AUTOECOLOGY, DIVERSITY, AND INTERNAL GENETIC STRUCTURE OF *Ocotea porosa* (NEES & MART.) BARROSO: SUBSIDIES FOR SEED COLLECTION

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ABSTRACT – Ecological restoration mitigates the negative effects of fragmentation and habitat loss. This practice allows the conservation of key species, such as *Ocotea porosa*, a tree native to the Araucaria Forest and extremely endangered. A key point in restoration projects is the source of seeds, as well as guidelines for collection. When carried out under technical criteria, the collection allows the maintenance of genetic diversity and adaptive potential in restoration plantations. Given the importance of seed source, genetic diversity, and adaptive potential, this study aimed to define areas and criteria for collecting seeds by characterizing the demography, genetics, and reproductive phenology of an *O. porosa* population. A plot of 16 hectares was installed in the municipality of Passos Maia, Santa Catarina, Brazil, and a demographic survey of trees with diameter at breast height (DBH) > 15 cm was carried out. Indices of diversity and internal genetic structure (IGS) were estimated using allozyme markers. The reproductive phenology of 67 individuals was evaluated during 8 months. The studied population showed a high density of individuals (10.7 ind. ha^{-1}) with normal diametric distribution. The phenological pattern of the species is regular, seasonal, and annual. The evaluated population showed high genetic diversity, high fixation index, and significant IGS up to 80 meters away. Based on these results, the evaluated fragment can be used as a seed collection area. It has high genetic diversity, density, and area size sufficient to contain several demes. In addition, it is highly recommended that the matrices be at least 80 meters apart to avoid the effects of significant IGS.

Keywords: Araucaria Forest; Ecological restoration; Spatial genetic structure.

AUTOECOLOGIA, DIVERSIDADE E ESTRUTURA GENÉTICA INTERNA DE Ocotea porosa *(NEES & MART.) BARROSO: SUBSÍDIOS PARA A COLETA DE SEMENTES*

RESUMO – A restauração ecológica é uma forma de mitigar os efeitos negativos da fragmentação e perda de habitat. Essa prática permite a conservação de espécies-chave, como ***Ocotea porosa****, uma árvore nativa da Floresta com Araucária e extremamente ameaçada de extinção. Um ponto fundamental em projetos de restauração é a fonte de sementes bem como as diretrizes para a coleta. Quando realizada sob critérios técnicos, a coleta permite a manutenção da diversidade genética e potencial adaptativo nos plantios de restauração. Diante do exposto, o objetivo do presente estudo foi caracterizar aspectos da demografia, genética e fenologia reprodutiva de uma população de* ***O. porosa****, visando gerar informações para definição de áreas e critérios para a coleta de sementes. Foi instalada uma parcela de 16 hectares no munícipio de Passos Maia, Santa Catarina*

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e realizado levantamento demográfico de árvores com Diâmetro à Altura do Peito (DAP) > 15 cm. Foram estimados índices de diversidade e de estrutura genética interna (EGI), utilizando-se marcadores isoenzimáticos. A fenologia reprodutiva de 67 indivíduos foi avaliada durante 8 meses. O fragmento estudado apresentou uma alta densidade de indivíduos (10,7 ind. ha^{-1}*), com uma distribuição diamétrica similar a normal. O padrão fenológico da espécie é sazonal regular e anual. A população avaliada apresentou uma alta diversidade genética, elevado índice de fixação, além de uma EGI significativa até 80 metros de distância. Conclui-se que o fragmento avaliado pode ser utilizado como área de coleta de sementes. O mesmo possui alta diversidade genética, densidade e tamanho de área suficiente para conter várias demes. Além disso, é altamente recomendado que as matrizes tenham no mínimo 80 metros de distância entre si, evitando os efeitos da EGI significativa.*

Palavras-Chave: Floresta de Araucária; Restauração ecológica; Estrutura genética espacial.

1. INTRODUCTION

The Atlantic Forest biome is a global hotspot of biological diversity that has become increasingly vulnerable as a result of significant losses through selective logging, fragmentation, and deforestation (SOS Mata Atlântica 2020). To mitigate the effects of habitat loss and fragmentation in this biome, restoration initiatives have intensified across various forest types found in the Atlantic Forest. For instance, the Brazilian Pact for Atlantic Forest Restoration, launched in 2009, aims to promote the restoration of 15 million hectares of forest by the year 2050 (Melo et al. 2013), having already recovered approximately 0.67 and 0.74 million hectares of forest between 2011 and 2015 (Crouzeilles et al. 2019).

