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Prospection of ISSR primers and population genetic characterization of *Paratecoma peroba* (Record) Kuhlm.

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Abstract

The objective of this study is to prospect ISSR primers (Inter Simple Sequence Repeats) and, further genetic characterization in *Paratecoma peroba*. For this, leaf samples of 20 individuals were collected in a forest fragment, located in a private area, close to the Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, in the city of Alegre, ES, Brazil. For this, 43 ISSR primers were tested, and 10 primers were selected, which provided a total of 91 bands, with 57 polymorphic bands, resulting in 62.63% polymorphism. The polymorphic information content (PIC = 0.27) indicated moderate informativeness of the primers and, therefore, they are efficient in studies with the species. However, the values found for genetic parameters such as the number of observed ($A_O = 1.68$) and effective ($A_E = 1.41$) alleles and, the genetic diversity indices of Nei ($H^* = 0.23$) and Shannon ($I^* = 0.35$) indicate the occurrence of homozygous *loci* and low genetic diversity in the population. On the other hand, the genetic structure evaluated by the Bayesian approach revealed the formation of three genetic groups distributed in all sampled individuals, inferring once again about the occurrence of *loci* in homozygosity. Therefore, the connection of neighbouring fragments and the establishment of individuals obtained from other sources could increase the genetic diversity of the population and reduce the possible effects of depression by inbreeding and genetic drift.

Introduction

Genetic characterization in forest species is essential to elucidate information about the genetic variability available to breeders and geneticists, aimed at the conservation and improvement of germplasm. DNA polymorphisms are important markers in genetic analyses, supporting to understand the molecular basis of inheritance, also providing knowledge about the different biological and ecological aspects of the species (Hoban *et al*., 2022).

The choice of a molecular marker as an effective genomic resource for population genetic studies stems from its effectiveness in identifying polymorphisms, genotyping yield, cost, ease of use, consistency and laboratory reproducibility (Nadeem *et al*., 2018). Among the markers, Inter Simple Sequence Repeat (ISSR) is widely used due to its low cost of analysis, multilocus nature and universal character, making it applicable to non-model species. Moreover, ISSRs are considered highly reproducible when consistent concentrations of primers, reagents and additives, PCR settings and scorer criteria are maintained (Ng and Tan, 2015).

The application of ISSR markers has supported knowledge about variability, diversity and genetic structure in populations of forest species. Emphasis on Brazil and its native forest species, such as *Astronium concinnum* (Vieira et al., 2022), *Dalbergia nigra* (Silva Júnior *et al*., 2022), *Eremanthus erythropappus* (Pádua *et al*., 2021), *Freziera atlantica* (Zorzanelli *et al*., 2022), *Plathymenia reticulata* (Souza *et al*., 2017), among others.

As previously described, the quality and standard of results in genetic studies can only be achieved with well-defined and standardized methodologies, with the preliminary selection of functional primers being an indispensable requirement for the use of those with the best amplification profile (Silva *et al*., 2015). Thus, primer prospection studies can guarantee cost and time reduction to obtain the results and, greater efficiency in the detection of genetic variability.

The species *Paratecoma peroba* (Record) Kuhlm. popularly known as peroba-do-campo, it is a tree of the Bignoniaceae family, endemic to Brazil, which has ecological and economic potential, and can be used in forest restoration projects and in timber industry (CNCFlora, 2022). Currently, the species is threatened with extinction due to its history of exploitation by selective cutting (Martinelli and Moraes, 2013; Fraga *et al*., 2019), which characterizes it as a potential species for conservation and improvement programmes.

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Furthermore, studies of genetic characterizations with *P. peroba* are scarce (França *et al.*, 2022) and there are still no specific molecular markers for the species, which justifies the previous selection of arbitrary and informative markers, such as the ISSR. Thus, the aim is to prospect ISSR primers and subsequent genetic characterization of a natural population of the *P. peroba* species. With this study, we hope to optimize the use of molecular tools and their associated methods, so that they can be efficiently applied in future work with this species. In addition, we seek to obtain the necessary information for the maintenance and proper management of the *in situ* population.

