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Impact of fire on the genetic variability of a natural population of *Stylosanthes hippocampoides* (Fabaceae) in Corrientes, Argentina

Efecto de los incendios sobre la variabilidad genética de una población natural de *Stylosanthes hippocampoides* (Fabaceae) en Corrientes, Argentina

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Abstract

Stylosanthes hippocampoides, a native legume forage species, was first studied by our research team in 2016 to assess genetic variability in natural populations in northeastern Argentina. Recurrent fires in the following years raised concerns about their effects on population genetics. This study aimed to evaluate and compare the genetic variability and structure of *S. hippocampoides* populations before and after fire events, and to infer whether such disturbances influence genetic diversity. Using Geographic Information System tools, we selected one fire-affected population (ASI) and one unaffected population (TC). Genetic analyses were conducted using inter-simple sequence repeat markers, and pre- and post-fire genetic statistics were compared. Greater genetic variability was observed between populations than within them. The TC population exhibited higher intra-population genetic diversity than the ASI population. Genetic variability decreased in ASI post-fire, while TC slightly increased. However, neither change was statistically significant. Genetic structure analysis consistently grouped individuals by population, regardless of fire exposure. These findings provide a foundation for future research on *Stylosanthes*, incorporating additional populations with varied fire histories and examining post-fire recovery processes.

Keywords
Stylo • NEA • ISSR • wildfires • forage legumes

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RESUMEN

Stylosanthes hippocampoides, una leguminosa forrajera nativa, fue estudiada por primera vez por nuestro equipo de investigación en 2016 para evaluar la variabilidad genética en poblaciones naturales del noreste de Argentina. Los incendios recurrentes en los años siguientes generaron interrogantes sobre sus efectos en la genética de las poblaciones. Este estudio tuvo como objetivo evaluar y comparar la variabilidad y la estructura genética de poblaciones de *S. hippocampoides* antes y después de los eventos de incendio, e inferir si tales perturbaciones influyen en la diversidad genética. Utilizando herramientas del Sistema de Información Geográfica, seleccionamos una población afectada por incendios (ASI) y otra no afectada (TC). Se realizaron análisis genéticos utilizando marcadores moleculares de inter secuencias simples repetidas, y se compararon las estadísticas genéticas anteriores y posteriores a los incendios. Se observó mayor variabilidad genética entre poblaciones que dentro de ellas. La población TC mostró una mayor variabilidad genética intrapoblacional que la población ASI. En ASI, la variabilidad genética disminuyó tras el incendio, mientras que TC mostró un ligero aumento; sin embargo, ninguno de los cambios fue estadísticamente significativo. El análisis de estructuración genética agrupó a los individuos por poblaciones, independientemente de la exposición al fuego. Estos resultados proporcionan una base para futuras investigaciones sobre *Stylosanthes,* incorporando poblaciones adicionales con historias de incendios variadas y examinando los procesos de recuperación post-incendio.

Palabras clave
Stylo • NEA • ISSR • fuego • leguminosas forrajeras

INTRODUCTION

Ecological disturbances, whether natural or anthropogenic, can alter key demographic and biological processes, ultimately affecting the distribution of genetic diversity within populations (4). Genetic variability influences all levels of biodiversity, from individual fitness and population viability to species adaptability to environmental change and even speciation. Consequently, the interaction between disturbances and genetic variability carries significant ecological and evolutionary implications (4, 19). Understanding how genetic variability fluctuates in populations exposed to recurrent disturbances is crucial for effective biodiversity conservation and management.

Since mid-2019, northeastern Argentina (NEA) has experienced negative monthly precipitation anomalies, leading to prolonged droughts (33). This situation, combined with fire use in agricultural practices as a vegetation management tool, created favorable conditions for wildfires (35). Notably, recurrent fires in the province of Corrientes affected 12% of its total area (30).

Stylosanthes Sw. (Fabaceae) comprises approximately 50 species adapted to tropical and subtropical regions of the Americas, ranging from the southern United States to Argentina. These species are valued for their use as forage (18), as well as for soil recovery (41) and ecological restoration (36). Eight *Stylosanthes* species are native to Argentina, most of which are found in the NEA (40). As part of a project to conserve native forage legumes in the NEA, our research team evaluated the genetic variability of *Stylosanthes hippocampoides* Mohlenbr. populations in 2016 (34). These populations were later affected by fires (30), providing an opportunity to examine potential genetic impacts.