Regardless of the scale of restoration, access to a high-quality seed source is essential to increasing the likelihood of successful plantings (Sebbenn 2002). However, even though seeds form the foundation of many restoration programs (Gann et al. 2019), they are often a limited resource (Pedrini and Dixon 2020). Therefore, seed collection must be carried out based on technical criteria to ensure the maintenance of genetic diversity and the evolutionary potential of restored populations (Breed et al. 2018). To achieve this, it is crucial to develop recommendations that determine the minimum number of source trees (Montagna et al. 2018c), the minimum distance between source trees (Tarazi et al. 2010), the ideal collection season (Luna-Nieves et al. 2017), and density of source trees (Montagna et al. 2018b). Implementing these guidelines will permit the capture of samples representing the genetic variability of one or more populations, thereby reducing the potential risks of introducing non-adapted genotypes and avoiding exogamy/endogamy depression (Stingemore and Krauss 2013).

The signature tree species of Santa Catarina, *Ocotea porosa* (Nees & Mart.) Barroso, more commonly known as “imbuia”, is a forest species belonging to the Lauraceae family. It is capable of reaching heights up to 20 meters and a diameter at breast height (DBH) of 50 to 150 cm (Meyer et al. 2013). Valued in the timber industry, it has suffered intense exploitation for decades (Reis et al. 2007), leading to a significant reduction in its population. This has resulted in the inclusion of the species in state (Santa Catarina 2014), national (Brazil 2014), and international (Varty and Guadagnin 1998) lists of threatened species.

Considering the demand for seeds of species used in restoration, this study evaluated demography, phenology, internal genetic structure, and genetic diversity in a population of *O. porosa*. We raised three questions. First, does the studied population exhibit sufficient demographic density and genetic diversity for seed collection? Second, what is the minimum distance for seed collection? Third, what is the recommended period for seed collection? The answers to these questions will allow us to establish the necessary technical criteria and guidelines for seed collection, as well as the delineation of seed collection zones for *O. porosa*. Moreover, it is anticipated that the data garnered from this study will yield significant implications for the conservation of this species.

2. MATERIALS AND METHODS

2.1. Study Area

The evaluated population is situated in the municipality of Passos Maia, located in the western mesoregion of Santa Catarina, Brazil (Figure 1). The forest fragment covers 227 hectares and is located on

