

Short-term increase in clonal propagules following disturbance in a natural population of *Eremanthus erythropappus*

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Abstract

Habitat fragmentation and management of natural populations of tree species may interfere with their dynamics of reproduction. *Eremanthus erythropappus* is a native pioneer tree from Cerrado and Atlantic Forest as well as their transition zones, that usually reproduces by sexual means, that is producing seeds, but can also induce propagules through asexual propagation. Management of natural populations of *E. erythropappus* for their use in cosmetics and other industrial applications, however, threaten the species survival and may alter the reproduction patterns of the species. Here we used six microsatellite loci to investigate whether management of a natural stand increased clonality rates in comparison to an unmanaged area. Significant increase in clonality levels, as detected from multilocus genotypes, were observed in the managed stand, although some clonality was also detected in the unmanaged area. Through the interpretation of the richness index (R), managed regenerants showed higher levels of clonality ($R = 0.293$), followed by managed adults ($R = 0.489$). Significant aggregation was also detected, indicating that closer individuals in distance could also be identical genotypes. The association index revealed some extent of linkage disequilibrium generated by clonality. Therefore, our data showed that clonality rates are significantly augmented when *E. erythropappus* is subjected to management. This may have further negative impact on the genetic diversity of natural populations of the species and for its long term conservation in their environment of origin. Management practices should take into account the extent of clonality they can induce in natural stands of the species.

Introduction

Habitat fragmentation is a major factor that threatens survival and reproduction of plant species, leading populations to become more isolated and reduced in size (Lienert, 2004). The destruction of natural sites in which tree species belong may often produce a disequilibrium in ecological interactions with pollinators (Mendes et al., 2022) and seed dispersers (Giombini et al., 2009). Studies that have addressed the current genetic diversity of allogamous tropical tree species through molecular markers frequently found high or moderate levels of genetic variation in natural populations (Collevatti et al., 2014, Souza et al., 2017, Muniz et al., 2022). But also becoming more common are studies documenting significant reduction in genetic diversity and changes in the genetic structure of populations when comparing conserved forests with sites that underwent habitat loss (Muniz et al., 2022) and fragmentation (Vranckx et al., 2012, González et al., 2020). A decay in genetic variation has also been described when analyzing succeeding generations, that is adult e juvenile trees (Waqar et al., 2021). Conservation geneticists claim that it is a matter of urgency that humankind should embrace strategies for the long term conservation of species and that an integrated view of knowledge and practices for conservation is timely (Pizzuto et al., 2021).

The reproduction of several tree species occurs through sexual means and it is often to find that trees have allogamous or mixed systems of mating. In the latter, both selfing and outcrossing occur in populations, with variable patterns of the relative contribution of selfing and outcrossing among species (Olson et al., 2016). Scarcely documented for woody tree species though, is the ability to reproduce by

both sexual and asexual means and its genetic consequences. Populations of *Betula humilis* from Poland showed significant clonality levels after 522 samples were screened with microsatellite markers and 86 genets were found, 76% of them sharing the same genotype with their nearest neighbor (Bona et al., 2019). Populations of the neotropical tree *Pentaclethra macroloba* were sampled in distinct elevational gradients in Costa Rica and also showed a degree of clonal propagation as revealed by microsatellites. However, clonal individuals were found in inundated swamp areas only, and those produced higher genetic structure in comparison to the other populations (Gaddis et al., 2014). An older study with populations of *Quercus chrysolepsis* attested clonal propagation through isozyme loci, where clustered trees belonged to single clones (Montalvo et al., 1997).

In the aforementioned studies, although clonality was present, population genetic statistics suggested that sexual reproduction was predominant. This was not the case of populations of the endangered tree *Elaeocarpus williamsianus*, that showed single clones in most populations, and two genets at maximum in the most protected areas in Australia, after analyses with microsatellites and RAPD markers (Rossetto et al., 2004). The same study discussed that the genetic differences among individuals was much lower than expected and low seed viability was also observed in the populations. The authors recognized a disturbance in the balance between sexual and vegetative reproduction in this species due to habitat fragmentation. Morris et al. (2004) reached a similar conclusion with *Fagus grandifolia* populations analyzed with ISSR markers, as the frequency of clonal reproduction indicated to be higher in more severely disturbed sites.