Genetic consequences of fire on plant populations are diverse and context-dependent, influenced by the scale and frequency of fire events, biological traits and adaptive strategies of the species involved (3). For instance, individuals of some species can survive fire and resprout, resulting in minimal effects on genetic variability. In contrast, other species depend on the formation of seed banks and the genetic variability they preserve for post-fire regeneration (37). Moreover, fires can fragment previously continuous populations, creating barriers that isolate subpopulations. Such fragmentation is expected to reduce gene flow and increase genetic differentiation among populations (9).

In this context, this study analyses the genetic variability and structure of a natural population of *S. hippocampouides* affected by fires, comparing it with a control population not exposed to such events. This would allow inferring whether these disturbances influence population genetic variability and structure. Considering the extent of recent fires in the region, the small population sizes of *S. hippocampoides*, and the time elapsed since such events, we hypothesize that the post-fire ASI population exhibits reduced genetic variability compared to pre-fire levels, likely due to a bottleneck effect. Additionally, we expect the genetic structure of the ASI population to remain unchanged, as the elapsed time may be insufficient for spatial genetic subdivision to occur.

MATERIALS AND METHODS

Stylosanthes hippocampoides is a perennial herb or sub-shrub with a prostrate to semi-erect growth habit, native to grasslands and savannas of Argentina, Brazil, Bolivia, Paraguay, and Uruguay (40). Its recorded lifespan is up to five years, with an estimated generation time of 6-12 months (23). *Stylosanthes* species are self-pollinating or predominantly self-pollinating (29).

Populations evaluated in this study were selected by overlaying geolocations of the populations assessed in 2016 by the research team (34) with fire-affected areas in Corrientes province, identified using historical satellite images from the FIRMS database (15). A fire was recorded at the ASI population site (27°27'58" S; 57°28'12" W) on September 30, 2020, with additional events occurring 0.96 km and 0.24 km away in July 2019. Based on this, the ASI population was selected. As a control, we selected a population within the Private Natural Reserve "Paraje Tres Cerros" (29°6'18" S; 56°56'10" W), which was confirmed to be unaffected by fire (figure 1, page XXX). The ASI site lies on a broad roadside shoulder along National Route 12 (km 1171), about 700 meters from the Santa Isabel stream, subject to human disturbance due to routine roadside maintenance. In contrast, the TC population is located within the protected reserve, on the northern slope of Nazareno Hill. This area, characterized by dry air, high solar radiation, and sparse herbaceous vegetation, is free from human disturbance.

Sample collection was conducted in February-March 2022. Following Silvestri *et al.* (2020), we sampled approximately 10 - 15 adult individuals from each population, ensuring at least 3 meters between individuals. Total DNA was extracted from 200 mg of dry leaves from 10-11 individuals per population, following the protocol of Doyle and Doyle (1987). DNA integrity was confirmed by electrophoresis on 0.8% agarose gel in 1× TAE buffer stained with ethidium bromide (10 mg/ml), and concentration was determined by spectrophotometry. Nuclear DNA was amplified using PCR reactions with the same ISSR primers and conditions described by Silvestri *et al.* (2020). Amplified products were separated on 2% agarose gels in 1× TAE buffer at 60 V for 2 h and stained with ethidium bromide (10 mg/ml). Bands were scored as present or absent to generate a binary matrix based on homologous DNA fragments. Genetic diversity within populations was estimated using the total number of bands, number of private bands, percentage of polymorphic loci, number of different alleles, number of effective alleles, expected heterozygosity, and Shannon's information index. These indices were compared between pre-fire and post-fire periods for each population.

To assess whether differences in genetic variability were statistically significant, we compared the Shannon index and expected heterozygosity using previously published data (34). Given the non-normal distribution of the data, the non-parametric Wilcoxon-Mann-Whitney test was applied (42). Analyses were performed in the R Studio environment (28). Total genetic differentiation was analyzed at the species level, population, and by population combined with collection period using analysis of molecular variance (AMOVA) (11). Nei's distance and genetic identity indexes (21) were calculated between individuals. Based on genetic distance values, Principal Coordinate Analysis (PCoA) was used to visualize the distribution of genetic variation within and among populations. All statistical analyses and diversity indices were computed using GenAlEx 6.5 (24). Genetic differentiation within and between populations was further assessed using Bayesian clustering in STRUCTURE 2.3.4 (26), applying the admixture model with correlated allele frequencies. The analysis used a burn-in of 50,000 iterations followed by 100,000 Markov chain Monte Carlo (MCMC) replications (12). The algorithm was run 10 times for each number of genetic clusters (K), ranging from 1 to 6. The optimal number of clusters (ΔK) was determined following the method of Evanno *et al.* (2005).