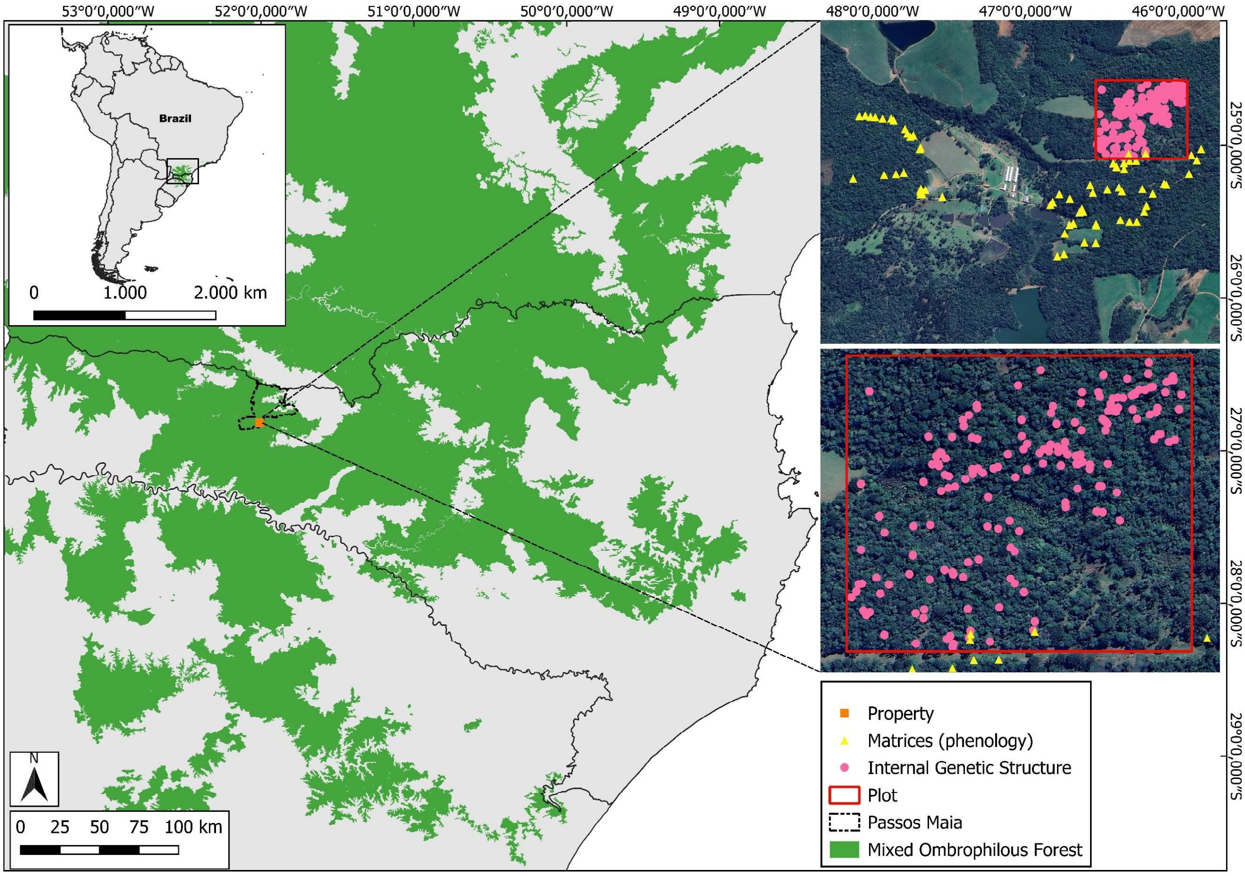

Figure 1 – Study site located in Passos Maia, Santa Catarina, Brazil.
Figura 1 *– Local do estudo, situado em Passos Maia, Santa Catarina, Brasil.*

private property, with 2,466 hectares acquired by the current owner in 1953. The region is within the Atlantic Forest biome and features vegetation characteristic of the phytogeographic domain of Mixed Ombrophilous Forest (Araucaria Forest).

2.2. Sampling

A demographic survey was conducted on a square plot measuring 400 x 400 meters, totaling 16 hectares (Figure 1). All individuals classified as adults (circumference at breast height > 41.7 cm, the smallest observed in a reproductive individual) were evaluated. Measured plants were tagged with an aluminum plate bearing a numerical identification and mapped with geographic coordinates. For demographic data analysis, the average density of adult individuals per hectare and dominance (basal area) were obtained, along with a graph depicting size distribution.

2.3. Genetic Data Collection and Analysis

The collection of leaf material for genetic diversity characterization was performed on all 171 adult individuals measured in the 16-hectare plot. Individuals were genotyped at the Laboratory of Developmental Physiology and Plant Genetics, Universidade Federal de Santa Catarina. Genetic characterization of the populations was carried out using isoenzymatic markers on starch gel (penetrose 30-13%), following the recommendations of Alfenas (1998). The buffer-electrode system used was Tris-Citrate pH 7.5 with the following isoenzymatic systems: malate Dehydrogenase (MDH, EC 1.1.1.37), superoxide Dismutase (SOD, EC 1.15.1.1), peroxidase (PRX, EC 1.11.1.7), shikimate dehydrogenase (SKDH, EC 1.1.1.25), phosphoglucomutase (PGM, EC 5.4.2.2), diaphorase (DIA, EC 1.8.1.4), glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), esterase

(EST, EC 3.1.1.1), glutamate dehydrogenase (GTDH, EC 1.4.1.2), and acid phosphatase (ACP, EC 3.1.3.2).

Furthermore, intrapopulational genetic diversity of the sampled adult individuals was characterized using the following indices: average number of alleles per locus ($\hat{A}$), average number of effective alleles per locus ($\hat{A}_e$=1/1-$\hat{H}_E$), number of alleles per polymorphic locus ($\hat{A}_p$), observed heterozygosity ($\hat{H}_O$), expected heterozygosity ($\hat{H}_E$) according to the proportions proposed by the Hardy-Weinberg Equilibrium (Nei 1978), and fixation index ($\hat{f}$). All indices were estimated using the software FSTAT (Goudet 2002) and GDA (Lewis and Zaykin 2001). The statistical significance ($p < 0.05$) of the $\hat{f}$ values was determined through 1000 allele permutations among individuals.

Characterizing the spatial distribution of adult genotypes was conducted using the estimation of the coefficient of coancestry between pairs of trees (θ_{xy}), as described by Loiselle et al. (1995) and obtained using the SPAGEDI 1.4 program (Vekemans and Hardy 2004). Confidence intervals (95%) for each parameter were obtained through 10000 bootstrappings. The neighborhood size (N_b) was estimated as N_b= -(1-θ_1)/b, where θ_1 represents coancestry estimated for the first distance class, and *b* is the slope of the regression curve of θ with respect to the logarithm of distance (up to 520 m) (Vekemans and Hardy 2004). Based on N_b and individual density, the deme area (or neighborhood area) in hectares was estimated as $deme = N_b/density$.

