No previous assessment of *P. dilatata* has concurrently examined genetic structure, morphological diversity, and habitat characteristics in populations with a shared regional geography. Examination of populations at this scale provided greater understanding of intraspecific variation for this species and of the environmental factors that may influence morphological and genetic variation. We note several novel results: (1) flower phenotypes are strongly associated with habitats, (2) there is a deep genetic divergence between small-flowered and large-flowered forms, (3) nevertheless, admixture has occurred between populations harboring different phenotypes and introgression is deeply rooted in some populations, and (4) whereas IBD and IBE both contribute to significant population genetic structure at regional scales, among closely spaced populations, environmental factors are stronger determinants of genetic structure.

4.1. Phenotypic variation in relation to environmental factors

Platanthera dilatata has long been recognized as a morphologically variable species (Luer, 1975; Sheviak, 2002). Within Southeast Alaska, *P. dilatata* populations have variable flower morphology, yet the phenotypes are partitioned by habitat (Figures 1, 2). Plants with inflorescences containing fewer flowers and flowers with shorter lips and spurs have a narrow habitat niche as they are restricted to muskeg bogs, whereas plants with larger inflorescences and flowers with longer lips and spurs have a broader habitat niche, occurring across a habitat gradient that is exclusive of muskeg bogs except at extremely high elevations.

The high estimates of P_{ST} for lip and spur lengths relative to F_{ST} is suggestive of divergent selection on flower morphology. When morphological variation is partitionable across populations, selection by pollinators has been documented as an underlying mechanism promoting its retention in *Platanthera bifolia* (Boberg et al., 2014), *Disa draconis* Sw. (Johnson and Steiner, 1997), and *Gymnadenia odoratissima* (L.) Rich. (Sun et al., 2014). Though

flowers of *P. dilatata* fit the primitive "settling moth" syndrome characteristic of Noctuidae moths (Hapeman and Inoue, 1997), regional differences in primary and secondary pollinators readily occur. This variation includes seven Noctuidae species and a Hesperiidae butterfly in Newfoundland, Canada (Boland, 1993), a Noctuidae species in Oregon, United States (Larson, 1992), three *Bombus* Latreille bumblebee species, a Noctuidae, and a Nymphalidae butterfly in British Columbia, Canada (Van der Voort et al., 2022), and three Noctuidae, a Sphingidae moth, and a butterfly in Southeast Alaska (this study; Bowles and Armstrong, 2021). Such variation in primary and secondary pollinator types and abundance could drive selection for variable flower morphology at regional and local scales.

Nectar spur length in *Platanthera* determines whether an insect can access nectar and how pollinia are attached and pollen are deposited on the stigma, and selection should shift spur length toward pollinators that maximize fitness (Boberg et al., 2014). Such selection could be rapid if pollinators remain constant and gene flow from other populations is infrequent. In this study, Noctuidae moths were the most commonly observed pollinators on both the small- and large-flowered forms at all elevations. The hawkmoth *Hyles gallii* (Sphingidae) was observed only on large-flowered plants at low elevations and appeared to carry greater pollen loads than did Noctuidae pollinators. Because hawkmoths have a longer proboscis (*ca.* 25 mm; Miller, 1997) than Noctuidae moths (< 11 mm; Zenker et al., 2011; Zhang et al., 2021), they may be more effective pollinators for longer-spurred orchids than are Noctuidae (Tao et al., 2018). Based on iNaturalist observations ($n = 27$), the median elevation at which *H. gallii* has been observed in SE Alaska is less than 50 m (range 1–285 m), much lower than the median elevation (215 m, range 10–600 m) of muskegs in the study area. If *H. gallii* is prevalent in non-muskeg habits, then selection for longer spurs is expected to drive flower morphology to match the most efficient pollinator (Johnson and Steiner, 1997; Boberg et al., 2014; Sun et al., 2014) despite counter-selection from the more frequent Noctuidae moth pollinators across the study area. By contrast, if plants in muskeg bogs are visited only by shorter tongued Noctuidae species, then selection is expected to drive flowers toward shorter spurs and lead to adaptation of those plants to muskeg habitat. Absence or rarity of Sphingidae moths at higher elevations could limit gene flow across an altitudinal gradient. Further work is needed to characterize pollinators and their selection for flower size and nectar spur length for *P. dilatata* across its distribution to test this hypothesis of localized selection.