Eremanthus erythropappus (DC.) Mac-Leishn is an Asteraceae member that reaches a shrub or a tree to 10m in height and 10cm in diameter. *E. erythropappus* flowering and fruiting occur from June to November in a natural distribution area located in the southeastern regions of the Central Plateau in Brazil in altitudes varying from 700 to 2400m. The species is common in colonies in the middle of secondary forests of the Atlantic Forest and in Cerrado (Brazilian savanna), being found in the states of Espírito Santo, Rio de Janeiro, Minas Gerais and São Paulo. Populars commonly refer to it as “candeia” or “pau de candeia” (candlestick) (MacLeish, 1987).

E. erythropappus has important economic applications due to the production of an essential oil containing α -bisabolol that is extracted for production of cosmetics and pharmaceuticals (de Oliveira et al., 2009). Essential oils of the species also showed acaricidal effect (Marchesini et al., 2021). *E. erythropappus* is also recommended for forest restoration, especially in degraded sites, as it is adapted to poor fertility and rocky outcrops (da Silva et al., 2007).

However, the intense habitat fragmentation and indiscriminate exploitation have led to reduction in population size throughout the species original habitat range (de Pádua et al., 2021). Such interference may result in allele losses, reduction of genetic diversity and changes in genetic structure over time. Prior study with adults and juvenile individuals from managed and unmanaged stands of *E. erythropappus* revealed high genetic diversity in both areas, however, managed stands showed changes in genetic structure possibly due to management (Rocha et al., 2021). In another study, cpDNA-RFLP markers

indicated a genetic structure between populations from the northern range of distribution and populations from the center-south (Rocha et al., 2020a). Using climate-based ecological modeling, a severe reduction in the range of distribution of *E. erythropappus* is predicted from their study.

Management of *E. erythropappus* populations may also increase the propagation of the species by vegetative means, with the production of sprouts mainly from the roots (Fonseca et al., 2021). Therefore, this work was developed under the hypothesis that forest management increases clonal propagation in comparison to non-managed stands. We used population genetics approaches to investigate whether managed and unmanaged areas differ in clonality and how the genetic diversity and structure might potentially be altered.

Material And Methods

Sampling areas

The study was conducted in Itamonte, southern Minas Gerais, Brazil. Two stands located 300m apart were sampled. One stand was protected (44°46'24"W; 44°46'25.6"W) while the other has undergone management (22°16'45"S; 22°16'44.6"S) so that *E. erythropappus* was the only remaining species within the area. Management occurred in 2009, occasion in which 1918 mature trees were removed, leaving a density of individuals at 3197 trees ha⁻¹. Additional thinning was conducted 40 months after that aiming for reduction in tree competition. The unmanaged site was selected in the proximity for the purpose of comparison with the managed stand. Both areas are located within the Serra da Mantiqueira Protected Area, in elevations that reach 2,798m.

Four plots (10 10m) were installed 50m apart in both managed and unmanaged stands. Each plot was centered on a seed tree to analyze possible clonal individuals. Samples were collected 60 months after the initial thinning. Samples were collected from adult and juvenile individuals. The criterion used for distinguishing adults and juveniles was the diameter at breast height (DBH), so that trees > 1.5m in height and > 5cm in DBH were considered adults. Meanwhile, individuals with measurement below those limits were sampled as juveniles. Further details on the location and sampling are described by Rocha et al. (2020a) in prior study characterizing the same area.

DNA extraction and genotyping

DNA extraction was conducted following CTAB protocol (Doyle and Doyle, 1987) and quantified using a NanoVue Plus™ spectrophotometer. DNA quality was checked using the A₂₆₀/A₂₈₀ ratio. Six microsatellite markers developed by Rocha et al., (2020b) (loci *Ere02*, *Ere03*, *Ere04*, *Ere07*, *Ere08* and *Ere14*) were genotypes for all samples following the specific steps described in Rocha et al. (2020a). PCR products were separated with capillary electrophoresis in an ABI 3730 Automated Sequencer (Life Technologies, Carlsbad, CA, USA). Genotyping was conducted with GeneMaker 1.9 V (Applied Biosystems). Identification and corrections for null alleles and genotyping errors were processed with Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004).

