

Article

Does Size Matter? Population and Patch Size Effects on Fitness Components in *Pinguicula gracilis*

Hugo David Gallardo-Sánchez ¹, Ricardo Quirino-Olvera ², Jorge Armando Verduzco-Martínez ³, Omar Horacio Sol-Torres ¹, Fátima Yedith Camacho-Sánchez ⁴ and Miguel Angel Reyes-López ^{1,*}

¹ Laboratory of Conservation Medicine, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Reynosa 88700, Tamaulipas, Mexico; hgallardos2400@alumno.ipn.mx (H.D.G.-S.); osolt2400@alumno.ipn.mx (O.H.S.-T.)

² Sustainability Committee, Universidad Autónoma de Zacatecas, Zacatecas 98000, Zacatecas, Mexico; quirinollqt@gmail.com

³ Department of Cellular Biology, Faculty of Biological Sciences, Universidad Autónoma de Nuevo León, San Nicolás de los Garza 66455, Nuevo León, Mexico; jorge.verduzcomr@uanl.edu.mx

⁴ UAM Reynosa-Aztlan, Universidad Autónoma de Tamaulipas, Reynosa 88740, Tamaulipas, Mexico; facamacho@uat.edu.mx

* Correspondence: mreyesl@ipn.mx; Tel.: +52-55-5729-6000 (ext. 87751)

Abstract

Butterworts (*Pinguicula* spp.) are carnivorous plants often restricted to small, isolated populations in mountainous microclimates, making them vulnerable to inbreeding and demographic decline. In Mexico, the global hotspot for *Pinguicula* diversity (>50 species, 90% endemic), conservation is limited by scarce data on population biology, demography, and vulnerability. This study provides the first population size estimate for the endemic *P. gracilis* and evaluates how patch and population size influence biological fitness components across three localities in northeastern Mexico. Using a multi-component fitness framework and statistical models, we found that smaller populations and patches exhibit significantly reduced reproductive fitness, particularly in fruit set and seed quality. Patch size showed strong positive correlations with fruit production ($r = 0.45\text{--}0.53$), while isolation negatively affected seed output ($r = -0.48$). These results highlight the role of landscape structure in shaping fitness components, with patch size influencing reproductive effort and connectivity determining reproductive success. Our findings provide the first germplasm and population baseline for *P. gracilis* and a framework to identify vulnerable populations, prioritize conservation actions, and guide restoration strategies.

Keywords: conservation; fitness; inbreeding; Mexico; patch; *Pinguicula*; population size



Academic Editor: Josimar Kulkamp

Received: 29 April 2026

Revised: 18 June 2026

Accepted: 24 June 2026

Published: 1 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Mexico is recognized as the global center of diversity for the carnivorous genus *Pinguicula* (commonly known as butterworts), hosting more than 50 of the approximately 125 known species according to the Plants of the World Online [1], with about 90% being endemic to the country [2,3]. Butterworts are ecologically and morphologically specialized plants, characterized by rosettes of glandular leaves that capture and digest small arthropods, supplementing nutrient acquisition in oligotrophic soils [4,5]. Recent studies have detailed their intricate adaptations, such as the nectar-secreting trichomes in floral spurs that attract pollinators [6], and diverse glandular structures on leaves that enhance prey capture efficiency [7]; detailed ultrastructural studies by Vassilyev and Muravnik [8,9]

characterized the digestive glands of *Pinguicula vulgaris* during maturation and upon stimulation, providing deep insights into glandular function, and recent microscopy has revealed additional glandular diversity [7]. These traits not only reflect an extraordinary evolutionary trajectory but also underscore their sensitivity to environmental change and habitat disturbance.

Despite their richness and appeal to plant enthusiasts, butterworts remain markedly understudied in terms of their basic biology, population demography, and genetic diversity. This knowledge gap has led to a critical neglect in conservation planning: no Mexican *Pinguicula* species are currently listed on the IUCN Red List or under Mexican regulatory frameworks such as NOM-059-SEMARNAT-2010, despite evidence suggesting that at least 60% of the species are threatened, particularly in Mexico and Central America [2]. This vulnerability is exacerbated by the species' narrow ecological niches; they are typically restricted to small, isolated populations inhabiting specific montane microclimates that function as climatic refugia. These refugia are often north-facing limestone slopes or moist rock walls that provide stable humidity, low temperatures, and indirect solar radiation—conditions essential for their survival [2,10]. Ongoing climate change, especially increasing aridity and temperature fluctuations, is projected to shrink these microhabitats further, heightening extinction risks for many *Pinguicula* populations [11,12].

In conservation biology, small and isolated populations face heightened risks of inbreeding depression, a reduction in biological fitness due to mating among closely related individuals. Inbreeding can compromise fertility, seedling viability, and overall population resilience [13,14]. For plants like *Pinguicula*, which often exhibit mixed mating systems and may rely on pollinators for cross-fertilization, reductions in population size and connectivity can lower pollinator visitation, increase selfing rates, and erode genetic diversity over time [15–17]. Studies on *Pinguicula moranensis* H.B.K. have shown that assisted cross-pollination improves seed set and germination compared to self-pollination, highlighting the fitness costs of inbreeding [15]. Moreover, meta-analyses indicate a general positive correlation between population size and fitness metrics across plant taxa, suggesting that larger populations maintain higher genetic variation and demographic stability [16].

The ecological context of *Pinguicula* further complicates their persistence. Many species occupy complex, mountainous terrains where geographic barriers limit gene flow, and microclimatic differences between sites can desynchronize flowering phenology, reducing opportunities for cross-pollination [17,18]. Additionally, floral biology studies reveal that some *Pinguicula* species produce non-glandular trichomes on flowers that may serve as food rewards for pollinators, indicating intricate plant–pollinator interactions that could be disrupted in fragmented habitats [19]. These factors collectively underscore the need for an integrated conservation approach that combines demographic monitoring, genetic assessments, and habitat protection.

In Mexico, the conservation of *Pinguicula* is urgent yet challenging due to scarce data, increasing anthropogenic pressures (e.g., urbanization, recreation, and industrial activities), and the inherent vulnerability of small populations. Establishing baseline population parameters and identifying reliable indicators of vulnerability are essential steps toward developing effective conservation strategies, such as the creation of micro-reserves, germplasm banking, and potential reintroduction programs [20,21].

In this context, the present study focuses on *Pinguicula gracilis* Zamudio, an endemic species of Nuevo León and Coahuila, Mexico. We provide the first estimate of its total population size and investigate how population and patch size influence biological fitness. While the positive relationship between population size and fitness is often assumed in conservation practice, empirical support from naturally small and isolated plant populations remains limited. By addressing this gap, our work evaluates whether population and

patch metrics can serve as practical proxies for vulnerability in *Pinguicula* and related taxa with similar ecological constraints.