The coancestry group was calculated as $\theta = \frac{0{,}5n(1+f) + \sum_{i=j}^{n}\sum_{i=0}^{n}\theta xy}{n^2}$ (Lindgren and Mullin, 1998), where *n* is the number of sampled individuals, $\hat{f}$ is the inbreeding coefficient for the parental population, and *θxy* is the coancestry coefficient between individuals. Subsequently, the effective size ($\hat{N}_e$) was estimated as $\hat{N}_e$= 0,5/Θ (Cockerham, 1969). Based on effective size, the minimum viable area (MVA) for the genetic conservation of an *O. porosa* population was estimated according to Lynch (1996), taking $\hat{N}_e$ = 1000 as a reference point indicating sufficient mitigation of the effects of deleterious mutations. Thus, the minimum viable area is given by $N_{e(ref)}/(\hat{N}_e/n)d$), where *n* is the sample size, and *d* is the density of reproductive individuals in the population.

2.4. Reproductive Phenology

Reproductive phenology was assessed monthly in 67 individuals between September 2021 and April 2022, using binoculars. The synchrony among population individuals was assessed using the presence/absence method, which indicates the percentage of individuals in each phenophase. Phenological events were classified as asynchronous (<20% of the population individuals in the phenophase), slightly synchronous (20-60% in the phenophase), and highly synchronous (>60% in the phenophase) (Bencke and Morellato 2002).

The evaluation of reproductive phenophase intensity followed the criteria of Fournier and Charpantier (1978). The intensity of each phenophase was estimated using a semi-quantitative interval scale of five categories (0 to 4): 0 corresponds to 0%; (1) presence of the event in a range of 1 to 25%; (2) presence of the event in a range of 26 to 50%; (3) presence of the event in a range of 51 to 75%; and (4) presence of the event in a range of 76 to 100%. Subsequently, the percentage of intensity was calculated according to Fournier (1974).

Relationships between the synchrony of assessed phenological events and climatological data (monthly averages of relative humidity, precipitation, mean temperature, maximum temperature, and minimum temperature) were investigated using Spearman's correlation. The level of significance was verified by a p-value < 0.05. Meteorological data were obtained from EPAGRI – Experimental Station of Ponte Serrada -SC. For statistical analyses and graph creation, R Studio 4.1 software was used.

3. RESULTS

3.1. Demographic Structure of Reproductive Individuals

In the 16 hectares, 171 reproductive individuals were measured, resulting in an average of 10.7 reproductive individuals per hectare. All individuals recorded above 15 cm in DBH were reproductive. The average DBH per hectare was 48.8 cm, ranging from 15 to 110.5 cm. The measured basal area was 2.2 m^2 ha^{-1}. Considering the distribution in diameter classes (Figure 2), we found a higher frequency of individuals in the intermediate DBH classes (between 35 cm and 65 cm), accounting for 69% of total individuals. The frequency of individuals decreases as the diameter classes increase, with the last four classes containing 4.8% of the individuals. It is