Materials and methods

Sampling and study location

Leaf samples of 20 individuals of *P. peroba* were collected in a forest fragment of approximately 5.9 ha, located in a particular area, close to the Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, Campus de Alegre, ES (IFES, Campus Alegre), district of Rive, Alegre, ES/Brazil, between the coordinates 20°45′14″ South latitude and 41°27′18″ West longitude (Fig. 1). The local climate, according to the Köppen classification, falls into the Cfa type (subtropical, with hot summer), with an average annual temperature of 20°C and annual precipitation around 1200 mm (Alvares *et al.*, 2013). In addition, the forest fragment has Seasonal Forest type vegetation, surrounded by areas of pastures and coffee plantations.

DNA extraction

Genomic DNA extraction was performed from leaf tissue of all sampled individuals, submitted to the CTAB method (Doyle and Doyle, 1987), adjusting the concentrations to 1% polyvinylpyrrolidone (PVP) and 2% cetyltrimethylammonium bromide (CTAB). The concentration and purity of the extracted DNA were determined by spectrophotometry using the NanoDrop 2000C equipment (Thermo Scientific), analysing the A_{260}/A_{280} ratio, satisfactory within the range of 1.8–2.0 as it indicates no contamination by proteins or phenolic compounds (Aguilar *et al.*, 2016).

Amplification by polymerase chain reaction (PCR) and electrophoresis

Initially, four individuals of the *P. peroba* species were randomly selected to compose the amplification tests, which used 43 ISSR primers (UBC's 802, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 818, 822, 824, 825, 827, 829, 833, 834, 836, 840, 842, 849, 852, 855, 856, 861, 862, 864, 865, 866, 867, 868, 870, 874, 876, 877, 878, 880, 884, 886, 887 and 891) developed by the University of British Columbia, Vancouver, Canada. The total reaction volume was 20 µl, containing 1× buffer (10 mM Tris-HCl pH 8.5 and 50 mM KCl), 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of primer, 1 unit of Taq DNA polymerase and about 50 ng of genomic DNA.

The following amplification conditions were used: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 45 s), annealing (52°C for 45 s) and elongation (72°C for 90 s), with a final extension of 72°C, for 7 min. The amplification products were separated by electrophoresis on 2% agarose gel in 1× TBE solution (Tris – Boric acid – EDTA), at a voltage of 100 V for approximately 4 h. Finally, the gels were stained by immersion in ethidium bromide solution (0.50 µg/ml) and photographed under UV light in a photodocumentator (ChemiDoc MP Imaging System – Bio Rad). The estimation of the size of the amplified fragments was obtained by using the molecular weight marker (Ladder) of 100 base pairs.

Data analysis

From the photodocumentation of the gels and based on the amplification profile, the selection of primers with the best bands sharpness patterns was performed. Subsequently, a binary matrix was generated, considering the presence (1) and absence (0) of amplified fragments, for which the following descriptive analyses were performed: Total number of bands (TNBs), number of polymorphic bands (NPBs), percentage of polymorphic bands (PPBs) per primer and the range of variation of bands (BVR). Thus, the selection of the ISSR primers for the study with the *P. peroba* species was carried out based on the sharpness of the bands and the identification of polymorphism. In addition to the descriptive analyses, the primers were also evaluated based on the polymorphic information content (PIC), calculated using the Genes program (Cruz, 2016).

To evaluate the genetic diversity contained in the population, the parameters called number of observed alleles (A_O), number of effective alleles (A_E), Nei genetic diversity index (H*) (Nei, 1978) and Shannon index (I*) (Shannon and Weaver, 1949) were calculated using the Popgene program (Yeh and Boyle, 1997). The genetic dissimilarity was evaluated based on the estimated values between the individuals pair by pair through the arithmetic complement of the Jaccard coefficient, resulting in a numerical matrix, used in the development of a dendrogram by the method of grouping unweighted arithmetic means (UPGMA), with an estimated cut-off point according to Mojena (1977), with the coefficient $k = 1.25$. To confirm the consistency between the dissimilarity values and the representation in the dendrogram, the cophenetic correlation coefficient (CCC) was calculated. All analyses were performed using the Genes program (Cruz, 2016).

Based on the dissimilarity matrix, principal coordinate analysis (PCoA) was performed using the Genes software (Cruz, 2016), to determine the relative position of the sample in a multidimensional space, graphically demonstrating the genetic structure of individuals within the population. In addition, the genetic structure was also evaluated using a Bayesian approach performed using the Structure 2.3 software (Falush *et al.*, 2007), establishing 20 runs for each K value, with the number of groups (K) varying between $K = 1$ and $K = 4$, totalling 10,000 Monte Carlo interactions via Markov Chains (MCMC). The number of genetic groups was determined by the *ad hoc* ΔK method proposed by Evanno *et al.* (2005) using Structure Harvester software (Earl and Vonholdt, 2012).