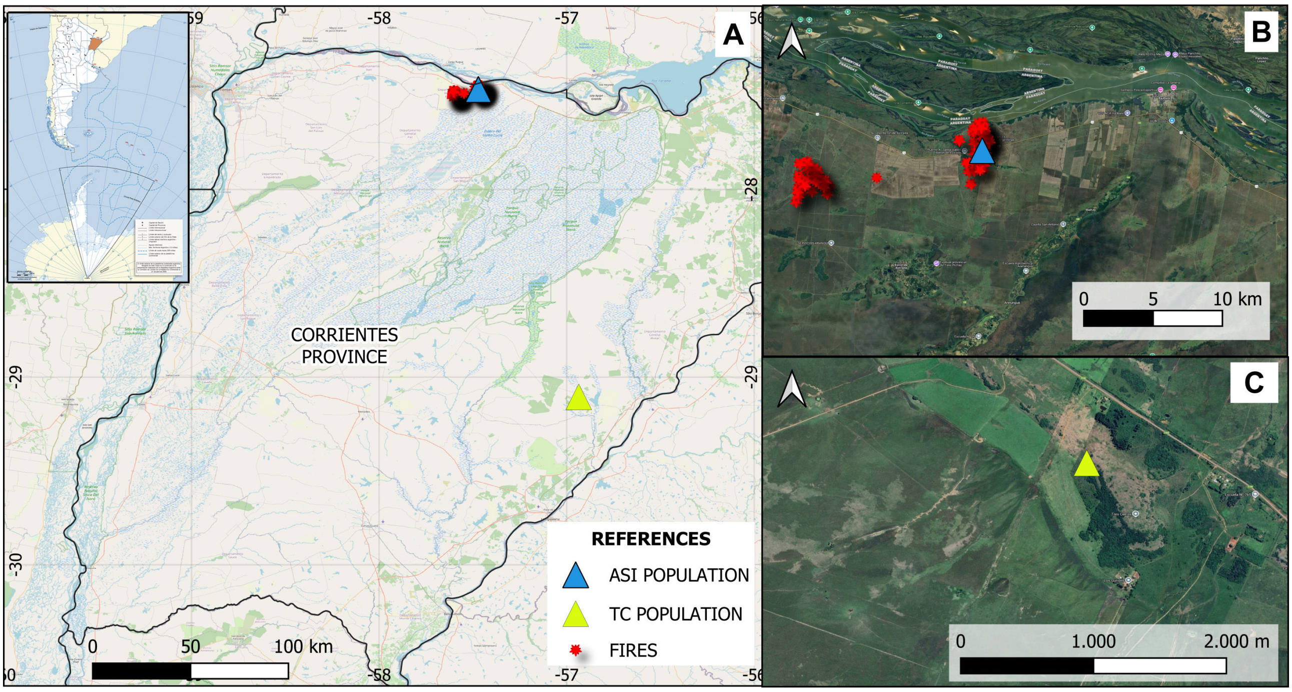

Figure 1. Location of *S. hippocampoides* populations under study and fire hotspots within a 15 km radius of the ASI population location in July 2019 and September 2020 (A). Satellite zoom of the location of the ASI population (B) and the TC population (C).

Figura 1. Localización de las poblaciones de *S. hippocampoides*, y focos de incendio en un radio de 15 km de la localización de la población ASI en julio de 2019 y septiembre de 2020 (A). Zoom satelital de la ubicación de la población ASI (B) y de la población TC (C).

Results

Genetic variability before and after fire

A total of 55 loci were scored in *S. hippocampoides*, of which 62.27 % were polymorphic. At the species level, expected heterozygosity (He) was 0.25, and the Shannon index (I) was 0.37. Intrapopulation genetic diversity was lower in the ASI population compared to TC population.