Determination of multilocus genotypes and multilocus lineages

Genetic variability estimates were calculated using GenAEx 6.501 (Peakall and Smouse 2006). Determination of multilocus genotypes (MLG) was performed with R package *poppr* (Kamvar et al., 2014) and *Rclone* (Bailleul et al., 2016). Individuals with the same genotype were assigned to the same MLG. To test for the occurrence of multilocus lineages (MLL), *Rclone* was used to compute a genetic distance matrix based on the original data, followed by two simulated datasets, one considering a theoretical distribution under sexual reproduction with 1000 replicates, and the other with the same parameters except for not considering selfing. Histograms of original and simulated datasets were plotted overlapped and visually inspected to establish a threshold distance for MLL discrimination. The threshold distance determination followed the definition of Meirmans and Van Tienderen (2004) as a valley into the distribution frequencies of the genetic distances among the three datasets. Moreover, we followed a similar approach to Bona et al. (2019) to further assess if replicates of the same MLG belonged to the same genet. Therefore, the probability that those individuals were the result of distinct sexual reproduction events (p_{sex}) was calculated in *Rclone*. Definition of MLG and MLL was also accompanied by the construction of an UPGMA dendrogram (Kamvar et al., 2015) based on Bruvo's genetic distances (Bruvo et al., 2004), using package *ape* (Paradis and Schlieps, 2019). To visualize the relationships among haplotypes, minimum spanning networks were constructed with Bruvo's distances using function 'bruvo.msn' in *poppr*.

Clonal diversity, size distribution and aggregation indexes

Assessment of clonality was further examined through calculation of clonal diversity indexes using the *Rclone* implemented function 'clonal_index': R , the richness index; D , the Simpson diversity index; and V , the Simpson evenness index. The R index ranges from 0 (all individuals belong to the same clone) to 1 (all individuals are genetically distinct). The distribution of the size of MLL was analyzed through linear regression and calculation of the Pareto index. Through the interpretation of the linear model, the higher the slope the greater the evenness of clone size distribution. The probability that spatially close individuals are identical genotypes, that is, they belong to the same clone, was assessed through computation of the aggregation index (A_c) (Bailleul et al., 2016).

Verification of departures from sexual reproduction

Poppr was used to calculate the index of association (I_A), originally developed by Brown et al. (1980). Determination of I_A and its significance from a permutation test assess if loci in a population are in linkage disequilibrium (Kamvar et al., 2014). The test is run under the null hypothesis that loci are not linked, which is generally expected for sexual populations. By rejecting this hypothesis, disequilibrium is detected, and it could be evidence of clonality in populations. I_A was calculated for all data and recalculated after removal of clonal replicates.

Results

Multilocus genotypes and multilocus lineages

In total, 249 individuals were sampled: 46 adults and 141 regenerants in the managed stand; 52 adults and 10 regenerants in the not managed area. The six loci analyzed showed an average of 2.67 alleles per locus and an expected heterozygosity of 0.431. After analyzing the genotypes of all individuals, 54 multilocus genotypes were detected. The highest number of MLG were determined from the regenerant individuals of the managed stand (MLG = 42), followed by not managed adults (MLG = 26), managed adults (MLG = 23), and not managed regenerants (MLG = 10) (Table 1). The highest frequencies of the same MLG were detected among the juvenile individuals of the managed area (Fig. 1a). MLG 47 was counted 20 times in this stand and 3 times among managed adults. MLG 47 was also present in not managed adults with one individual. MLG 48 was the second most frequent in managed adults (5 counts) and juveniles (15 counts), however it was not present among the individuals of the not managed stand. The occurrence of MLG implicated in genetically grouped individuals, based on Bruvo's distance, as shown from the dendrogram, indicating putative multilocus lineages (Fig. 1b). Additionally, the calculation of P_{sex} values supplied further confidence that the MLG were composed by genotypes belonging to the same lineage. A full table of pairwise P_{sex} calculations is presented in Supplementary Table 1.

The R index ranged from 0.293 in managed regenerants to 0.778 in not managed regenerants (Table 1). Therefore, managed regenerants showed higher levels of clonality ($R = 0.293$), followed by managed adults ($R = 0.489$). Yet, the Simpson's diversity index (D) showed high clonal heterogeneity, being higher than 0.90 in all stands. In turn, the aggregation index (A_c) was generally low, but significant in managed regenerants, managed adults and not managed adults, that is, meaningful aggregation was found according to the statistical criterion ($P < 0.05$) for those stands (Table 1). The highest aggregation index value was found in managed regenerants (7.4%), although still numerically low.

Moreover, a detailed analysis of the four plots of the managed stand allowed the identification of distinct levels of clonality within each plot. Taking the case of managed regenerants only, Fig. 2 shows the coordinates of each sample and their distribution in the four plots. Individuals belonging to the same multilocus genotypes are frequently located in proximity of each other, suggesting some level of aggregation and an uneven distribution of size. In plots 3 and 4, it is noticeable the aggregation of a few MLGs (Fig. 2), which is also consistent with the significant aggregation indexes, respectively, of 21.7% and 5.3% (Table 2). Levels of clonality were also higher in plot 3, as indicated by the R index (0.28). A minimum spanning network of the haplotypes of managed regenerants also showed that individuals of plot 3 tended to belong to fewer and genetically closer haplotypes (Fig. 3). The P_{sex} values statistically supported 15 MLG in plot 3 and 28 in plot 4. In plot 3, the lineage with more representation was composed by 15 individuals, while that number was of 8 at maximum in plot 4 (Supplementary Material 2).