Results

Selection and efficiency of ISSR primers

Tests performed for the 43 ISSR primers allowed the identification of different amplification patterns, ranging from clear to no amplification. Therefore, only primers with clear amplification were selected (Fig. 2), and only the bands visible to the naked eye were counted, so that the analysis could be reproduced in future studies.

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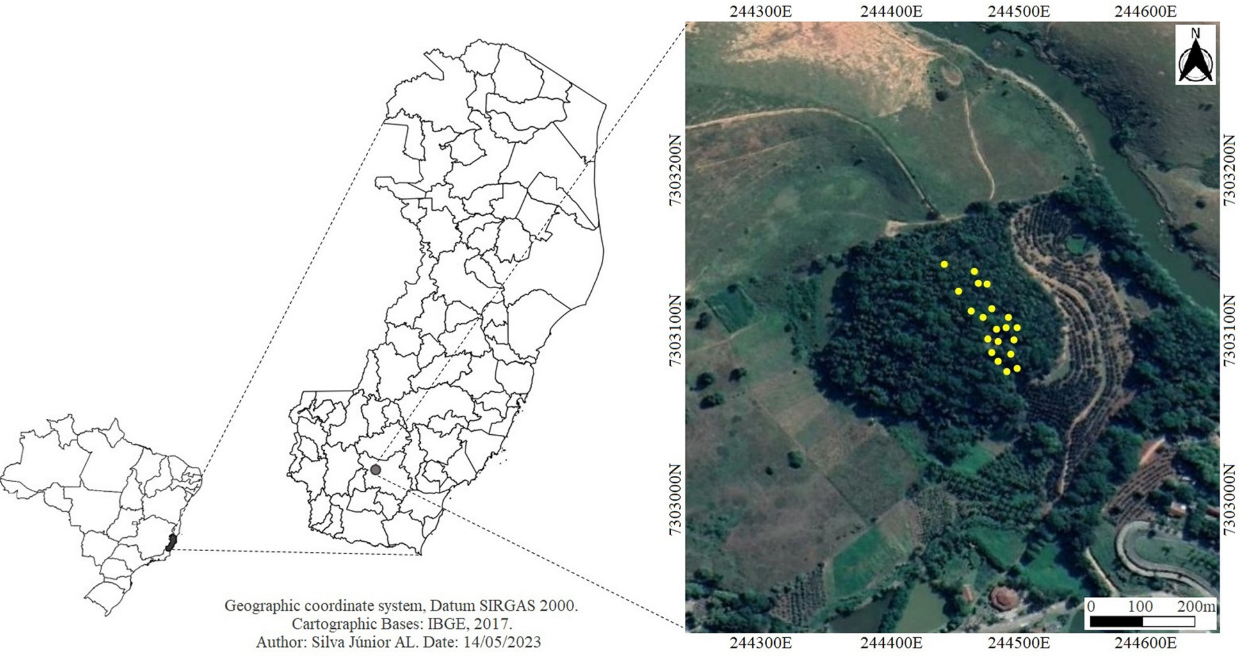

Figure 1. Location map of the study area and selection of individuals of the species *Paratecoma peroba* (in yellow).

Among the total number of primers evaluated, 10 were selected to compose the genetic-population analyses. A total of 91 bands were identified, of which 58 were polymorphic, resulting in 62.63% polymorphism. The UBC 836 primer presented the highest total number of bands (TNB), while the UBC's 810 and 815 primers showed the lowest TNB values. The primer that presented the highest percentage of polymorphic bands (PPB) was UBC 880, with the lowest percentage observed for the primer UBC 807. The values for the polymorphic information content (PIC) ranged from 0.18 to 0.35 for the primers UBC 868 and