Based on the same populations and molecular markers, comparisons between pre-fire (2016) and post-fire (2022) periods showed generally similar values. In the ASI population, I, He, and PLP values decreased from 0.20 to 0.17, from 0.13 to 0.11, and from 39.26% to 30.91%, respectively. Conversely, in the TC population, these indices increased: I from 0.17 to 0.23, He from 0.11 to 0.15, and PLP from 33.33% to 38.18%. The Na values increased in both populations, from 0.90 to 1.11 in ASI and 0.74 to 1.24 in TC. The Ne values slightly decreased in ASI (from 1.22 to 1.19) and increased in TC (from 1.20 to 1.27). The TNB decreased in both populations, from 69 to 44 in ASI, and from 56 to 47 in TC. The NPB remained constant in ASI (8 bands) but increased in TC from 1 to 11 bands (table 1, page XXX). However, differences in I and He between pre- and post-fire periods for each population were not statistically significant according to the Wilcoxon-Mann-Whitney test (42) (ASI: $w = 3997$, $p = 0.3393$; TC: $w = 3452$, $p = 0.374$).

Table 1. Descriptive statistics of genetic variability of each population before (pre-fires, year 2016) and after fire events (post-fires, year 2022).

Tabla 1. Comparación de los estadísticos descriptivos de la variabilidad genética de cada población antes (pre incendios, año 2016) y después de los incendios (post incendios, año 2022).

N: Number of individuals, TNB: total number of bands, NPB: number of private bands, Ne: number of effective alleles, Na: number of different alleles, I: Shannon's information index, He: expected heterozygosity, PPL: percentage of polymorphic loci, ±: standard deviation. The genetic data of the populations from the pre-fire period were obtained from Silvestri *et al.* (2020).

N: Número de individuos, TNB: número total de bandas, NPB: número de bandas privadas, Ne: número de alelos efectivos, Na: Número de alelos diferentes, I: Índice de Shannon; He: Heterocigosis esperada, PPL: Porcentaje de Loci Polimórficos, ±: Desviación estándar. Los datos genéticos de las poblaciones del periodo pre-incendios fueron obtenidos de Silvestri *et al.* (2020)

Descriptive statistics	**ASI population**		**TC population**	
	Pre-fires (2016)	**Post-fires (2022)**	**Pre-fires (2016)**	**Post-fires (2022)**
N	9	11	8	10
TNB	69	44	56	47
NPB	8	8	1	11
Ne	1.22 (±0.03)	1.19 (±0.04)	1.20 (±0.03)	1.27 (±0.05)
Na	0.90 (±0.08)	1.11 (±0.09)	0.74 (±0.08)	1.24 (±0.09)
I	0.20 (±0.02)	0.17 (±0.03)	0.17 (±0.02)	0.23 (±0.04)
He	0.13 (±0.02)	0.11 (±0.02)	0.11 (±0.02)	0.15 (±0.03)
PPL (%)	39.26	30.91	33.33	38.18

Genetic structure analysis

AMOVA results showed that genetic variation was higher among populations than within them, when grouping by population alone and by population with collection period (table 2). Genetic distances between ASI2016 and ASI2022 and between TC2016 and TC2022 were 0.05 and 0.01, respectively, with corresponding genetic identity values of 0.95 and 0.998. PCoA based on individual genetic distances revealed that the first three coordinates accounted for 61.71% of total variation, with coordinates 1 and 2 explaining the largest proportion (56.73%). Coordinate 1 separates ASI and TC individuals. ASI individuals showed greater dispersion than those from TC. However, individuals within each population could not be grouped by collection period (pre- and post-fire) (figure 2, page XXX).

Bayesian clustering identified two groups with the highest ΔK observed at $K = 2$. Individuals showed high membership coefficients to a single group corresponding to their geographic population, ASI (combined from pre- and post-fire individuals) and the TC (also including individuals from both periods) (figure 3, page XXX).

Table 2. Hierarchical analysis of the genetic variability distribution estimated by AMOVA.

Tabla 2. Análisis jerárquico de la distribución de la variabilidad genética estimada por AMOVA.

Est. Var**:** Estimated Variance component, %: Percentage of total variance, Φst= Phi value for each cluster, *p < 0.05.

Est. var= Componente de la varianza, **%**= Porcentaje de la varianza total, Φ***st***= Valor de *phi* para cada agrupación, *significativo p < 0,05.