The Pareto index was significant in the managed stands (adults and juveniles), as well as in not managed adults. Pareto's was not significant in not managed regenerants (Table 1). The lowest slope

(0.602) was computed from the managed regenerant group, suggesting more heterogeneity in clone size within this stand. When calculated only within plots of the managed regenerants, no significant Pareto indexes were found.

Table 1
– Clonality indexes.

Site	<i>N</i>	<i>G</i>	<i>R</i>	<i>D</i>	<i>Ac</i>	<i>Pareto index</i>	<i>I_A</i>	<i>r_D</i>
Managed adults	46	23	0.489	0.956	0.028*	1.067*	0.2043*	0.0512*
Managed regenerants	141	42	0.293	0.949	0.074*	0.602*	0.1177*	0.0296*
Not managed adults	52	26	0.490	0.966	0.062*	1.031*	0.1602*	0.0402*
Not managed regenerants	10	8	0.778	0.956	-0.047	1.322	0.2334*	0.0587*
Joint analysis	249	54			0.100*		0.0943*	0.0237*
*Significant at $P < 0.05$								

Table 2
– Clonality indexes for the managed regenerants plots.

Plot number	<i>N</i>	<i>G</i>	<i>R</i>	<i>D</i>	<i>Ac</i>	<i>Pareto index</i>	<i>Pareto P value</i>
1	9	6	0.625	0.917	0.065	0.585	-
2	24	11	0.435	0.862	-0.087	0.510	0,095
3	51	15	0.280	0.874	0.217*	0.454	0,001
4	55	28	0.500	0.959	0.053*	0.947	0,001

Further evidence of clonality was found through significant indexes of association (I_A and r_D) found in all stands (Table 1). Figure 4 shows the association index significance ($p = 0,002$) when all genotypic data of the managed regenerants were submitted to 999 permutations. However, when the data were clone corrected, non-significant ($p = 0,989$) association was found, which fits with a random sexual population in linkage equilibrium. That is, the presence of clonality produced linkage disequilibrium within each stand.

Discussion

Our data revealed that management of a natural population of *E. erythropappus* increased the number of individuals with identical genotypes, as identified from microsatellite markers. Individuals at initial stages of development, here referred to as regenerants, were more frequently grouped into the same multilocus genotype in the managed stand. Nevertheless, the identification of multilocus genotypes among adult

individuals within the managed stand also indicated clonality to some extent. Moreover, even among individuals genotyped within the unmanaged stand, a noticeable degree of clonality was detected, as inferred from the analyses.

The increase in clonal propagules after environmental disturbance might be linked to injuries caused to plants within the stand, especially to the root system. The injury might promote the emission of new shoots by physiological mechanisms leading to accumulation of solutes to the damaged tissue (Fonseca et al., 2021). Such mechanism along with seeds deposited on soil may drive the species to rapidly reoccupy cleared areas after deforestation, due to its pioneer ecological behavior (Souza et al., 2007). However, with the gradual recovery of disturbed areas, *E. erythropappus* populations tend to decline as other species structure their distributions in those areas (Souza et al., 2007). This may explain the reduced number of individuals at the adult stage within the sampled areas, including the managed stand. Yet, even at adult stages, some clonality is detected.

The ability to induce propagules by roots may explain the aggregation patterns observed from our data, especially among the regenerants within the managed plots. The spatial proximity of identical genotypes is corroborated by prior study that found a tendency of coancestry among juveniles at the first class of distance in the same managed area (Rocha et al., 2021). Another study of spatial genetic structure at a fine-scale was conducted by Pádua et al. (2016), that also detected significant coancestry in nine sites of candeia. But they attributed the genetic relatedness to factors such as limitations in seed dispersal, pollen flow, habitat disturbance and wind intensity. More specifically, our study adds clonality as a contributing factor with significant interference in the genetic structuring within populations.

The factor clonality may also be considered in future examination of genetic structure for candeia at a more general scale. For instance, Pádua et al. (2021) analyzed a broader range of distribution of *E. erythropappus* populations in discontinuous forest remnants. Using a landscape genetics approach with ISSR markers, high genetic differentiation was detected among populations (more than 30%) and it was positively correlated with the geographic distances among the populations. As our data revealed the production of spatial genetic structure within a few distances, further management of populations may accelerate the genetic differentiation among distant populations of *E. erythropappus*.