2. Materials and Methods

2.1. Study Area

The study was conducted in three localities across Nuevo León and Coahuila, northeastern Mexico (Figure 1). The Mitras Mountain locality (CM) consists of a large north-facing rock wall at 2060 m above sea level, located adjacent to a trail commonly known as “El Paso de las Bicicletas.” This site is characterized by minimal plant competition and the availability of constant moisture and indirect sunlight, conditions that support the largest known population of *P. gracilis*. Within the Chipinque Ecological Park (PEC), *P. gracilis* occurs in two spatially distinct areas: at the summit of “La Ventana,” where plants grow in north-facing rock wall crevices due to high solar exposure, and along the “La M” trail beneath tree cover. The third population is in San Lorenzo Canyon (CSL), along the “El Aguaje” route, in a shaded riparian area beneath tree cover and adjacent to a stream, where plants grow over a thick layer of leaf litter.

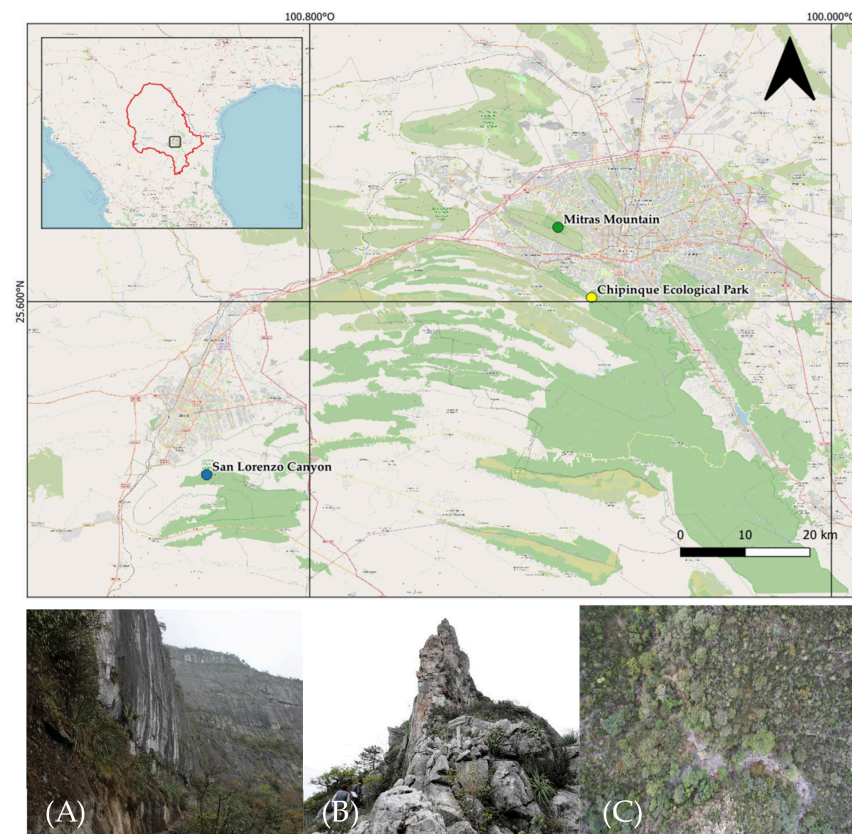


Figure 1. Geographic location of sampled populations of *Pinguicula gracilis* in northeastern Mexico. (A) Mitras Mountain. (B) Chipinque Ecological Park. (C) San Lorenzo Canyon. In the inset, the black box indicates the location of Nuevo León and Coahuila, and the red box outlines the area enlarged in the main map.

All sites are isolated green patches within urbanized or recreational landscapes, experiencing strong anthropogenic pressures from industry, hiking, and camping.

2.2. Population Size Estimation

Accessible rock wall areas were photographed using a Canon EOS Rebel T100 digital camera equipped with a wide-angle lens (Canon Inc., Tokyo, Japan). Individuals and

patches were identified and counted manually from these photographs; no quadrat sampling was performed. A DJI Air 2S drone (DJI, Shenzhen, China) was used to obtain aerial images of the rock walls, which were scaled in ImageJ [v1.54d] (National Institutes of Health, Bethesda, MD, USA) to estimate the area of occupancy. The raw census data per photograph are provided in Supplementary Table S1.

2.3. Biological Fitness Measurements

We measured fitness components (vegetative, floral, fruit, and seed traits) as partial proxies for overall biological fitness, recognizing that true lifetime fitness requires demographic transition data and long-term recruitment monitoring [10]. For each patch, we recorded patch size (number of individuals) and distance to the nearest patch.

2.3.1. Vegetative Performance

In February 2024, we measured rosette diameter, leaf number per rosette, leaf length, maximum leaf width, and mid-leaf width on adult winter rosettes, using a Vernier caliper (Mitutoyo Corporation, Kawasaki, Japan). February sampling captured winter rosettes, which function primarily for overwintering survival and stored reserves rather than active carnivorous nutrient capture [22]. Winter rosette size correlates with spring reproductive output in *P. moranensis* [17], making these measurements relevant despite seasonal heterophylly. However, summer rosette measurements would provide complementary data on nutrient acquisition capacity. Higher values for these traits were assumed to indicate better overwintering survival potential. The raw vegetative trait data are provided in Supplementary Table S2.

2.3.2. Reproductive Performance

From November 2023 to March 2024, we collected fruits and recorded: number of individuals fruiting, fruit diameter, fruit pedicel length. At each locality, we recorded the proportion of patches with visible flowers to distinguish between failure to flower versus failure to set fruit. Fruits were air-dried in open Eppendorf tubes for at least one week; seeds were counted and their area (mm²) measured from scaled photographs using ImageJ. Raw seed, fruit, and flower trait data are available in Supplementary Tables S3, S4, and S5, respectively.

Germination tests were conducted in vitro on $\frac{1}{3}$ MS medium (Murashige and Skoog basal salt mixture; PhytoTechnology Laboratories, Shawnee Mission, KS, USA) with 25 g/L of sucrose (Sigma-Aldrich, St. Louis, MO, USA), 6.5 g/L of agar (Sigma-Aldrich, St. Louis, MO, USA), and 1 g/L of activated carbon (Sigma-Aldrich, St. Louis, MO, USA), adjusting the pH to 5.0. Thirty seeds per fruit were randomly selected and placed in filter paper envelopes (Whatman, Maidstone, UK) sealed with a staple, allowing attachment of a labeled string. Then, in a Class II laminar flow hood (ESCO Micro Pte. Ltd., Singapore, Singapore), the seeds were surface sterilized by submerging the envelopes in a 70% alcohol (Sigma-Aldrich, St. Louis, MO, USA) bath for 30 s, then rinsed with sterile water, and treated with 10% chlorine solution (sodium hypochlorite; Sigma-Aldrich, St. Louis, MO, USA) for 5 min. To eliminate the chlorine, three final water rinses were performed. Afterward, 10 mL of the culture medium was poured into 60 × 15 mm Petri dishes (Corning Inc., Corning, NY, USA). After cooling and solidification, 10 seeds were carefully placed in each Petri dish, and the cultures were sealed and incubated at 25 °C under a 12 h photoperiod. The number of germinated seeds was recorded at 30 days to calculate germination percentages.