A strong correspondence between phenotype and soil conditions has not been previously noted for this species and suggests the possibility that other factors might also influence phenotypic variation. While phenotypic plasticity often underlies phenotypic variation that aligns with environmental differences (Schlichting, 1986), it has rarely been documented for flower traits (Sultan, 2000; Pélabon et al., 2011), and the alpine population HMA also occurs in a muskeg-like habitat at high elevation yet retains a large-flowered phenotype similar to populations at lower elevations. This suggests that flower size is not a plastic trait, and that soil fertility does not influence flower size. An alternative hypothesis is that the small-flowered phenotype is a stress tolerant poor competitor that is adapted to the skewed soil chemistry of muskeg habitat. Although bog habitats may appear to have adequate base concentrations, most nutrients are bound in OM in peat soils and are not available for plant uptake, especially under acidic conditions (Verhoeven, 1986; Vitt and Chee, 1990). Indeed PHS, which was relatively high in muskeg (Figure 2E), was negatively correlated with CEC ($r = -0.4046$, $p = 0.0403$). Mycorrhizal fungi may increase efficiency of mineral uptake in peat soils and could provide a competitive advantage as well as a favorable germination site for orchids in these habitats (Rasmussen, 1995). Unlike *Ceratobasidium*, which can utilize N from both ammonium and nitrate, *Tulasnella* requires ammonium as a N source (Fochi et al., 2017). Ammonium is the predominant form of N in dissolved nutrient concentrations in bogs and fens our study area, but it is much more highly concentrated in fens (Fellman et al., 2008). This could explain the greater presence of *Tulasnella* in fens in our study area and might suggest that the larger phenotype uses these fungi. However, it is unknown whether obligate relationships exist between the large and small *P. dilatata* phenotypes and different fungal species. Other habitats associated with the large-flowered phenotype also tended to have greater fertility and association with the *Tulasnella* fungus. If these mycorrhizal fungi occur in different environments because of nutrient availability (Fochi et al., 2017; Thixton et al., 2020) and have strong relationships with *P. dilatata* phenotypes, then they could reinforce their habitat selection. Given the importance of mycorrhizae to orchid life history, research to understand the potential for mycorrhizae to impose selection on orchid phenotypes would also be useful.

4.2. Concordance between morphological differentiation and genetic differentiation

Whereas previous studies identified significant morphological and genetic divergence in *P. dilatata* at broad geographic scales, they have not previously correlated genetic differentiation with phenotypic divisions (Wallace, 2003a; Adhikari and Wallace, 2014). The allelic variation reported in this study provides the strongest indication yet that a shared evolutionary history connects phenotypically similar populations and distinguishes these from phenotypically dissimilar populations. The genetic dataset has also revealed some major differences between the phenotypic groups. For example, small-flowered populations that are not strongly admixed have lower allelic variation and heterozygosity and greater population differentiation compared to large-flowered populations. These results suggest greater isolation, which could occur due to lower density of populations and reduced gene flow over widely spaced muskeg habitats, as non-forest habitats cover only 17% of the landscape in Southeast Alaska.

The deep genetic divergence in phenotypic groups may also reflect historical divergence, perhaps associated with Pleistocene refugia in this area (Carrara et al., 2007; Marr et al., 2008; Geml et al., 2010; Shafer et al., 2010). The Alexander Archipelago of Southeast Alaska contains more than 2,000 individual islands and stretches across 16,000 km of coastline (Carrara et al., 2007), giving the region's extensive topographical and geographical complexity that undoubtedly influences gene flow and population isolation. The impact of the last glacial period was heterogenous across Southeast Alaska, with numerous refugia proposed along the western edges of the Alexander Archipelago and exposed areas of the continental

shelf (Carrara et al., 2007). More recently, successional changes in coastal vegetation were associated with uplift following the Little Ice Age between 1770–1790 (Motyka, 2003). With isostatic changes continuing to occur, extensive uplift meadows may have rapidly developed in the area (e.g., Auffret and Cousins, 2018). While these habitats could represent an earlier successional stage relative to muskeg, they may harbor older genetic lineages if they persisted during glaciation. Phylogeographic studies would be useful to understand the evolutionary and historical connections among populations in the study area and the presence of multiple refugia within the Alexander Archipelago or dispersal from other refugia in northwestern North America.