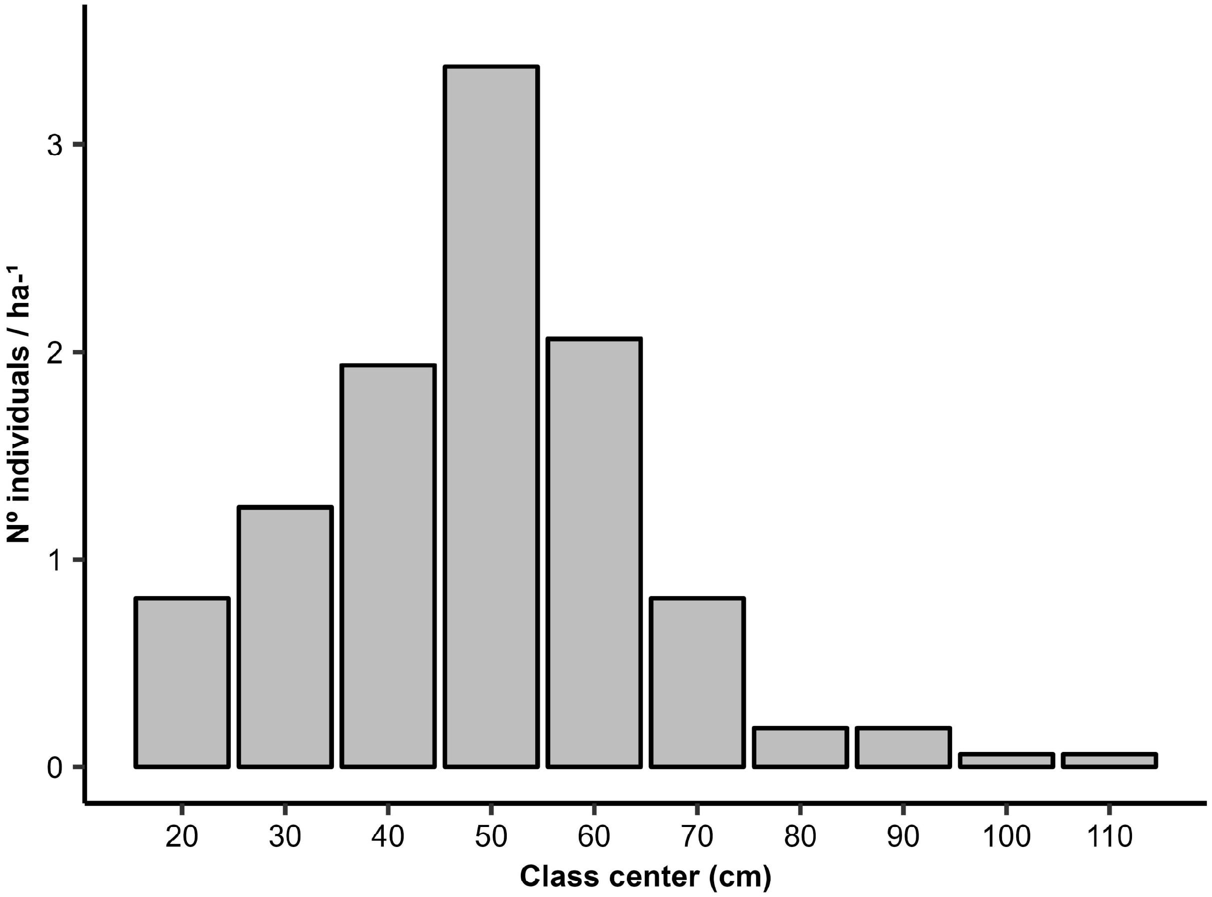

Figure 2 – Frequency distribution in diameter classes for reproductive individuals in a 16-hectare area of an *Ocotea porosa* population in Passos Maia, SC.
Figura 2 *– istribuição de frequência em classes diamétricas para indivíduos reprodutivos em uma área de 16 hectares de uma população de* ***Ocotea porosa*** *em Passos Maia – SC.*

evident that the frequency of individuals per diameter class follows the trend of a normal distribution.

3.2. Genetic Diversity

In the studied population, 30 different alleles were sampled. The average number of individuals analyzed per locus was 160. The mean number of alleles per polymorphic locus ($\hat{A}_p$) was 3.22, while the mean number of alleles per locus (Â) was 3, differing by about 51% from the mean effective alleles per locus ($\hat{A}_e$), which was 1.5. The genetic diversity ($\hat{H}_E$) found in the analyzed population was 0.3. This value substantially differed from the observed heterozygosity ($\hat{H}_O$), which was 0.181. This discrepancy between $\hat{H}_E$ and $\hat{H}_O$ resulted in a fixation index ($\hat{f}$) of 0.379, statistically different from zero. From the fixation index, the effective number of individuals was estimated to be 103, approximately 40% less than the total sample. Finally, the estimated MVA for genetic conservation of the population was 56.2 hectares.

3.3. Internal Genetic Structure

The analyzed population exhibits positive and significant coancestry ($\hat{\theta}_{xy}$) up to 80 meters between individuals (Figure 3). Additionally, starting from 260 meters, the graph shows that coancestry values become negative and statistically different from zero ($p > 0.05$). Based on coancestry data, a neighborhood size (N_b) of 116.5 individuals was estimated. Since the study site