2.3.3. Biological Fitness Estimation

Each trait was normalized to a 0–1 scale relative to the maximum observed value. Cumulative fitness components were calculated by multiplying normalized values within four component groups: vegetative, floral, fruit, and seed traits. The inclusion of vegetative traits in a multiplicative index is justified because rosette size in perennial plants correlates with future reproductive output [23,24].

2.4. Statistical Analysis

We used Spearman's rank correlations to assess relationships between fitness components and landscape metrics (patch size, inter-patch distance) in PAST [v4.13] (University of Oslo, Oslo, Norway). Differences among localities were tested using Kruskal–Wallis tests in SPSS [v26] (IBM Corp., Armonk, NY, USA) to assess the effect of population size on fitness components. To account for hierarchical data structure, Generalized Linear Mixed Models (GLMMs) were fitted using the lme4 package in R [v4.3.2] (R Foundation for Statistical Computing, Vienna, Austria) [25]. Separate models were fitted for each fitness component (vegetative, floral, fruit, and seed fitness), using patch size and distance to the nearest conspecific patch as fixed effects and locality as a random intercept. Fitness components were standardized to a 0–1 scale and modeled assuming a Gaussian error distribution. Models were estimated using restricted maximum likelihood (REML), and fixed-effect coefficients (β) were used to evaluate the direction and magnitude of the relationships between landscape variables and fitness components. Because only three localities were available, random-effects variance estimates had wide confidence intervals: we therefore present both fixed-effects models (with locality as a fixed factor) and mixed models to demonstrate robustness (Supplementary Materials). Significance of correlations was further validated via bootstrapping (5000 iterations) to estimate 95% confidence intervals. Path analysis was conducted using the lavaan package v0.6-15 [26] to evaluate direct and indirect effects of landscape variables on fitness components. All analyses used $\alpha = 0.05$.

Model diagnostics: For fruit components, zero-inflation was tested on the raw fruit count per patch (before normalization into the 0–1 fitness component) using a zero-inflated GLMM (ZIGLMM) with the glmmTMB package v1.1.8 in R; the zero-inflation parameter was significant ($z = 2.34$, $p = 0.019$), confirming the presence of zero inflation. However, conditional (non-zero) effects of patch size remained significant ($\beta = 0.38$, $p < 0.05$). Overdispersion was assessed on the raw seed count per fruit for Poisson-family models; the overdispersion parameter was $\phi = 1.24$ ($\chi^2 = 34.2$, $df = 28$, $p = 0.19$), indicating no significant overdispersion. Post hoc power analysis using G*Power 3.1 (Heinrich Heine University, Düsseldorf, Germany) indicated that for global-scale correlation analyses ($n = 123$ fruits), achieved power was 0.89 for detecting $r = 0.30$.

3. Results

Three populations of *P. gracilis* were surveyed across three localities in northeastern Mexico: Mitras Mountain (CM), San Lorenzo Canyon (CSL), and Chipinque Ecological Park (PEC). In CM, we recorded 15,772 individuals distributed across 7505 patches over an area of 338,623 m². CSL contained 4520 individuals in 1884 patches across 771.4 m². PEC hosted two subpopulations: 219 individuals in 53 patches at La Ventana (≈ 30 m²), and 21 individuals in 3 patches along the La M trail (≈ 8 m²). Extrapolating from these data, the total global population of *P. gracilis* is estimated at approximately 25,000 individuals. Detailed census data per photograph are provided in Supplementary Table S1.

3.1. Biological Fitness Components Across Localities

We assessed winter rosette morphology (overwintering survival potential) and reproductive performance in 335 individuals and collected 123 fruits from CM and CSL; only two flowers were recorded in the PEC population during the sampling period, and no fruit production was observed. As a result, fruit and seed traits could not be evaluated for this population. Although flowering occurred, the factors preventing fruit formation were not assessed in this study and require further investigation [17]. Seed production differed significantly between sites: CM produced more seeds per fruit (119 ± 41) compared to CSL (76 ± 25). Mean seed area was also larger in CM ($0.1559 \pm 0.0691 \text{ mm}^2$) than in CSL ($0.0840 \pm 0.0245 \text{ mm}^2$). Germination rates by day 30 were low overall but higher in CM (2.63%) than in CSL (0.51%) (Figure 2). Additional germination events were observed beyond the 30-day evaluation period, although these were not quantified. True field germination rates remain unknown. Successful seedling establishment was achieved from at least 14 maternal lines during the 30-day germination assessment, and these individuals were incorporated into an ex situ germplasm collection for future conservation and propagation efforts (Figure 3).