The clonality levels observed from our genotyping were also implicated in the detection of significant linkage disequilibrium, as revealed from significant values of the association index. After correcting the data for clonality, association was no longer detected, and the data suggested that the population could reproduce mainly by sexual means. However, it must be emphasized that even in adult stages of both managed and unmanaged stands, significant aggregation and association were found.

Since prior studies recognized the existence of spatial genetic structure within populations of *E. erythropappus* (Pádua et al., 2016, Rocha et al., 2021), high genetic differentiation among remnants located in discontinuous areas (de Pádua., 2021) and projected population decline over future generations (Rocha et al., 2020a), this study puts at stake the current management practices within natural sites of the species. Pádua et al. (2021) recommended that four operational units should be

implemented for the conservation of the species, as four genetically distinct groups were detected and geographically correlated in the populations sampled.

Here, we want to provoke a more specific conservation thinking, within each site of management. Our data indicated that intensive management induced clonal propagation more than would be expected by natural means, and some clonality also exists towards adult stages of the species. Therefore, we recommend that management should be reduced in intensity, keeping higher densities of individuals than the usual practices. Nevertheless, the appropriate management intensity is yet to be studied and should be carefully considered for further strategies aimed at conservation and breeding of this important species.

Declarations

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

LFR and DC conceived the experimental design and conducted the genotyping. ERK and DC analyzed the data. LFR, ERK and DC have contributed to the written manuscript. All authors have read and contributed to the final version of this manuscript.