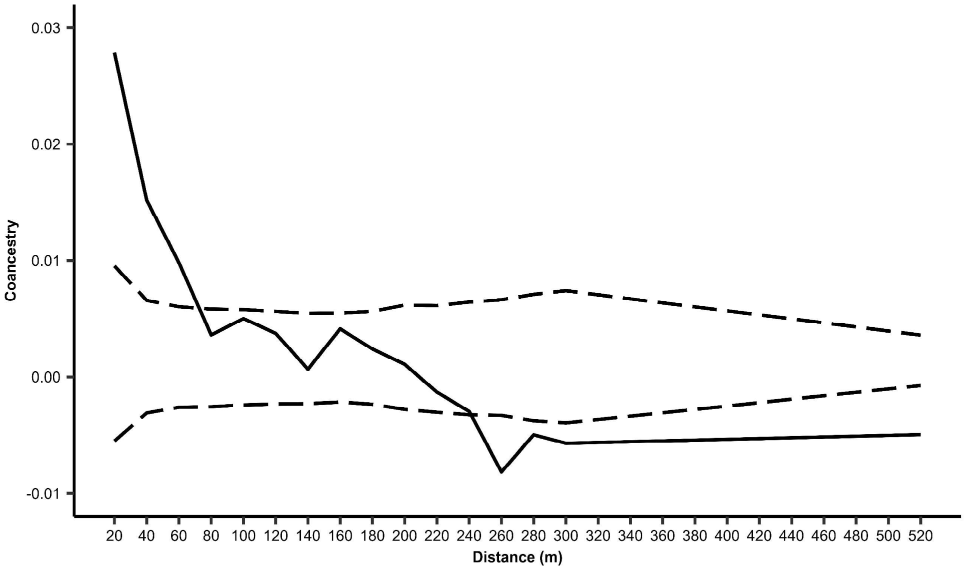

Figure 3 – Internal genetic structure for a population of reproductive *Ocotea porosa* individuals in Passos Maia, SC. Dashed lines represent the confidence interval (95%). Solid line represents the mean values of coancestry.

Figura 3 *– Estrutura genética interna para uma população de indivíduos reprodutivos de **Ocotea porosa** em Passos Maia - SC. Linhas tracejadas representam o envelope de confiança (95%). Linha sólida representa os valores médios de coancestria.*

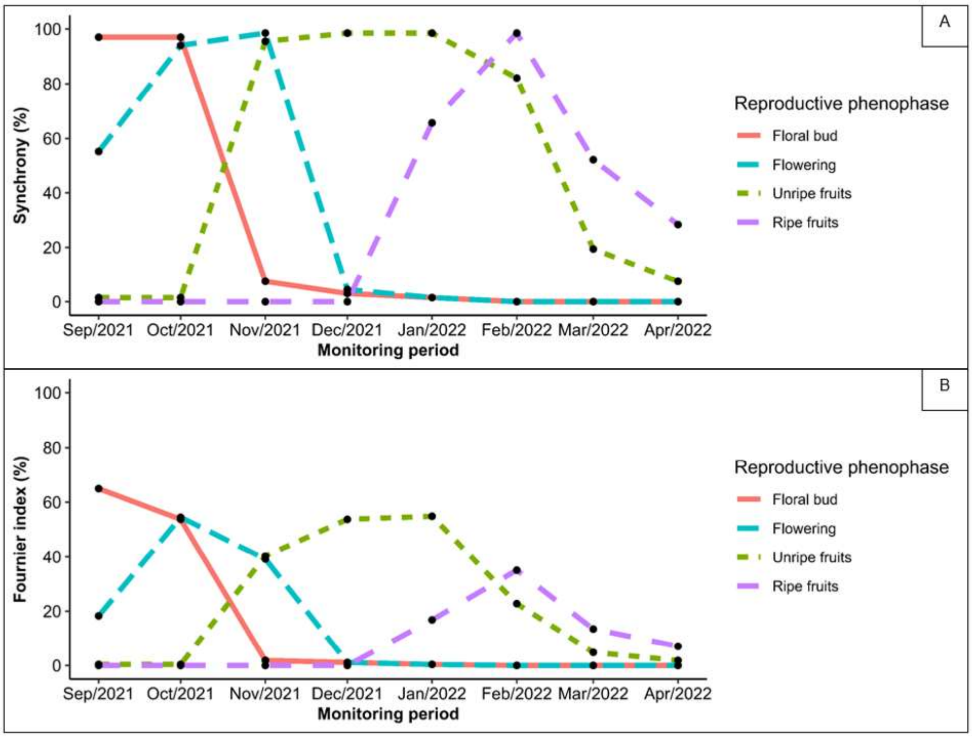

Figure 4 – Phenophase activity (A) and phenophase intensity (B) for an *Ocotea porosa* population in Passos Maia, SC.

Figura 4 *– Atividade de fenofases (A) e intensidade de fenofases (B) para **Ocotea porosa** no Município de Ponte Serrada – SC.*

has a density of 10.7 reproductive individuals/ha, the estimated deme area was 10.9 hectares.

3.4. Reproductive Phenology

In all phenophases, synchrony levels exceeding 60% were recorded (Figure 4A). The initiation of floral bud emergence occurred in September (Figure 4A) and reached peak synchrony in the same month. Similarly, flowering began in September but peaked in November. The first green fruits were observed in November, and peak synchrony occurred between December and January. Fruit maturation was recorded from January to April, with the highest synchrony in February.