Grouping	**Source**	**Est. Var.**	**%**	**Φ*st***
Populations (ASI and TC)	Among Populations	7.59	66	0.656*
	Within Populations	3.99	34	
	Total	11.58	100	
Populations + periods (ASI2022. ASI2016. TC2022 and TC2016)	Among populations + periods	5.070	56	0.560*
	Within populations + periods	3.989	44	
	Total	9.058	100	

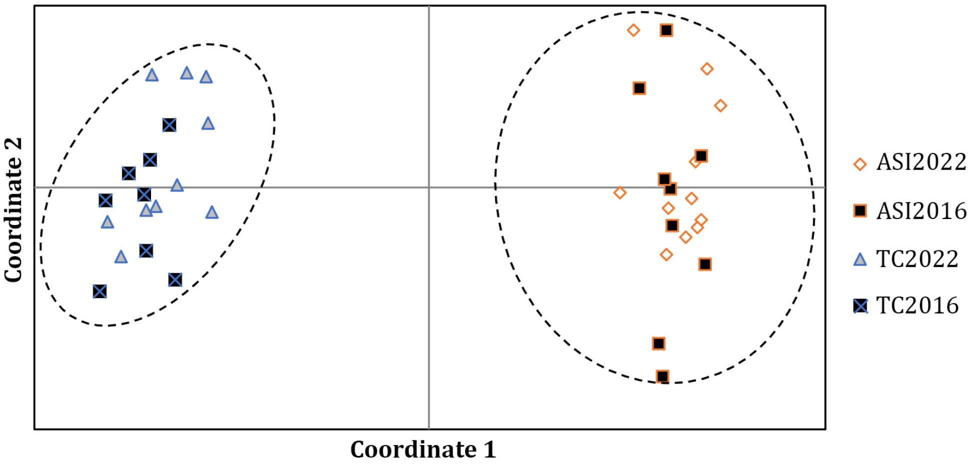

Figure 2. Principal Coordinate Analysis (PCoA) according to Nei's genetic distance between individuals of the ASI2022, ASI20116, TC2022 and TC2016 populations of *S. hippocampoides.*

Figura 2. Análisis de Coordenadas Principales (PCoA) de acuerdo a la distancia genética de Nei entre individuos de las poblaciones ASI2022, ASI20116, TC2022 and TC2016 de *S. hippocampoides.*

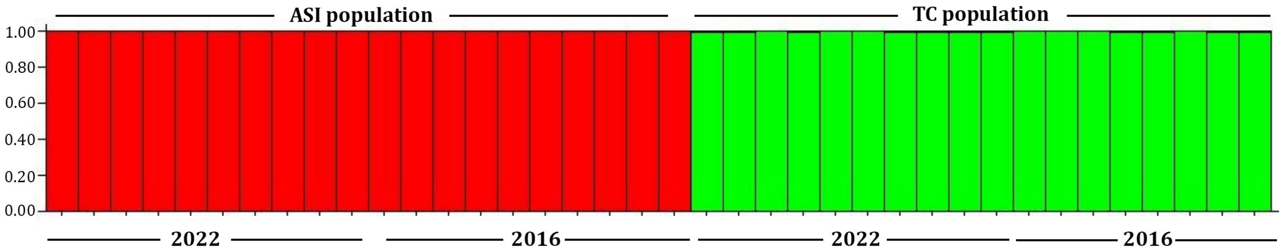

Figure 3. Bayesian clusters inferred by STRUCTURE of the populations under study of *S. hippocampoides*. Each individual is represented by a single vertical bar. Bars are partitioned into K = 2 components that represent each individual's proportional assignment to one of the genetic clusters. The populations and the collection period are indicated above and below the figure, respectively.

Figura 3. Agrupaciones Bayesianas inferidas por STRUCTURE de las poblaciones en estudio de *S. hippocampoides*. Cada individuo está representado por una barra vertical. Las barras están divididas en K = 2 componentes que representan la proporción del genoma de cada individuo asignada a un grupo genético. Las poblaciones y los periodos de colección se indican por encima y por debajo del gráfico, respectivamente.

DISCUSSION

Our results show high genetic variability in *S. hippocampoides*, relative to values reported for this species using ISSR markers (33) and random amplified polymorphic DNA markers (17). Higher genetic diversity has been observed in other *Stylosanthes* species using ISSR markers (1, 6). These variations may result from differences in sampling and inherent genetic variability among species. For instance, Silvestri *et al.* (2020) analyzed eight populations, while we included only two. Kazan et al. (1993) used four individuals per population, whereas we analyzed ≥10 individuals per population. AMOVA results indicated that genetic variation is greater among populations than within populations, consistent with previous studies on *Stylosanthes* species using ISSR (1, 6, 34) and other molecular markers (5, 31, 39). This is expected given the predominant self-fertilizing reproduction of most *Stylosanthes* species. The total number of bands (TNB) in both populations was lower than in the pre-fire evaluation, while the number of private bands (NPB) increased in the TC population and remained stable in the ASI population. These differences may be due to variations in the number of populations analyzed and differences in band interpretation between studies. We assessed two geographically distant populations, whereas Silvestri *et al.* (2020) included eight populations, two of which were from the "Tres Cerros" Natural Reserve. Since only one of these populations is included in our current analysis, the increase in NPB in the TC population can be attributed to the absence of nearby populations in both evaluations, whereas the NPB in the ASI population remained unchanged.