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Figures

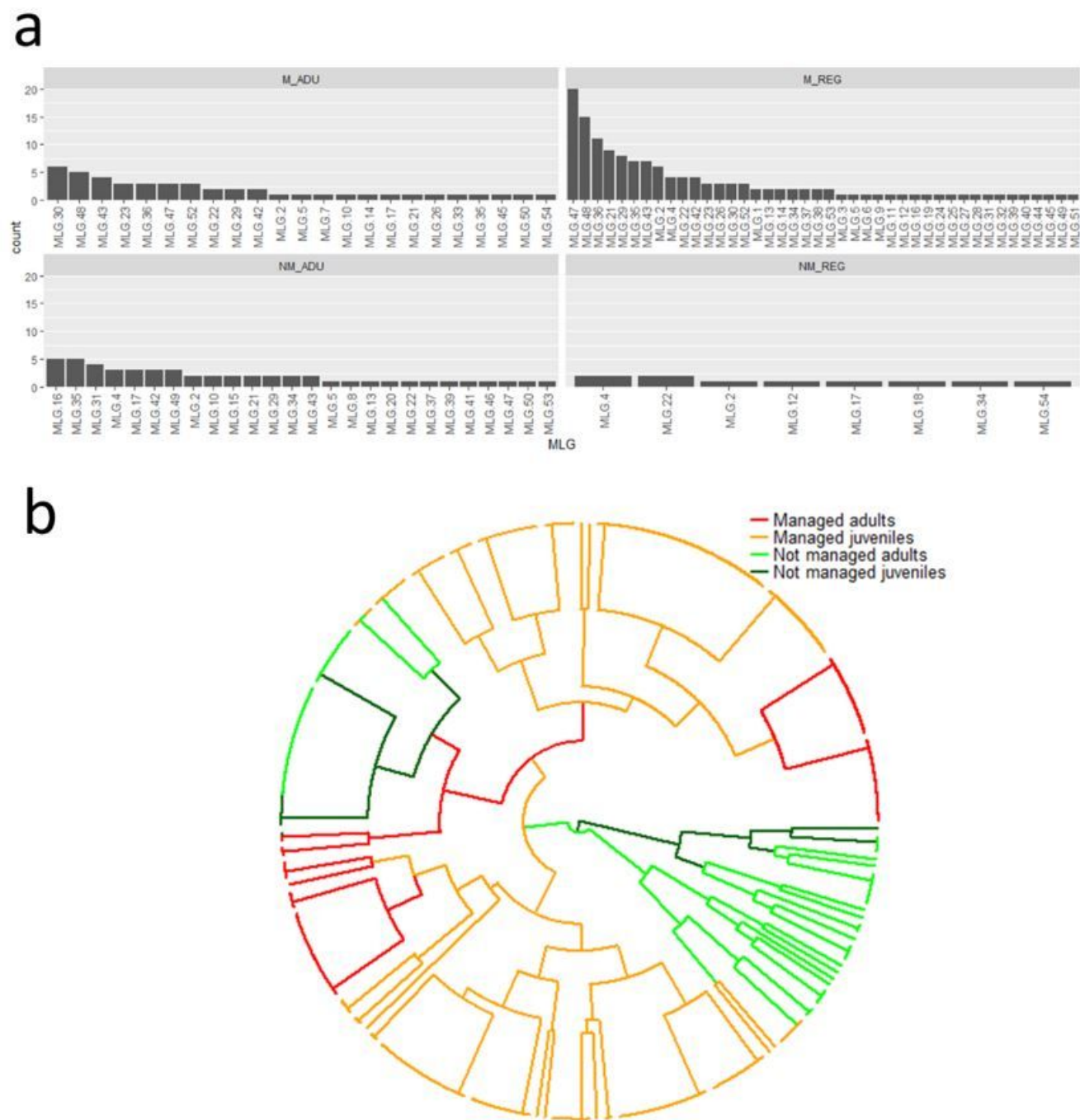


Figure 1

Identification of multilocus genotypes (MLG) in four natural sites of *Eremanthus erithropappus*. (a) Frequency of each MLG identified per site. M_ADU: managed adult individuals; M_REG: managed regenerants; NM_ADU: not managed adults; NM_REG: not managed regenerants. (b) UPGMA dendrogram based on Bruvo's genetic distance.