Figure 4B shows the intensities of each reproductive phenophase assessed using the Fournier method. As also shown in Figure 4B, the floral bud event was most intense in September, while the intensity of flowering reached its peak in October. Subsequent phenophases, i.e., unripe fruits and ripe fruits, had their peak intensities in January and February, respectively. The spearman correlation results were significant only for the phenophases of unripe fruits versus maximum temperature (0.87), average temperature (0.93), and precipitation (-0.70), as well as the positive correlation between ripe fruits and minimum temperature (0.79).

4. DISCUSSION

4.1. Demographic Structure of Reproductive Individuals

The evaluated population exhibited a density of reproductive individuals consistent with most values reported in the literature, specifically those considering the inclusion of DBH ≥ 15 cm. According to data from the Forest and Floristic Inventory of Santa Catarina (Montagna et al. 2018a), the average density of *O. porosa* populations in Santa Catarina (SC) is 12.8 ind.ha^{-1}, a value similar to that reported by Schaaf et al. (2006) (9.2 ind.ha^{-1}). However, various publications by Klein on the flora and vegetation of Santa Catarina described the understory of Araucaria Forest in the plateau region as extensively dominated by *O. porosa* (Klein 1974), reaching up to 90% in abundance (Klein 1960).

The reduction in density values can be attributed to historical exploitation of the species. As reported by Klein (1979), Santa Catarina developed under the strong influence of the timber industry, especially after World War II, and *O. porosa* was the most exploited species. The present study was conducted on private property, which, according to the owner, was the site of a sawmill prior to 1953. This timber activity continued until the mid-1990s, after which the fragment remained "untouched." Nonetheless, the presence of sawmills was common in this region, giving evidence of exploitation into the mid-20th century (Moretto 2017).

4.2. Genetic Diversity

The estimated results indicate that the evaluated population possesses high genetic diversity ($H_e = 0.3$) when compared to the average for long-lived perennial woody species (0.149) (Hamrick and Godt 1990). Based on results reported by other authors (Tarazi et al. 2010; Reis et al. 2012), high levels of genetic diversity are expected for species of the *Ocotea* genus. The observed heterozygosity is considered low, indicating an excess of homozygotes. The fixation index (f) is considerably higher than fixation indices reported in other studies on the *Ocotea* genus (Tarazi et al. 2010; Montagna et al. 2018), suggesting a deviation in the frequency of heterozygotes from what is expected under Hardy-Weinberg equilibrium.

Intensive historical exploitation and distribution in severely anthropogenic environments are factors contributing to the high $\hat{f}$ value (Reis et al. 2012), a trend described for other species (de Sousa et al. 2020; Mariot et al. 2020). For example, in a study of six plant species, Lauterjung et al. (2019) concluded that heavily exploited species in the past exhibited concerning genetic indices. Another factor that may have influenced a high $\hat{f}$ value is the significant genetic structure found in the evaluated population (Bittencourt and Sebbenn, 2009; Meirmans 2015).

The high estimated and significantly positive fixation index resulted in a reduction in the $\hat{N}_e$. However, even with the reduction in $\hat{N}_e$, the estimated minimum viable area (56.2 ha) was smaller than the size of the studied fragment (227 ha), as well as the property (2,466 ha). Therefore, based on a reference size of 1,000 individuals (Lynch 1996) and the estimated $\hat{N}_e$, the study site is capable of supporting a minimum number of individuals able to sufficiently mitigate the effects of genetic drift and allow for seed collection. Similar results were described by Montagna et al. (2018b) for two populations of *O. catharinensis* in the state of Santa Catarina.

4.3. Internal Genetic Structure

Significant IGS has been reported for the genus *Ocotea* in various studies, and with different spatial scales of significance. Bittencourt (2007) found significance within 0-19 meters, Tarazi et al. (2010) within 0-80 meters, and Montagna et al. (2018b) within 0-60 meters. In contrast, Kageyama et al. (2003) found no significant structuring. Expanding comparisons to the Lauraceae family, studies by Chung et al. (2000), Chung et al. (2003), and Hardy et al. (2005) showed the absence of significant structuring. It should be noted that studies on IGS for *Ocotea* species tend to indicate genetic structuring that extends upward of 100 meters.

Several factors may contribute to the estimated IGS in the present study. The mating system of the species is mixed with a predominance of outcrossing, but *O. porosa* flowers also exhibit self-compatibility, allowing for self-pollination. The reported pollination distance for the species is considered limited, primarily for short-flight insects such as *Frankliniella gardeniae* (Danieli-Silva and Varassin 2013). According to Wessinger (2021), this increases the likelihood of cross-pollination between flowers on the same plant and between neighboring plants, leading to genetic structuring. Species pollinated by insects are more likely to exhibit genetic structuring compared to those pollinated by vertebrates (Melo and Franceschinelli 2016). Furthermore, a pattern of aggregated distribution (Bittencourt 2007) coupled with low population density (Montagna et al. 2018a) facilitates restricted gene flow to spatially close individuals, resulting in increased endogamy (Goncalves et al. 2022).