Following natural or artificial fire events, population genetic variability may decrease, increase, or remain unchanged. A decrease is the least commonly reported outcome (43), while increases or stability are more frequent (3, 27, 32). In our study, the ASI population showed no statistically significant changes in genetic diversity indices after fire. Genetic diversity often requires multiple generations to show significant shifts, depending on the intensity and frequency of disturbances and the degree of population fragmentation (2).

The maintenance of genetic variability may result from adaptations that mitigate fire negative effects. Like many Fabaceae species, *Stylosanthes* seeds have hard, impermeable coats that require scarification at 80°C or exposure to surfaces reaching 120°C to break dormancy and enable germination (7, 13). This trait supports the formation of persistent seed banks below the soil surface, which can store genetic material from recent and earlier pre-fire generations. Seed bank formation has been documented in *S. hippocampoides* (23), and considering its generation time and the interval between our evaluations, this mechanism is a plausible explanation for the observed genetic stability. Such a strategy enables post-fire regeneration from multi-generational seed banks rather than a few surviving individuals (3). Additionally, *Stylosanthes* species are known for drought tolerance and high colonization capacity (18), traits that promote rapid recovery after fire events.

The ASI population, located along a roadside subject to frequent burning and mowing, may be adapted to such disturbances. Banks *et al.* (2013) suggest that populations in frequently disturbed areas may rely on these conditions for persistence, as disturbance can enhance resource availability and shape community composition. In this context, the ASI population may exhibit fire adaptation, with a strong colonizing response enabled by heat-softened seeds and reduced competition. This interpretation aligns with Gardener (1980), who found that *Stylosanthes* cultivars from arid environments were more fire-resistant than those from humid coastal areas.

Bayesian clustering analysis showed a high degree of individual assignment to a single cluster, corresponding to their respective geographic populations. This pattern, along with the separation of populations along different axes in the PCoA, supports the observed genetic differentiation. The presence of private bands in both populations further indicates strong genetic identity. These findings suggest that the two sampled sites represent genetically structured and distinct populations. However, no signs of fragmentation were detected in the genetic structure of the fire-affected ASI population. Fire effects on genetic structure are often spatially heterogeneous (22), and seed bank depletion may vary depending on fire intensity and frequency (25, 38). Such dynamics can influence demographic connectivity and affect the distribution of genetic diversity within and among populations. Despite this, few studies have compared the genetic composition of seed banks and adult populations in fire-prone ecosystems (3, 9, 25). Gardener (1980) found that most *Stylosanthes* cultivars possessed sufficient seed reserves for regeneration, with the number of seedlings in each regenerating line correlating with seed quantity in the soil. Nevertheless, direct comparisons between the genetic profiles of pre-fire adults and post-fire seedlings remain scarce, making it difficult to determine whether seed banks fully preserve the genetic composition of earlier generations. The lack of changes in genetic structure in the ASI population may be explained by several biological traits of the species, such as its predominantly self-fertilizing reproduction, which limits within-population genetic variability, a short generation time that allows a rapid regeneration; a large and persistent seed bank; and the relatively brief time between fire events and post-fire sampling, which may not have been sufficient for genetic subdivision to emerge.

Given the increasing frequency and intensity of fires across the natural range of *S. hippocampoides* in recent years (14, 20, 30), future research should expand on this study by including more populations with contrasting fire histories. Determining whether post-fire individuals originate from soil seed banks or surviving adults would also be valuable. Such information is essential for informing germplasm collection and designing effective conservation strategies.

CONCLUSION

Our results showed no statistically significant changes in the genetic variability indices of the ASI population after fire, nor evidence of fragmentation or alterations in its genetic structure. However, as only one fire-affected population was analyzed, our capacity to fully assess the species' response to fire is limited. This study should therefore be viewed as a preliminary step. Future research should include additional populations with contrasting fire histories and examine whether post-fire individuals originate from soil seed banks or surviving adults. Given the increasing frequency and intensity of fires in recent years in Argentina, driven by human activities and intensified by climate change, such studies are crucial for developing effective conservation strategies for native species.

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