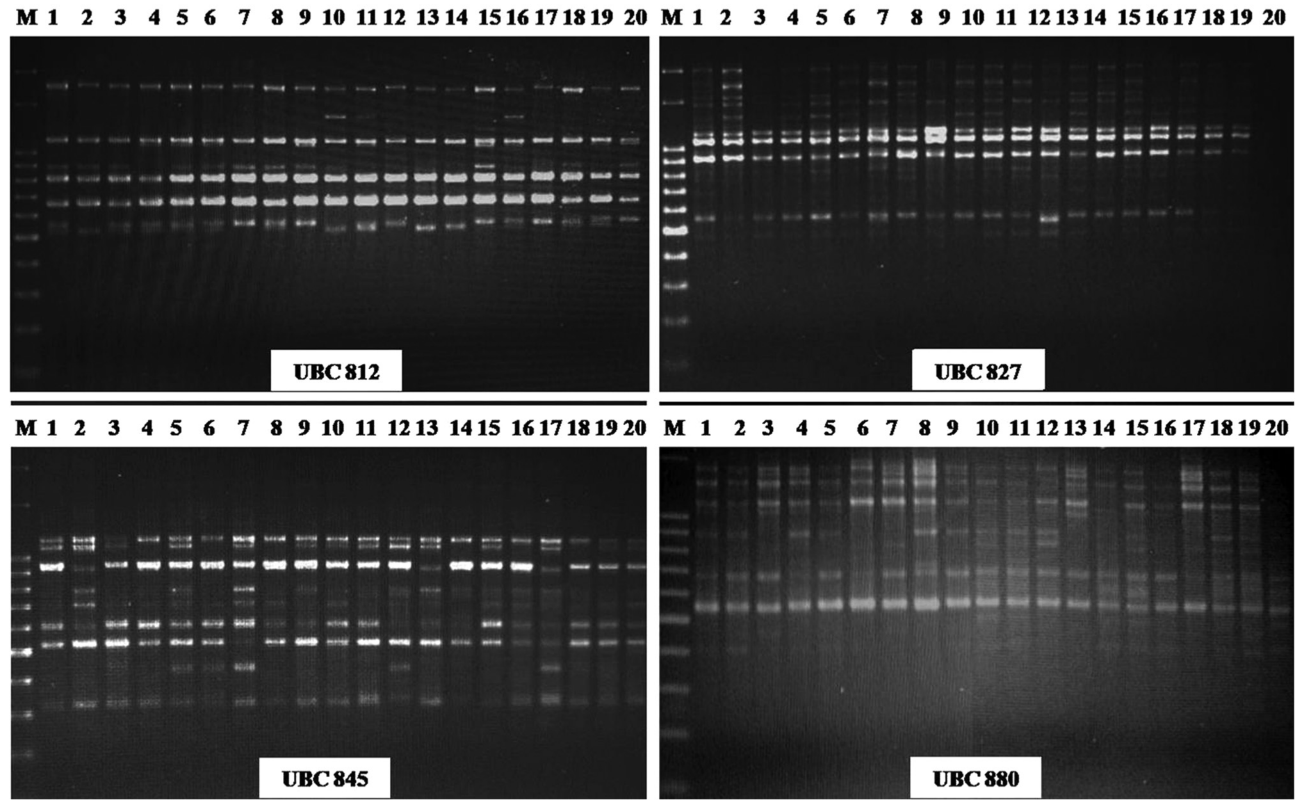

Figure 2. Amplification sharp profile for 20 individuals of the *Paratecoma peroba* species via ISSR primers (UBC's 812, 827, 845 and 880).

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Table 1. Descriptive analyses of the ISSR primers selected for evaluation in a population of the species *Paratecoma peroba*

Primers	*Sequence (5′-3′)	TNB	NPB	PPB (%)	BVR (max–min)	PIC
UBC 807	(AG)8T	9	4	44.44	980–200	0.33
UBC 810	(GA)8T	5	3	60.00	2080–400	0.23
UBC 811	(GA)8C	9	6	66.66	2080–400	0.29
UBC 812	(GA)8A	8	4	50.00	2080–310	0.28
UBC 815	(CT)8G	5	3	60.00	1600–550	0.24
UBC 827	(AC)8G	12	8	66.66	2080–480	0.35
UBC 836	(AG)8YA	13	9	69.23	1200–320	0.33
UBC 845	(CT)8TT	12	8	66.66	2080–320	0.25
UBC 868	(GAA)6	9	5	55.55	1080–480	0.18
UBC 880	(GGAGA)3	9	7	77.77	2080–500	0.25
Total	–	91	57	62.63	–	0.27

TNB, total number of bands; NPB, number of polymorphic bands; PPB, percentage of polymorphic bands; BVR, range of variation of bands determined by molecular weight marker of 100 bp; PIC, polymorphic information content. *H = (A, T or C); R = (A or G); V = (A, C or G) and Y = (C or T).

UBC 827, respectively. The PIC value considering the total set resulted in a value of 0.27 (Table 1).