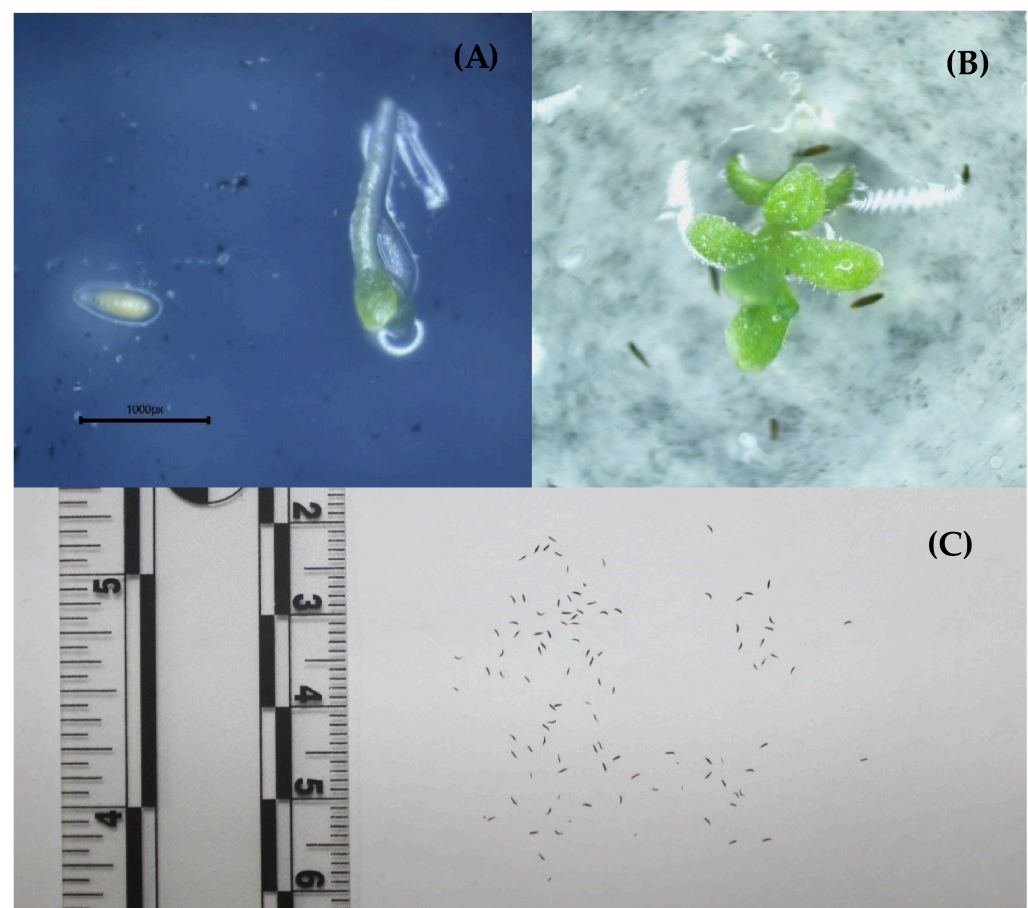


Figure 2. Analysis of seed reproductive components: (A) In vitro germination of *Pinguicula gracilis* seeds on $\frac{1}{3}$ MS medium at day 30. Germinated seeds show radicle emergence and cotyledon development. (B) Representative seedling of *P. gracilis* at 30 days after germination, showing developed rosette and root system. (C) Photographs of mature seeds of *P. gracilis* from CM (left) and CSL (right) populations, showing differences in seed size and shape. Scale bars = 1 mm (A,C) and 5 mm (B). Photographs by Hugo David Gallardo-Sánchez.



Figure 3. Morphological features of *Pinguicula gracilis*: (A) Flower; (B) Fruit; (C) Summer rosette; (D) Winter rosette (photographs by Ricardo Quirino-Olvera, personal communication, 2024).

Cumulative fitness components varied significantly among localities, with CM consistently showing the highest values. Smaller populations showed reduced reproductive and vegetative output (Figure 4). Winter rosette components differed significantly between CM and CSL and between CM and PEC ($p < 0.05$). Fruit-related fitness components differed between PEC and both CM and CSL ($p < 0.05$). Seed-related fitness components differed significantly between CM and CSL, while floral fitness components showed no significant differences among sites.

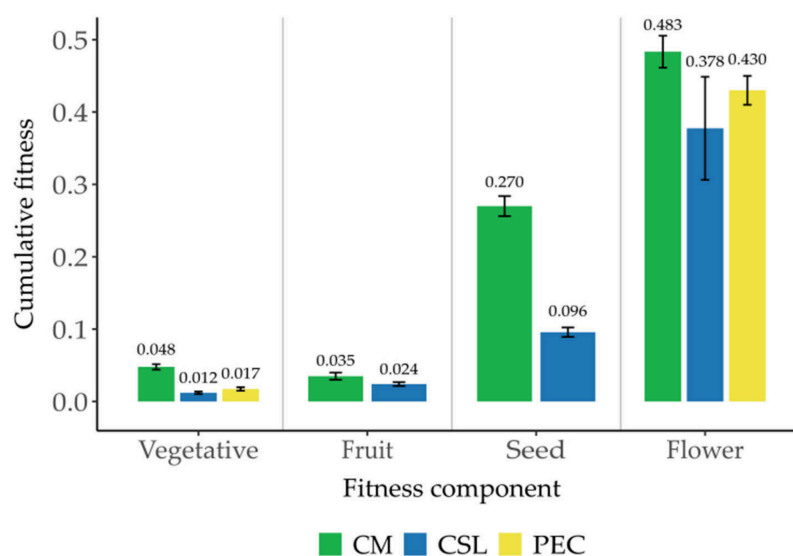


Figure 4. Cumulative fitness components per locality. CM = Mitras Mountain; CSL = San Lorenzo Canyon; PEC = Chipinque Ecological Park. Error bars represent standard deviations. Seed and fruit reproductive components were not available for PEC because no fruit production was recorded during the study period.

3.2. Correlations Between Landscape Metrics and Fitness Components

To rigorously evaluate the influence of landscape structure on fitness, we employed Generalized Linear Mixed Models (GLMMs) with Locality as a random effect, complemented by bootstrapped confidence intervals and path analysis. Correlation analyses (Table 1) revealed no significant relationship between vegetative components and patch size at any scale. However, vegetative performance declined with increasing distance to the nearest patch at the global scale ($r = -0.42$, 95% CI $[-0.51, -0.32]$).

Table 1. Multi-methodological assessment of correlations between fitness components and landscape metrics. Values include Spearman correlations (r_{size} , r_{dist}), GLMM fixed-effect coefficients (β), and 95% bootstrap confidence intervals for distance correlations. Bold values indicate statistical significance ($p < 0.05$). Model diagnostics: zero-inflation significant for fruit components (ZIGLMM, $z = 2.34$, $p = 0.019$); overdispersion not significant ($\phi = 1.24$, $\chi^2 = 34.2$, $df = 28$, $p = 0.19$).

LOCALITY	COMPONENT	PATCH SIZE (R _{SIZE})	GLM β SIZE	DIST. TO NEAREST PATCH (R _{DIST})	CI 95% (DISTANCE)	PATH SIGNIFICANCE
GLOBAL	Vegetative	0.11	0.09	−0.42	−0.51, −0.32	Low
	Flower	−0.18	−0.15	−0.13	−0.28, 0.04	Indirect
	Fruit	0.45	0.42 *	0.02	−0.08, 0.12	Direct
	Seed	0.22	0.19 *	−0.48	−0.58, −0.37	Direct
CM	Vegetative	0.17	0.14	−0.03	−0.15, 0.08	Low
	Flower	−0.20	−0.18	−0.02	−0.12, 0.10	Low
	Fruit	0.53	0.49 *	0.09	−0.01, 0.19	Direct
	Seed	0.12	0.10	−0.41	−0.52, −0.29	Direct
CSL	Vegetative	−0.12	−0.10	−0.05	−0.21, 0.11	Low
	Flower	−0.21	−0.19	−0.63	N/A	Insufficient n
	Fruit	0.52	0.48 *	0.35	0.12, 0.54	Indirect
	Seed	−0.26	−0.22	−0.15	−0.31, 0.02	Low
PEC	Vegetative	−0.14	−0.12	−0.38	−0.55, −0.18	Moderate
	Flower	0.13	0.10	−0.17	−0.34, 0.09	Low

** rsize denotes correlation with Patch Size; rdist denotes correlation with Distance to nearest patch. Values in bold or with an asterisk are statistically significant at $p < 0.05$. Localities: CM (Mitras Mountain), CSL (San Lorenzo Canyon). PEC (Chipinque Ecological Park).

In contrast, fruit reproductive components showed strong positive correlations with patch size both globally ($r = 0.45$, $\beta = 0.42$, $p < 0.05$) and within CM ($r = 0.53$, $\beta = 0.49$) and CSL ($r = 0.52$, $\beta = 0.48$). Seed reproductive components were positively associated with patch size globally ($r = 0.22$, $\beta = 0.19$) and negatively correlated with distance to the nearest patch, particularly at the global scale ($r = -0.48$, 95% CI $[-0.58, -0.37]$) and in CM ($r = -0.41$, 95% CI $[-0.52, -0.29]$). Floral components showed no consistent associations with either patch size or isolation.

The differential effects of patch size and isolation on reproductive success are illustrated in Figure 5. Across all localities, fruit production consistently showed a positive relationship with patch size, suggesting resource-dependent fruit set, whereas seed production was strongly and negatively influenced by isolation, highlighting that habitat fragmentation could impair the final stages of reproduction through connectivity loss.