4.3. Hybridization between divergent phenotypes

While most populations we studied have at least one admixed sample (Figures 4A,B), the extensive and cryptic introgression that characterized several small-flowered populations was unexpected as these populations are morphologically similar to other small-flowered populations sampled in muskeg bogs. While other studies have reported cryptic introgression, for example in *Protea* L. (Mitchell and Holsinger, 2018) and in *Lomatia* R. Br. (McIntosh et al., 2014), these studies also found hybrids with both genetic and morphological intermediacy. In our study, morphologically intermediate populations were not readily detected when averaged across samples. Nevertheless, at the individual level, statistical outliers representing larger flowers were observed in muskeg populations and could indicate admixed individuals due to pollinator-mediated gene flow from large-flowered populations (M.L. Bowles, unpublished data).

Phillips et al. (2012) suggested that seed dispersal between populations at regional scales (e.g., < 250 km) is likely common, but gene flow might be more limited at larger geographic scales. Our analyzes indicated that genetic distance among populations reflects isolation by distance at large scales but not at small scales, consistent with the patterns described by Phillips et al. (2012). The low incidence of admixture detected by STRUCUTRE and NewHybrids in the four hybrid populations suggests that introgression may have occurred swiftly and early in their history. Given the commonality of small-flowered populations in muskeg, we suggest it is more likely that these small-flowered populations were colonized from other small-flowered populations, rather than large-flowered populations. If gene flow occurred early in their establishment and was not maladaptive, then it would persist in the growing population. The alternative explanation for the genetic similarity of hybrid populations to large-flowered populations, that they originated from large-flowered colonizers of muskeg bogs that subsequently evolved smaller flowers, seems less likely in the absence of a functional basis for variation in flower size due to climate or soils.

The contemporary presence of large-flowered populations in more diverse habitats may indicate greater historical abundance across the landscape compared to small-flowered populations and muskeg habitats (Auffret and Cousins, 2018). To produce extensive and cryptic introgression in the small-flowered populations, large-flowered plants would need to be nearby and accessible to pollinators to facilitate repeated introgression and backcrossing with newly colonized small-flowered populations in the area. All but one of the hybrid populations sampled (GI) are located within 250 km of a large-flowered population in the study area, which is consistent with the maximum distance for seed dispersal that was suggested by Phillips et al. (2012).

While seed dispersal may have led to colonization of small-flowered forms in areas containing large-flowered populations, pollinators must be the agents of gene flow leading to introgression. Different spur lengths are expected to reduce cross-pollination between phenotypes, but not to prevent it. Because hawkmoths are strong fliers that may easily cross between habitats, gene flow may be more easily mediated from larger flowers to smaller flowers. Noctuidae moths can also transport pollen long distances (Hendrix et al., 1987), but this may be more likely during migration. Whereas pollinaria adhere to the proboscis of both pollinators, they would adhere closer to the eyes of Noctuidae moths visiting longer-spurred flowers of *P. dilatata* (Figure 3) than for hawkmoths. In short-spurred flowers, positioning of pollinaria closer to the proboscis tip for hawkmoths might facilitate contact with the column leading to successful cross-pollination. Thus, even occasional visits to these populations by hawkmoths carrying pollinia from long-spurred populations could have long-lasting impacts because an orchid pollinarium contains enough pollen to fertilize thousands of ovules. Many inter-specific hybrids are known within *Platanthera* (Wallace, 2003b; Brown, 2004; Alcantara et al., 2006; Brown et al., 2008; Wettewa et al., 2020; Hartvig et al., 2022), indicating that spur length does not consistently prevent cross-pollination and pollinators readily move pollen between species.

4.4. Genetic structure and factors influencing gene flow

Factors determining genetic structure may vary across the landscape and across spatial scales. We expected that across the extent of the study area, which is nearly 500 km, geographic distance would be important because of limited gene flow. By contrast, within the areas where seed dispersal can occur over shorter distances or pollinators are capable of flying between sites, environmental factors are expected to be more important determinants of genetic structure. In the study area, both elevation and habitat differences might influence gene flow at varying scales. When considering all populations, both geographic distance and habitat (i.e., elevation, soils, and climate) are significant predictors of genetic distance. Nevertheless, consistent with our hypothesis, geographic distance explained more of the observed variation in genetic distances than environmental factors did (Table 3; Supplementary Figure S2). The extensive topographic variation of Southeast Alaska could impose barriers to gene flow if orchid seeds are not able to move between mountains and the orchids are adapted to soil types or interact with other organisms, e.g., mycorrhizae or pollinators, that are themselves restricted by environmental factors.