Genetic diversity

Regarding the individually evaluated *loci*, the values for A_O ranged from 1.44 (UBC 807) to 1.88 (UBC 880), while the A_E values ranged between 1.28 (UBC 868) and 1.57 (UBC 827). The genetic diversity indices of Nei (H*) and Shannon (I*) ranged from 0.16 (UBC 868) to 0.31 (UBC 827) and 0.26 (UBC's 810 and 868) to 0.43 (UBC 827), respectively. Considering all the primers used in the characterization of the genetic diversity of *P. peroba*, the values for A_O, A_E, H* and I* were 1.68, 1.41, 0.23 and 0.35, respectively, confirming low genetic diversity for the evaluated population (Table 2).

Genetic dissimilarity

The dendrogram showed the formation of three genetic groups, with one group with 16 individuals and two groups containing 2 individuals, respectively (Fig. 3(a)). The CCC was 0.77, showing consistency between the dissimilarity matrix and the dendrogram obtained.

Genetic structure

PCoA revealed the structuring of individuals within the population, making it possible to spatially identify the genetic proximity and distance between them. It is possible to observe, by PCoA, the genetic proximity of the pairs of 3×4 and 19×20 individuals. In addition, there is a greater genetic divergence of individual 2, as it is more isolated from the others (Fig. 3(b)).

Through the Bayesian approach, three genetic groups were identified ($K = 3$) (Fig. 4(a)), distributed among the individuals of the population (Fig. 4(b)).

Discussion

Selection of molecular markers based on band visualization capacity and amplification pattern is commonly used in plant species studies to avoid the use of poorly informative or false-positive primers (Silva *et al*., 2015). In the case of *P. peroba*, there is a lack of developed molecular markers, making the utilization of universal markers like ISSR beneficial for conservation and improvement studies of the species.

A study on *Plathymenia reticulata* showed that 10 ISSR primers resulted in a 64.74% polymorphism rate (Souza *et al*., 2017), which is similar to the findings of the current study. Similarly, another study on *Jacaranda mimosifolia* found an 83.02% polymorphism rate (Liu *et al*., 2022). Both studies employed ISSR primers, with the primers UBC 868 and UBC 836 identified a higher percentage of polymorphism in *P. peroba* (55.55 and 69.23%) compared to *P. reticulata* (40%) and *J. mimosifolia* (50%), respectively.

The difference between the percentage of amplification is expected to occur between different species, as previously demonstrated, and also between populations of the same species, since in the latter case different ecological and evolutionary factors act to

Table 2. Parameters of genetic diversity estimated, by *loci* and for the population of the species *Paratecoma peroba*, using ISSR markers

Primers	A_O	A_E	H*	I*
UBC 807	1.44	1.33	0.19	0.27
UBC 810	1.60	1.29	0.17	0.26
UBC 811	1.66	1.46	0.25	0.36
UBC 812	1.57	1.38	0.20	0.31
UBC 815	1.66	1.32	0.19	0.30
UBC 827	1.66	1.57	0.31	0.43
UBC 836	1.69	1.53	0.29	0.42
UBC 845	1.73	1.38	0.23	0.35
UBC 868	1.75	1.28	0.16	0.26
UBC 880	1.88	1.44	0.27	0.41
Total	1.68	1.41	0.23	0.35

A_O, number of alleles observed; A_E, number of effective alleles; H*, Nei genetic diversity index; I*, Shannon index.