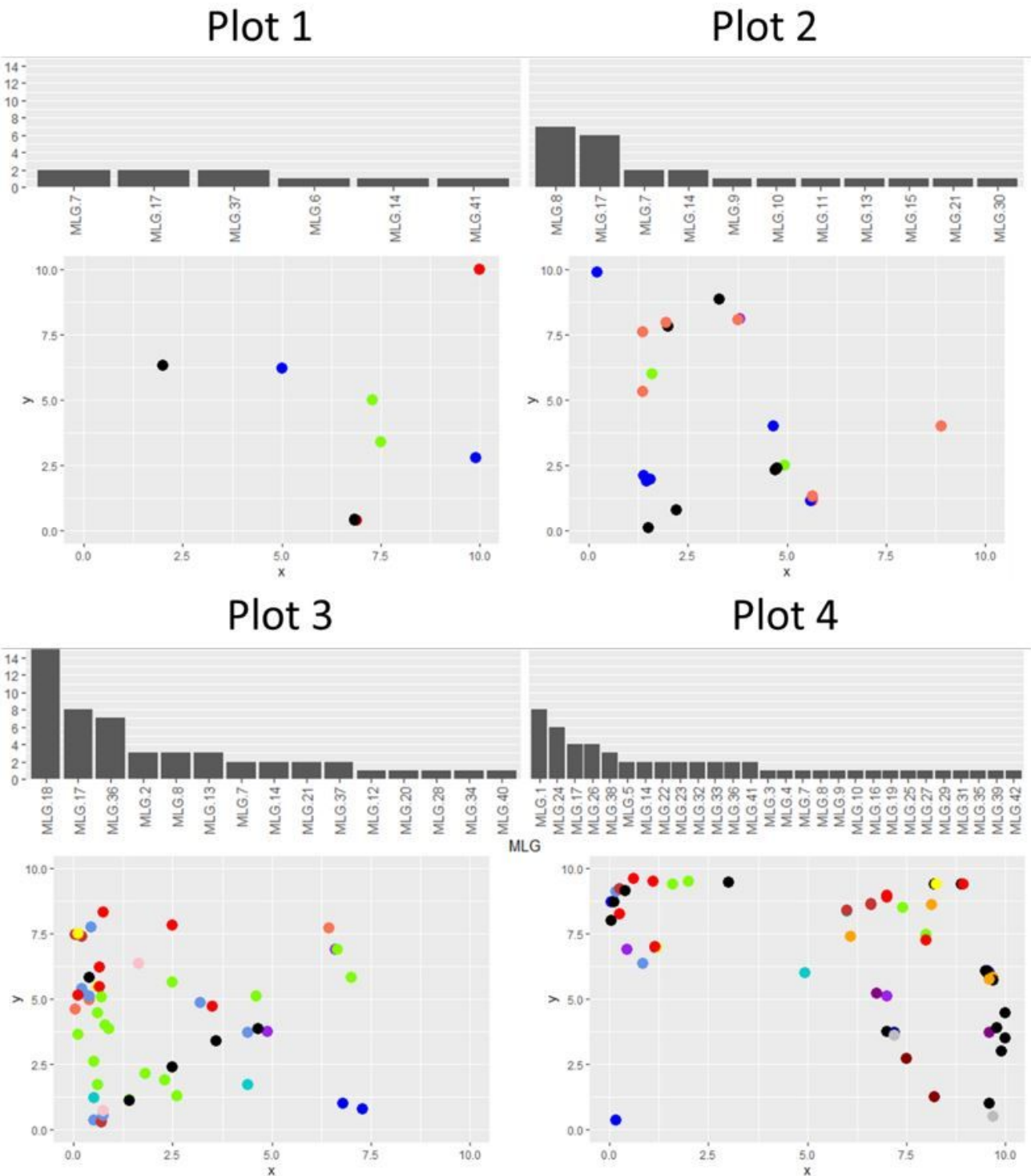


Figure 2

Frequency and spatial distribution (x and y coordinates) of multilocus genotypes (MLG) of regenerant individuals of a managed stand of *Eremanthus erythropappus*. Colors were assigned for each MLG and independently for each plot.

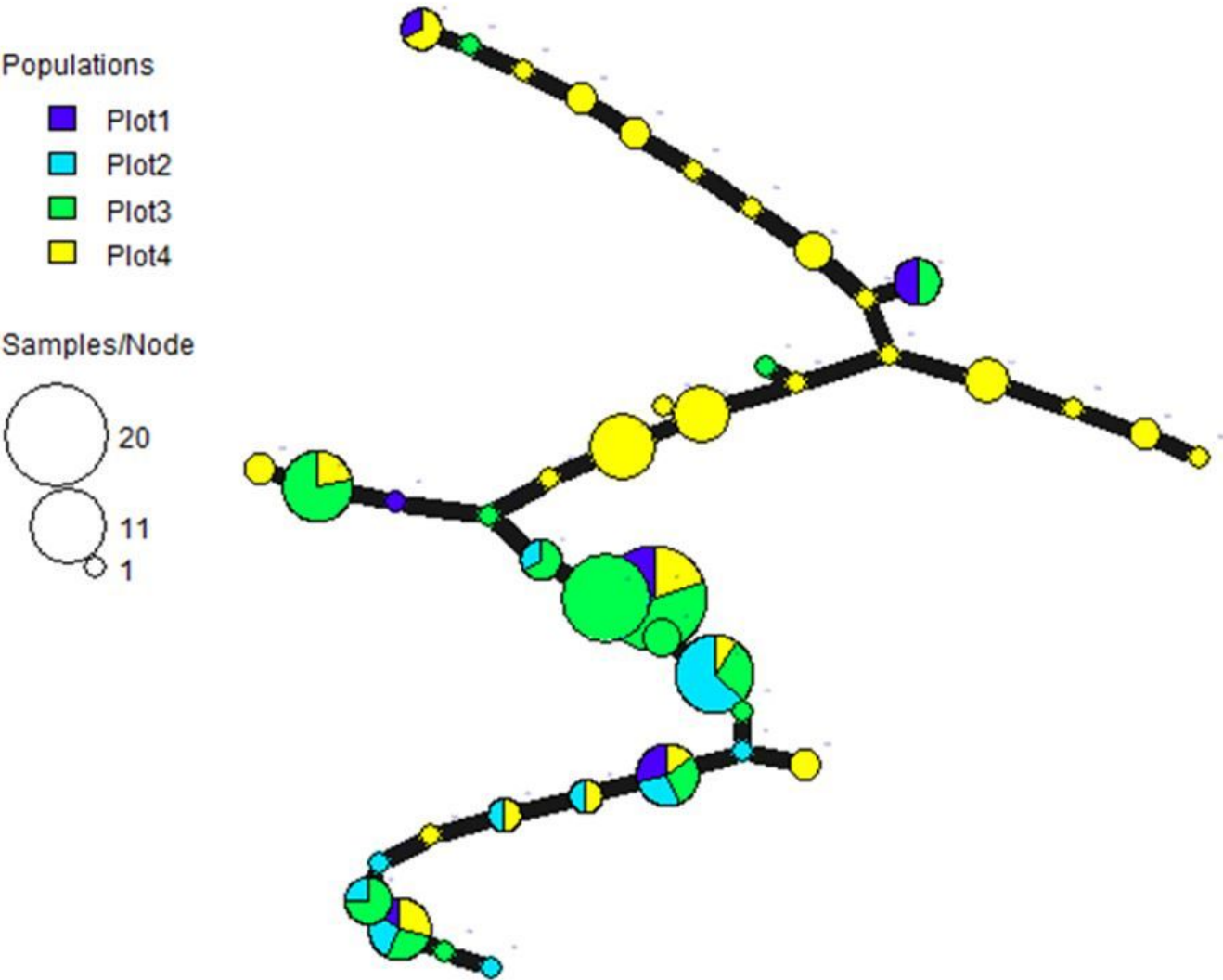


Figure 3

Minimum span network of multilocus genotypes (MLG) of regenerant individuals of a managed stand of *Eremanthus erythropappus*.