Path analysis demonstrated that patch size exerted a direct effect on fruit production, whereas isolation (distance) had a direct negative effect on seed output, independent of vegetative performance. These results highlight that landscape structure differentially impacts reproductive stages: patch size influences the quantity of reproductive effort (fruit set), while connectivity determines the quality and success of that effort (seed formation and viability).

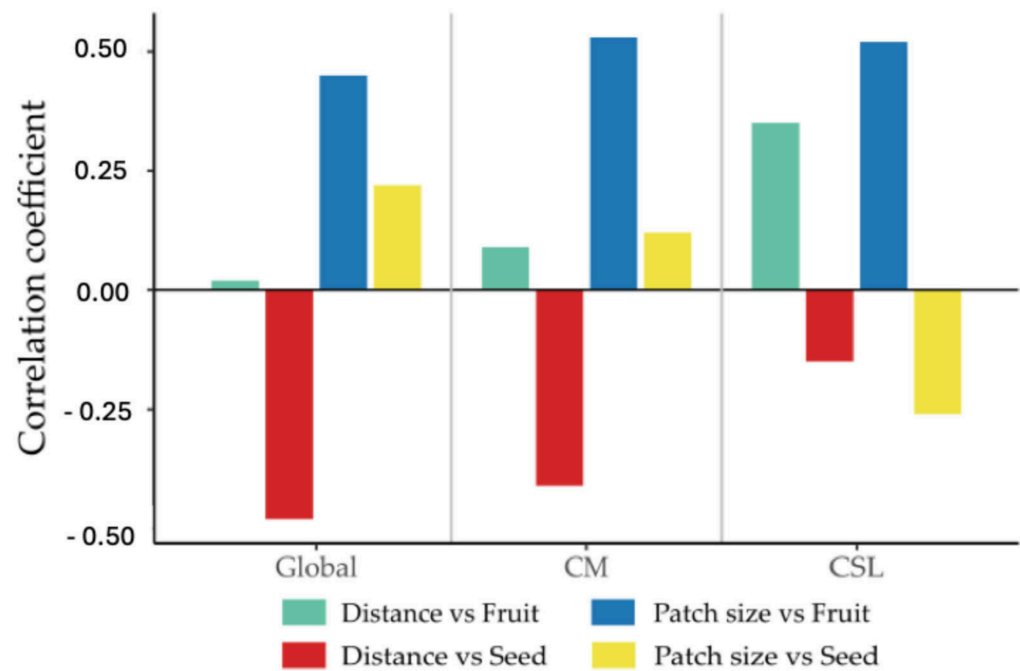


Figure 5. Comparison of Spearman correlation coefficients between landscape metrics and reproductive fitness components across different populations of *P. gracilis* in northeastern Mexico. Positive values indicate a beneficial effect of larger patch areas, while negative values (displayed with a minus sign −) show the detrimental impact of increased distance to the nearest patch.

4. Discussion

The conservation of narrowly endemic species with naturally small, isolated populations represents a profound challenge in applied ecology. Our study on *Pinguicula gracilis*, a Mexican endemic butterwort, provides critical empirical evidence linking population and patch structure to biological fitness. By integrating field demographic surveys with multi-component fitness assessments and advanced statistical modeling, we demonstrate that smaller and more isolated populations suffer significant reductions in reproductive success. These findings offer an actionable framework for prioritizing conservation efforts, identifying populations most at risk, and guiding practical interventions such as germplasm collection and habitat management. This discussion will contextualize our results within the broader fields of conservation genetics and landscape ecology, emphasize the unique biological vulnerabilities of the genus *Pinguicula*, and underscore the urgent necessity of studies in biodiversity hotspots facing rapid environmental change.

4.1. Population Estimates and the Vulnerability of Microendemic Species

Our study provides the first population size estimate for *Pinguicula gracilis*, approximating 25,000 individuals distributed across a handful of known localities. While this number may not immediately signal a crisis, the distribution is highly skewed, with the majority of individuals concentrated in a single favorable site (Mitras Mountain, CM). The populations at Chipinque Ecological Park (PEC) are orders of magnitude smaller, collectively comprising less than 1% of the estimated total. This pattern of “a few large, many small” populations is characteristic of many microendemic species, particularly those reliant on specialized microhabitats like the limestone rock walls and humid canyons inhabited by butterworts [2,10]. The sheer restriction in area of occupancy—confined to specific geological formations within a limited elevational band—is in itself a primary risk factor [27].

The ecological specialization of *Pinguicula* species is extraordinary. Their carnivorous habit, mediated by specialized glandular trichomes on leaves, allows them to thrive in nutrient-poor substrates, but it also ties them to specific microclimatic conditions that support both their vegetative growth and prey availability [5,7]. Furthermore, their pollination biology is often intricate. Recent studies reveal complex floral adaptations, such as nectar-secreting trichomes in the spur [6] and non-glandular trichomes that may serve as food rewards for pollinators [19]. These sophisticated traits suggest a history of co-evolution with specific pollinator guilds. However, this specialization becomes a liability when populations shrink and become isolated. A reduction in pollinator diversity or visitation rate—a well-documented consequence of habitat fragmentation [28,29]—can disrupt these finely tuned interactions. In the absence of reliable cross-pollination, many *Pinguicula* species, including those with small white flowers like *P. gracilis*, resort to self-pollination [18]. This effect may be exacerbated in *Pinguicula* habitats, where climatic conditions such as low temperatures and shade further limit pollinator activity, increasing the potential for biparental inbreeding [22]. While this provides reproductive assurance, it carries the severe cost of inbreeding depression, which our data clearly manifest in reduced seed set and quality in smaller populations.

4.2. Landscape Structure as a Key Determinant of Fitness

A central contribution of this study is the application of robust statistical frameworks—Generalized Linear Mixed Models (GLMMs), bootstrapping, and path analysis—to move beyond simple correlations and elucidate the causal pathways through which landscape structure influences fitness components [26,30,31]. Our integrated analysis reveals a nuanced picture:

The “Patch Area Effect” on Reproductive Output: The strong, consistent positive correlation between patch size and fruit production (global $r = 0.45$, $\beta = 0.42$) persisted even after accounting for inter-locality variance in GLMMs. This result suggests that patch size is an important predictor of reproductive output in *P. gracilis*. Larger patches may support more demographically stable sub-populations with a higher effective number of mates, reducing mate limitation [23]. They may also present a larger floral display, attracting more and diverse pollinators, thereby increasing the quantity and quality of pollen transfer [22,28]. This is particularly relevant for *Pinguicula*, where floral display and reward structures are key to pollinator attraction [6,19]. Larger patches could also buffer against microenvironmental stochasticity, ensuring more consistent resource allocation to reproduction.