At a smaller spatial scale encompassing the central populations, *ca.* 50 km north-to-south, we found that geographic distance was not predictive of genetic distance. This suggests that seeds and/or pollinators readily move about populations at this scale. By contrast,

environmental factors were found to significantly influence genetic distance, indicating the presence of habitat barriers to successful movement. This is expected if the small and large-flowered populations are adapted to different habitats or are limited by symbiotic partners that are themselves adapted to these differing habitats as noted for larger geographic scales. The difference in the pattern of genetic structure across spatial scales demonstrates a complexity of landscapes in how they influence population connectivity. While these results suggest that orchid seeds and or pollen may readily move about, we are unable to discern the relative importance of these factors for gene flow. Additionally, the complex history of this region has undoubtedly impacted the patterns observed today, but without a phylogeographic context we also cannot account for how historical factors have influenced the genetic structure of *P. dilatata* in this region. Future studies that test hypotheses about the locations of glacial refugia are important foci for future studies of this species across western North America.

4.5. Taxonomic implications

With four habitats and two phenotypic groups, eight unique combinations could characterize *P. dilatata* in Southeast Alaska. Yet, we found only five of these combinations as small-flowered plants are restricted to muskeg bogs and large-flowered plants are rarely found in these habitats. The deep genetic divergence between groups of populations supports the inference that there are multiple evolutionary lineages in the study area. Nevertheless, placing these lineages within the current taxonomy of this species is difficult. Plants from Southeast Alaska have a mean spur length that exceeds lip length (Figure 3A), which would place all of them in var. *leucostachys*. Yet, the range of spur and lip lengths measured on plants in the study area (spurs: 6.5–9 mm for small-flowered and 9–12 mm for large-flowered; lips: 5.25–7.25 mm for small-flowered and 7–9.5 mm for large-flowered) encompasses or exceeds the lengths described for the three varieties by previous authors (Sheviak, 2002; Wallace, 2003a; Sears, 2008; Adhikari and Wallace, 2014; Supplementary Table S6) but lie primarily within vars. *dilatata* and *leucostachys*. These morphological measurements are not consistent with varietal circumscriptions by Sheviak (2002) or the suggestion that three varieties occur in Southeast Alaska.

An additional consideration in metric comparisons among studies is the presence of artifacts associated with measurement methods. It is difficult to measure nectar spur length because they are falcate; thus, intact spurs will appear shorter than flattened spurs. The source of the flowers for measurement (i.e., fresh, dried, or spirit-preserved) also influences measurements as preservation can introduce distortions (Bateman et al., 2013), and spur length has been reported to increase over the flowering season in individual plants (Sheviak, 2002).

Taxonomic revision of *P. dilatata* is warranted because the division of three varieties is inadequate to explain the variation encountered in many areas of the distribution. Furthermore, ecological or pollination studies should explicitly include morphological measurements of samples, rather than simply giving a varietal designation, as this would provide more transparency in morphological variability of studied populations. Such data would also contribute to a greater ability to synthesize variation at local scales, which is needed to evaluate the cohesiveness of *P. dilatata* populations and to quantify the geographic scale of discord in morphological and genetic divergence.

5. Conclusion

By studying genetic, morphological and habitat diversity at the regional scale in *P. dilatata* we have identified novel patterns, yet consistency with previous studies on this species. Strong genetic divergence between flower groups suggests the presence of distinct evolutionary lineages within Southeast Alaska. Evidence of bidirectional gene flow between flower forms, nevertheless, indicates that they are not reproductively isolated. Although orchid seeds are considered capable of long-distance gene flow, our results indicate that gene flow most readily occurs only at shorter geographic distances, perhaps <50 km. Environmental factors also contribute significantly to genetic structure and could reflect adaptations of the orchids themselves to these habitats or adaptations of their symbiotic partners. Further studies are needed to understand the evolution of adaptation in this species and its phylogeographic history. *Platanthera dilatata* should be considered a model system for understanding the process of diversification in temperate orchids.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary files, further inquiries can be directed to the corresponding author.

Author contributions

LW and MB conceived of the study, collected data, wrote the manuscript, and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1085938/full#supplementary-material

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