The estimated neighborhood size (116.5 individuals) in the present study is higher than that reported by Bittencourt (2007) (62 individuals) and Montagna et al. (2018b) (44 and 48 individuals). On the other hand, the deme size measured in our study (10.9 hectares) is larger than that reported by Tarazi et al. (2010) (5 and 6 hectares) and Bittencourt (2007) (2.3 hectares), but intermediate in relation to that described by Montagna et al. (2018b) (8.8 and 11.7 hectares). Although recommendations emphasize the importance of preserving large areas (Bittencourt 2007), the estimates of deme size demonstrate that even small fragments (<30 hectares) can contain more than one deme and, therefore, contribute to the species conservation.

4.4. Reproductive Phenology

The observed phenological pattern for the evaluated *O. porosa* population aligns with that found in other studies reporting on the reproductive phenology of the species. In spite of differences in the duration of phenophases, the overall reproductive cycle of *O. porosa*, as reported in the present study, is consistently corroborated in the literature, i.e., beginning in August (Bittencourt 2007) and ending in April (Seubert 2017). Variations in phenological pattern among individuals and populations of the same species are common in tree Lauraceae species dispersed by birds (Wheelwright 1986). Additionally, general consensus holds that the phenological pattern of *O. porosa* is considered regular and annual (Rêgo et al. 2006), and the results of the present study support these observations.

Although not detected through correlation analysis, it was observed that the onset of flowering is related to an increase in temperature (average, maximum, and minimum) and precipitation. For example, the peak intensity of flowering coincided with the month of highest rainfall (October). Studies conducted across different formations in the Atlantic Forest domain have demonstrated a seasonal pattern for the flowering period, with an increase in this phenophase during the transition from the colder, drier period to the warmer and rainier months, typically occurring between September and January (Funch et al. 2002; Cascaes et al. 2013). This happens because phenology is determined by climatic and physiological factors (Rodarte et al. 2007).

4.5. Seed Collection

The results of this research have important implications for seed collection in *O. porosa* populations. Despite the difference in estimated density (10.7 individuals.ha^{-1}) from that reported by Klein (1974), who suggested a population reduction, the total area of the fragment is still large enough to maintain multiple demes and minimum viable populations. Furthermore, despite the positive and significant fixation index, the diversity values are considered high. Based on these results, it is reasonable to conclude that the studied population constitutes an important site for seed collection.

According to IGS results, seed collection should respect a minimum distance of 80 meters between plants to reduce the chances of relatedness among parent plants. To maximize the capture of genetic diversity, it is recommended that seeds be collected from individuals separated by a minimum distance of 240 meters. Other factors, such as type of pollinator (short flight), dispersal, mating system (mixed), distribution (aggregated), and historical use (population reduction), reinforce the adoption of distance criteria for *O. porosa* seed collection.

Collecting seeds at the right time is important because fruits collected from the ground are more likely to contain old, moldy, or insect-infested seeds (Pedrini et al. 2020). Given this context and based on the results of reproductive phenology, it is reasonable to suggest that the recommended time for fruit collection would be between January and February, the period of peak fruit maturity.

5. CONCLUSION

The evaluated population has a high density of individuals, high genetic diversity, a high fixation index, and significant spatial genetic structure up to 80 meters apart. Based on these results, it can be concluded that the forest fragment can be used as a seed collection area for O. porosa. The recommended time for collection is between January and February. These results have relevant and novel implications for O. porosa seed collection practices, especially for ecological restoration purposes. Additionally, estimates of minimum viable area (MVA) and deme size are important indicators for O. porosa conservation, which is distributed in environments with intense anthropogenic activity.

AUTHOR CONTRIBUTIONS

Conception of the idea: Igor de Carvalho Aguiar Rodrigues, Tiago Montagna. Field data collection: Igor de Carvalho Aguiar Rodrigues, Giulia Fabrin Scussel, Alison Bernardi, Ana Kelly de Sousa Silva de Carvalho. Genetic analyzes in the laboratory: Igor de Carvalho Aguiar Rodrigues, Giulia Fabrin Scussel, Alison Bernardi, Peggy Thalmayr, Juliana Machado Ferreira. Data analysis: Igor de Carvalho Aguiar Rodrigues, Tiago Montagna. Manuscript writing: Igor de Carvalho Aguiar Rodrigues. Manuscript review: all authors discussed the results and contributed to the final version of the manuscript. Research supervision and coordination: Tiago Montagna, Adelar Mantovani.

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