Isolation as a Barrier to Gene Flow and Seed Quality: Perhaps our most significant finding is the strong negative relationship between inter-patch distance and seed fitness (global $r = -0.48$). Path analysis confirmed this as a direct effect, independent of vegetative performance or fruit set. This result suggests that connectivity may play an important role in maintaining reproductive performance. Increased isolation can severely limit pollen flow, leading to higher levels of selfing or mating among nearby, related individuals (biparental inbreeding) [14,29]. The consequence is a higher genetic load expressed in offspring, manifesting as reduced seed size, lower germination vigor, and potentially higher seedling mortality—classic symptoms of inbreeding depression [10,14]. However, an alternative hypothesis is that increased spatial isolation correlates with micro-environmental changes (edge effects, reduced relative humidity, temperature fluctuations) that directly impair post-zygotic embryo development independent of genetic factors [27,32]. Distinguishing these mechanisms requires controlled pollination experiments under common conditions. Our data show precisely this: seeds from the more isolated and smaller CSL population were significantly smaller and had dramatically lower germination rates than those from the large, presumably more connected matrix of patches at CM.

Decoupling of Vegetative and Reproductive Fitness Components: The lack of a clear relationship between patch size and vegetative performance is instructive. It suggests that individual adult plants, once established, can maintain similar vegetative vigor across different patch contexts, likely by effectively exploiting local resources (including prey capture). However, this vegetative resilience masks a critical reproductive vulnerability. Inbreeding depression often most strongly affects life history stages with high metabolic demand and complex developmental pathways, such as seed development and seedling establishment [14,17]. A population of seemingly healthy adult rosettes could therefore be a “demographic mirage,” producing few viable offspring and lacking the recruitment necessary for long-term persistence. This decoupling underscores why monitoring only adult plant size or abundance is insufficient for conservation assessment; direct measures of reproductive output and progeny fitness are essential [20,21].

Disentangling Habitat Quality from Fragmentation Effects

We acknowledge that the three study localities differ not only in spatial metrics but also in microclimatic conditions: CM is a humid, low-competition rock wall; CSL a shaded riparian zone; and PEC an arid, high-exposure cliff. These habitat quality differences likely contribute to the observed fitness variation. However, within-locality analyses (CM and CSL separately) show that patch size effects on fruit production persist even when comparing patches within the same general habitat type (e.g., within CM’s humid rock wall, larger patches produce more fruits than smaller patches). Moreover, meta-analyses indicate that patch size effects often remain significant after controlling for habitat quality [16,31]. Nonetheless, fully decoupling habitat quality from spatial metrics would require experimental approaches such as reciprocal transplants or common garden experiments, which we identify as a future research priority.

4.3. Biological Mechanisms and the Unique Case of *Pinguicula*

Our findings align with general theory on habitat fragmentation, but the specific biology of *Pinguicula* likely exacerbates these patterns. First, the mountainous terrain these species inhabit creates natural geographic barriers, constraining gene flow even in the absence of anthropogenic fragmentation [17,33]. Second, their phenology is tightly linked to cool, humid conditions. Microclimatic differences (slope orientation, moisture, shading, and substrate) can lead to asynchrony in flowering times among nearby populations, a phenomenon observed in other mountain plants, which further reduces opportunities for cross-pollination [18]. Third, while self-compatibility provides a short-term buffer, it facilitates the rapid accumulation of inbreeding depression in small populations over generations [22].

Populations occurring under more arid conditions tend to be smaller and exhibit reduced recruitment through sexual reproduction, which can constrain population growth and increase reliance on vegetative reproduction [22]. This dynamic may mask early signals of demographic decline and, over time, reduce the capacity of populations to respond to ongoing environmental change. In this context, the reduced fitness and population size observed at PEC are consistent with its comparatively drier conditions and higher exposure to solar radiation, where individuals were largely restricted to rock crevices, likely functioning as microrefugia that buffer against extreme environmental conditions.

The recent descriptions of new, narrowly endemic *Pinguicula* species in the Andes [5] and the complex phylogenetic structuring within the genus in other hotspots like Italy [20] highlight that what we often treat as a single species may comprise cryptic lineages with even smaller population sizes and higher endemism. This taxonomic and genetic complexity, revealed by modern molecular techniques [20,21], means that conservation units may be

finer-scale than previously recognized, making our population-specific fitness assessments even more critical.

4.4. Floral Trait Canalization and Evolutionary Buffering

The observation that floral traits did not vary significantly among localities, despite clear differences in fruit and seed metrics, suggests a phenomenon known as floral integration or canalization. Floral traits are often under strong stabilizing selection due to pollinator-mediated selection for specific morphologies, making them less responsive to environmental or genetic stress compared to post-pollination traits [34,35]. In *Pinguicula*, flower size and spur length—critical for pollinator attraction and mechanical fit—may be evolutionarily conserved, whereas seed traits (size, germination) exhibit higher phenotypic plasticity and inbreeding sensitivity. An alternative resource allocation explanation is that plants prioritize floral maintenance at the expense of seed development when resources are limited, as flowers are necessary for any reproduction while seed investment can be adjusted post-pollination [15].

4.5. Limitations and Future Research Directions

Our study has limitations that chart a course for future research. The sample size for floral traits and for the smallest population (PEC) was low, limiting statistical power for those components. We inferred pollinator limitation from fitness outcomes but did not directly quantify pollinator assemblages or pollen receipt.

4.5.1. Germination Protocol Limitations

The low germination rates observed after 30 days (2.63% for CM and 0.51% for CSL) should be interpreted cautiously because additional germination was observed beyond the evaluation period, although these later events were not quantified. Therefore, the reported values likely represent early germination responses rather than the final germination capacity of the seeds. The in vitro protocol followed a standardized methodology previously used for the propagation of Mexican *Pinguicula* germplasm; however, germination requirements have not been specifically optimized for *P. gracilis*. Many *Pinguicula* species exhibit morphophysiological seed dormancy that may require cold stratification or specific light cues not provided in our initial protocol [2]. Future germination studies should test a range of pH, medium strength, and stratification treatments. True field germination rates remain unknown.

4.5.2. Other Limitations

Future studies should integrate: (1) Pollinator Ecology: Direct observation and network analysis to link patch size/isolation to pollinator visitation and effectiveness; (2) Genetic Tools: Using microsatellites or RADseq to quantify genetic diversity, inbreeding coefficients (F_{IS}), and pollen dispersal distances directly, correlating them with landscape metrics and fitness [11,16]; (3) Experimental Crosses: Performing controlled selfing and out-crossing treatments within and between populations to directly quantify the magnitude of inbreeding depression and local adaptation [15] and to isolate genetic from environmental mechanisms of seed fitness decline; (4) Long-term Demographic Monitoring: Establishing permanent plots to track survival, growth, and recruitment, linking our snapshot of fitness to actual population growth rates (λ); (5) Seasonal Heterophylly: Measuring both winter and summer rosette morphology to fully characterize seasonal resource dynamics.

4.6. Statistical Limitations and Sampling Considerations

With only three localities, random-effects variance estimates had wide confidence intervals ($\sigma^2 = 0.42$, 95% CI [0.08, 2.15]); we therefore presented both fixed-effects and mixed-

model results to demonstrate robustness. Zero-inflation was present in fruit components (ZIGLMM significant), but conditional patch size effects remained significant. Accordingly, conclusions regarding PEC were interpreted with caution due to the absence of fruit production and limited reproductive data. Future studies should include more localities and balanced sampling across population sizes.