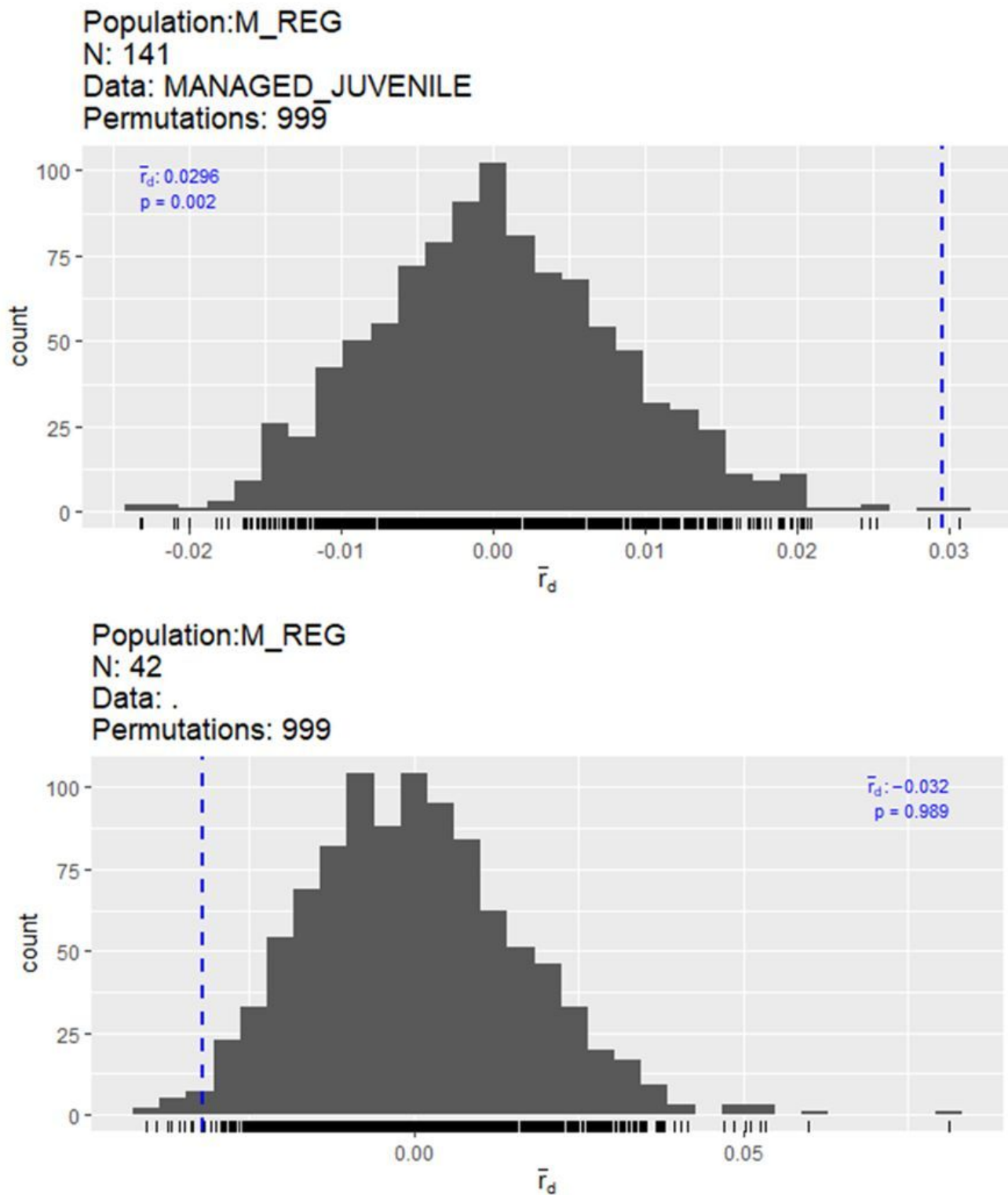


Figure 4

Index of association frequency distribution in the managed regenerant site before (upper graphic) and after correction (lower graphic) for clonality.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial1.docx](#)
- [SupplementaryMaterial2.xlsx](#)