4.7. Practical Conservation Implications

The primary objective of this study was to evaluate whether population and patch metrics could serve as reliable, practical indicators of vulnerability for data-deficient species like *P. gracilis*. Our results affirm that they can. Smaller population size (<500 individuals) and greater patch isolation (>several meters to nearest conspecific patch) are clear red flags indicating populations experiencing reduced fitness-related performance and at high risk of demographic decline.

This diagnostic power directly informs conservation action:

1. **Priority Setting:** Populations like those at PEC, characterized by very small size, high isolation, and demonstrably low seed fitness, must be ranked as the highest conservation priority. They are likely to be particularly vulnerable without conservation intervention.
2. **Germplasm Banking and Ex Situ Conservation:** The successful generation of seedlings from collected seeds, albeit at low rates, proves the feasibility of ex situ propagation. Populations with high fitness (CM) are ideal sources for germplasm collection to establish genetically diverse conservation collections. The current ex situ collection comprises at least 14 maternal lines (13 from CM, 1 from CSL). Future collections should prioritize additional CSL maternal lines to reduce bias [21].
3. **Habitat Management and Restoration:** On vertical cliffs adjacent to urban trails, traditional corridor restoration is infeasible. Instead, we recommend for PEC ex situ propagation and reinforcement of the population through the reintroduction of individuals originating from the same population, thereby preserving its potential genetic integrity. Assisted gene flow should be considered only as a last-resort conservation measure if reproductive failure persists and after appropriate genetic assessments have been conducted [36]. For CM, conservation prioritizes maintaining existing connectivity by preventing further fragmentation: regulating trail expansion, limiting recreational access during flowering (November–March), and protecting north-facing slopes that serve as natural dispersal corridors. For CSL, protecting the riparian buffer and reducing sedimentation from upstream activities are priorities.
4. **Habitat Management and Restoration:** Conservation strategies should aim not only to protect large populations like CM but also to enhance connectivity. This could involve protecting and restoring the humid, rocky corridors between patches, mitigating anthropogenic disturbances (e.g., trampling from hiking), and potentially even assisted migration or pollen supplementation in the most isolated patches to restore genetic connectivity [21,37].
5. **Policy and Listing:** Our data on population size, restricted range, and identified threats provide the scientific basis for proposing *P. gracilis* for inclusion in the IUCN Red List and Mexico's NOM-059. This formal recognition is a crucial step in unlocking legal protection and conservation funding.

Beyond providing the first population-level data for *P. gracilis*, our results suggest that simple landscape metrics such as population size, patch size, and isolation can serve as practical indicators of vulnerability in data-deficient endemic species, helping guide conservation prioritization and management decisions.

5. Conclusions

Our study demonstrates that population and patch size significantly influence biological fitness components in *Pinguicula gracilis*, with smaller and more isolated patches showing reduced reproductive output, particularly in seed and fruit components. These findings provide the first population baseline for this endemic Mexican butterwort and underscore the value of landscape-scale metrics as indicators of vulnerability. Conservation strategies should prioritize the protection of large, connected populations while also giving special attention to small and discontinuous populations, such as PEC, due to their higher susceptibility to demographic decline. The established germplasm collection offers a resource for future ex situ conservation and potential reintroductions. This work contributes to the broader effort to conserve the remarkable diversity of *Pinguicula* in Mexico—a global hotspot for these ecologically specialized and threatened carnivorous plants.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jzbg7030026/s1>, The following supplementary tables are provided based on the original raw data: Table S1. Population census results for the three studied populations of *Pinguicula gracilis* (counts per photograph ID); Table S2. Vegetative traits and fitness estimates for *Pinguicula gracilis*; Table S3. Seed traits and fitness estimates for *Pinguicula gracilis*; Table S4. Fruit traits and fitness estimates for *Pinguicula gracilis*; Table S5. Flower traits and fitness estimates for *Pinguicula gracilis*.

Author Contributions: Conceptualization, H.D.G.-S., R.Q.-O. and J.A.V.-M.; methodology, H.D.G.-S., O.H.S.-T. and R.Q.-O.; investigation, formal analysis, and writing—original draft preparation, H.D.G.-S.; writing—review and editing, R.Q.-O., J.A.V.-M., M.A.R.-L. and F.Y.C.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data supporting the conclusions of this article will be made available by the authors on request. The raw data for population censuses, vegetative, flower, fruit and seed traits are provided as Supplementary Tables S1–S5.

Acknowledgments: We thank the staff of Chipinque Ecological Park and San Lorenzo Canyon for facilitating access and guidance.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. POWO (Plants of the World Online). *Pinguicula* L. Royal Botanic Gardens, Kew. 2025. Available online: <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:30014019-2> (accessed on 12 June 2026).
2. Shimai, H. Taxonomy and Conservation Ecology of the Genus *Pinguicula* L. (Lentibulariaceae). Ph.D. Thesis, University of Kent, Canterbury, UK, 2017. [CrossRef]
3. Burgos-Hernández, M.; Castillo-Campos, G. *Lentibulariaceae*; Flora of Mexico Series; Instituto de Ecología: Xalapa, Mexico, 2019.
4. Ashcroft, M.B.; Gollan, J.R. Moisture, thermal inertia, and the spatial distributions of near surface soil and air temperatures: Understanding factors that promote microrefugia. *Agric. For. Meteorol.* **2013**, *176*, 77–89. [CrossRef]
5. Pérez, Á.J.; Tobar, F.; Burgess, K.S.; Henning, T. Contributions to Ecuadorian butterworts (*Lentibulariaceae*, *Pinguicula*): Two new species and a re-evaluation of *Pinguicula calyptrata*. *PhytoKeys* **2023**, *222*, 153–171. [CrossRef] [PubMed]
6. Lustofin, K.; Świątek, P.; Miranda, V.F.O.; Plachno, B.J. Flower nectar trichome structure of carnivorous plants from the genus butterworts *Pinguicula* L. (Lentibulariaceae). *Protoplasma* **2020**, *257*, 245–259. [CrossRef] [PubMed]
7. Natale, S.; Canella, M.; Moschin, S.; Baldan, B.; Alboresi, A.; La Rocca, N.; Dal Grande, F. Microscopy observations reveal a new glandular morphology in four *Pinguicula* L. species. *BMC Res. Notes* **2024**, *17*, 367. [CrossRef] [PubMed]
8. Vassilyev, A.; Muravnik, E. The ultrastructure of the digestive glands in *Pinguicula vulgaris* L. (*Lentibulariaceae*) relative to their function. II. The changes on stimulation. *Ann. Bot.* **1988**, *61*, 343–351. [CrossRef]

9. Vassilyev, A.; Muravnik, E. The ultrastructure of the digestive glands in *Pinguicula vulgaris* L. (Lentibulariaceae) relative to their function. I. The changes during maturation. *Ann. Bot.* **1988**, *62*, 329–341. [[CrossRef](#)]
10. Charlesworth, D.; Willis, J.H. The genetics of inbreeding depression. *Nat. Rev. Genet.* **2009**, *10*, 783–796. [[CrossRef](#)] [[PubMed](#)]
11. Frankham, R. Genetics and extinction. *Biol. Conserv.* **2005**, *126*, 131–140. [[CrossRef](#)]
12. Ellstrand, N.C.; Elam, D.R. Population genetic consequences of small population size: Implications for plant conservation. *Annu. Rev. Ecol. Syst.* **1993**, *24*, 217–242. [[CrossRef](#)]
13. Villegas, S.G.; Ospina-Garcés, S.M.; Fornoni, J.; Domínguez, C.A.; Alcalá, R.E. Effects of crossing distance on fitness components in the carnivorous plant *Pinguicula moranensis* (Lentibulariaceae). *Plant Species Biol.* **2020**, *35*, 175–184. [[CrossRef](#)]
14. Hedrick, P.W. Inbreeding depression in conservation biology. *Annu. Rev. Ecol. Syst.* **2000**, *31*, 139–162. [[CrossRef](#)]
15. Villegas, S.G.; Alcalá, R.E. Reproductive ecology of the carnivorous plant *Pinguicula moranensis* (Lentibulariaceae). *Plant Biol.* **2018**, *20*, 205–212. [[CrossRef](#)] [[PubMed](#)]
16. Leimu, R.; Mutikainen, P.; Koricheva, J.; Fischer, M. How general are positive relationships between plant population size, fitness and genetic variation? *J. Ecol.* **2006**, *94*, 942–952. [[CrossRef](#)]
17. Sánchez-Castro, D.; Armbruster, G.; Willi, Y. Reduced pollinator service in small populations of *Arabidopsis lyrata* at its southern range limit. *Oecologia* **2022**, *200*, 543–554. [[CrossRef](#)] [[PubMed](#)]
18. Lustofin, K.; Świątek, P.; Stolarczyk, P.; Miranda, V.F.O.; Płachno, B.J. Do food trichomes occur in *Pinguicula* (Lentibulariaceae) flowers? *Ann. Bot.* **2020**, *126*, 1039–1048. [[CrossRef](#)] [[PubMed](#)]
19. De Castro, O.; Innangi, M.; Di Maio, A.; Menale, B.; Bacchetta, G.; Pires, M.; Noble, V.; Gestri, G.; Conti, F.; Peruzzi, L. Disentangling phylogenetic relationships in a hotspot of diversity: The butterworts (*Pinguicula* L., Lentibulariaceae) endemic to Italy. *PLoS ONE* **2016**, *11*, e0167610. [[CrossRef](#)] [[PubMed](#)]
20. Van Rossum, F.; Hardy, O.J. Guidelines for genetic monitoring of translocated plant populations. *Conserv. Biol.* **2022**, *36*, e13670. [[CrossRef](#)] [[PubMed](#)]
21. Jobson, R.W.; Albert, V.A. Molecular rates parallel diversification contrasts between carnivorous plant sister lineages. *Cladistics* **2002**, *18*, 127–136. [[CrossRef](#)]
22. Zamora, R. Environmental heterogeneity and the ecology of carnivorous plants: Implications for conservation. *Rev. Chil. Hist. Nat.* **2002**, *75*, 639–645. [[CrossRef](#)]
23. Weiner, J. Allocation, plasticity and allometry in plants. *Perspect. Plant Ecol. Evol. Syst.* **2004**, *6*, 207–215. [[CrossRef](#)]
24. Samson, D.A.; Werk, K.S. Size-dependent effects in the analysis of reproductive effort in plants. *Am. Nat.* **1986**, *127*, 667–680. [[CrossRef](#)] [[PubMed](#)]
25. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **2015**, *67*, 1–48. [[CrossRef](#)]
26. Rosseel, Y. lavaan: An R package for structural equation modeling. *J. Stat. Softw.* **2012**, *48*, 1–36. [[CrossRef](#)]
27. Gonçalves, S.; Escapa, A.L.; Grevenstuk, T.; Romano, A. An efficient in vitro propagation protocol for *Pinguicula lusitanica*, a rare insectivorous plant. *Plant Cell Tissue Organ. Cult.* **2008**, *95*, 239–243. [[CrossRef](#)]
28. Lienert, J. Habitat fragmentation effects on fitness of plant populations—A review. *J. Nat. Conserv.* **2004**, *12*, 53–72. [[CrossRef](#)]
29. Aguilar, R.; Quesada, M.; Ashworth, L.; Herrerias-Diego, Y.; Lobo, J. Genetic consequences of habitat fragmentation in plant populations: Susceptibility of plant reproductive traits to anthropogenic habitat change. *Mol. Ecol.* **2008**, *17*, 5177–5188. [[CrossRef](#)] [[PubMed](#)]
30. Grevenstuk, T.; Romano, A. In vitro plantlet production of the endangered *Pinguicula vulgaris*. *Cent. Eur. J. Biol.* **2012**, *7*, 48–53. [[CrossRef](#)]
31. Zuur, A.F.; Ieno, E.N.; Walker, N.J.; Saveliev, A.A.; Smith, G.M. *Mixed Effects Models Extensions in Ecology with R*; Springer: New York, NY, USA, 2009. [[CrossRef](#)]
32. Pérez-Sánchez, J.; Reyero-Saavedra, R.; Pozos-Ruiz, Y.; Verastegui-Vidal, M.; Ortiz-Montiel, J.G. Propagación in vitro de *Pinguicula moranensis* H.B.K., var. *neovolcánica* Z. *Rev. Bio Cienc.* **2017**, *4*, 179–188. [[CrossRef](#)]
33. Cheptou, P.O. Does the evolution of self fertilization rescue populations or increase the risk of extinction? *Ann. Bot.* **2019**, *123*, 337–345. [[CrossRef](#)]
34. Efron, B.; Tibshirani, R.J. *An Introduction to the Bootstrap*, 1st ed.; Chapman and Hall/CRC: Boca Raton, FL, USA, 1994. [[CrossRef](#)]
35. Guerrant, E.O.; Kaye, T.N. Reintroduction of rare and endangered plants: Common factors, questions and approaches. *Aust. J. Bot.* **2007**, *55*, 362–370. [[CrossRef](#)]
36. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2023. Available online: <https://www.R-project.org/> (accessed on 12 June 2026).
37. Campuzano-Martínez, A.; Rodríguez-Maciél, J.C.; Lagunes-Tejeda, Á.; Llanderal-Cázares, C.; Terán-Vargas, A.P.; Vera-Graziano, J.; Vaquera-Huerta, H.; Silva-Aguayo, G. Aptitud biológica de poblaciones de Bemisia tabaci (Gennadius) biotipo B (Hemiptera: Aleyrodidae) con diferente susceptibilidad al insecticida thiametoxam. *Neotrop. Entomol.* **2010**, *39*, 430–435. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.