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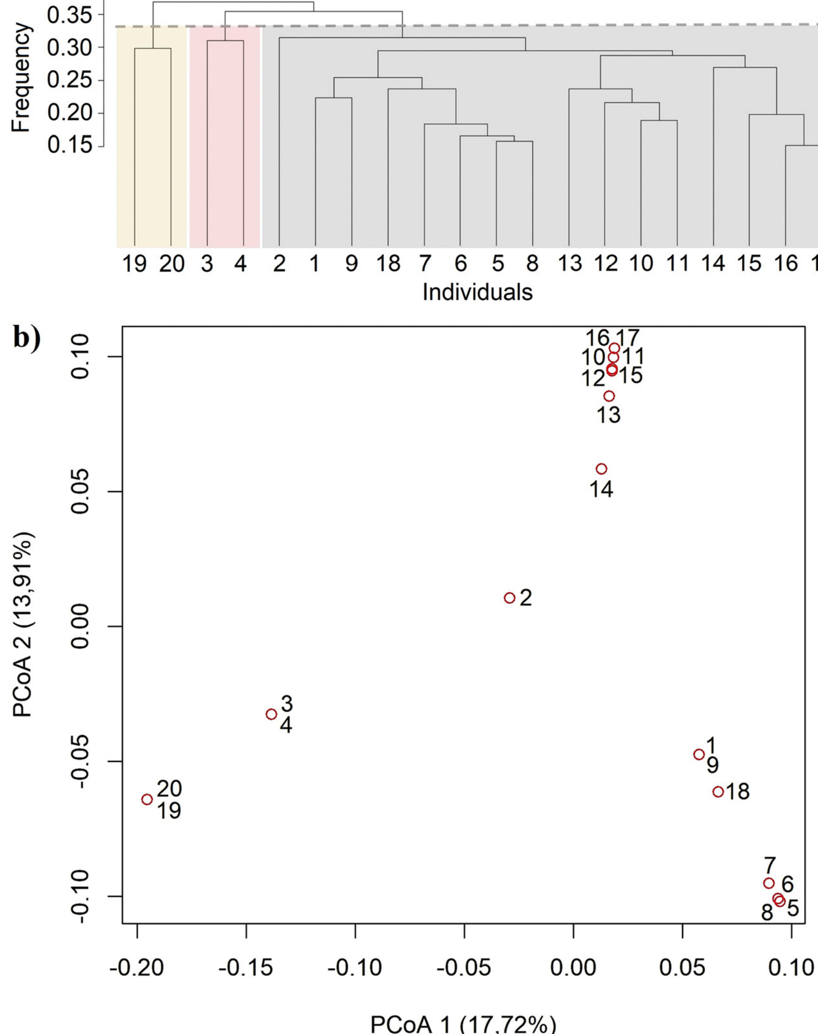

Figure 3. Analysis of dissimilarity and genetic structure for individuals in a population of the species *Paratecoma peroba*. (a) Dendrogram of genetic dissimilarity, obtained by the UPGMA method with cut-off point (PC): 0.33. (b) Principal coordinate analysis (PCoA) for the total sample of individuals.

shape the genetic variability among individuals. However, we emphasize the reproducibility of ISSR markers when applied to the same species, as long as the concentrations of primers, reagents and additives, PCR settings and scoring criteria are maintained (Ng and Tan, 2015).

Individually, the evaluated primers were classified as having low to moderate informativeness. However, when considering the collective PIC value for the entire set of primers, they demonstrated moderate informativeness (Tatikonda *et al*., 2009). Therefore, these primers are efficient for genetic characterization studies involving *P. peroba*.

Most Bignoniaceae species are diploid with up to two distinct alleles per locus (Gentry, 1980). The observed number of alleles ($A_O = 1.68$) may be influenced by loci with similar alleles, while the number of effective alleles ($A_E = 1.41$) suggests the presence of alleles that do not contribute effectively, indicating loci with homozygosity.

Homozygosity in the evaluated population of *P. peroba*, where open pollination occurs (Lins and Nascimento, 2010), can result from the inbreeding or self-pollination. Inbreeding refers to the natural or artificial crossing of related individuals, leading to reduced fitness due to increased homozygosity, known as inbreeding depression, primarily caused by the expression of deleterious alleles (Howard *et al*., 2017). Consequently, inbreeding depression effects can limit the maintenance of the *P. peroba* population as generations progress.

The values for Nei's genetic diversity ($H^* = 0.23$) and Shannon's index ($I^* = 0.35$) indicate low genetic diversity contained in the population. According to Lewontin (1972), the values for the Shannon index range from 0 to 1, indicating a variation from low (0) to high (1) genetic diversity. The small size of the population in a fragmented forest, along with factors like genetic drift, contributes to the low genetic diversity (Basey *et al*., 2015).

The low genetic diversity can also be attributed to the population's geographic isolation and ecological factors such as insect pollination (Gentry, 1974) and anemochory-mediated seed dispersal (Lins and Nascimento, 2010). Insect pollination limits pollen and allele exchange in forest fragments located at greater distances, while anemochory tends to disperse seeds closer to the parent tree (Warneke *et al*., 2021).

To expand the genetic diversity of the *P. peroba* population, strategic actions such as creating ecological corridors to connect neighbouring fragments, potentially harbouring *P. peroba* representatives and introducing individuals from other sources are essential. Genetic diversity encompasses the sum of loci and

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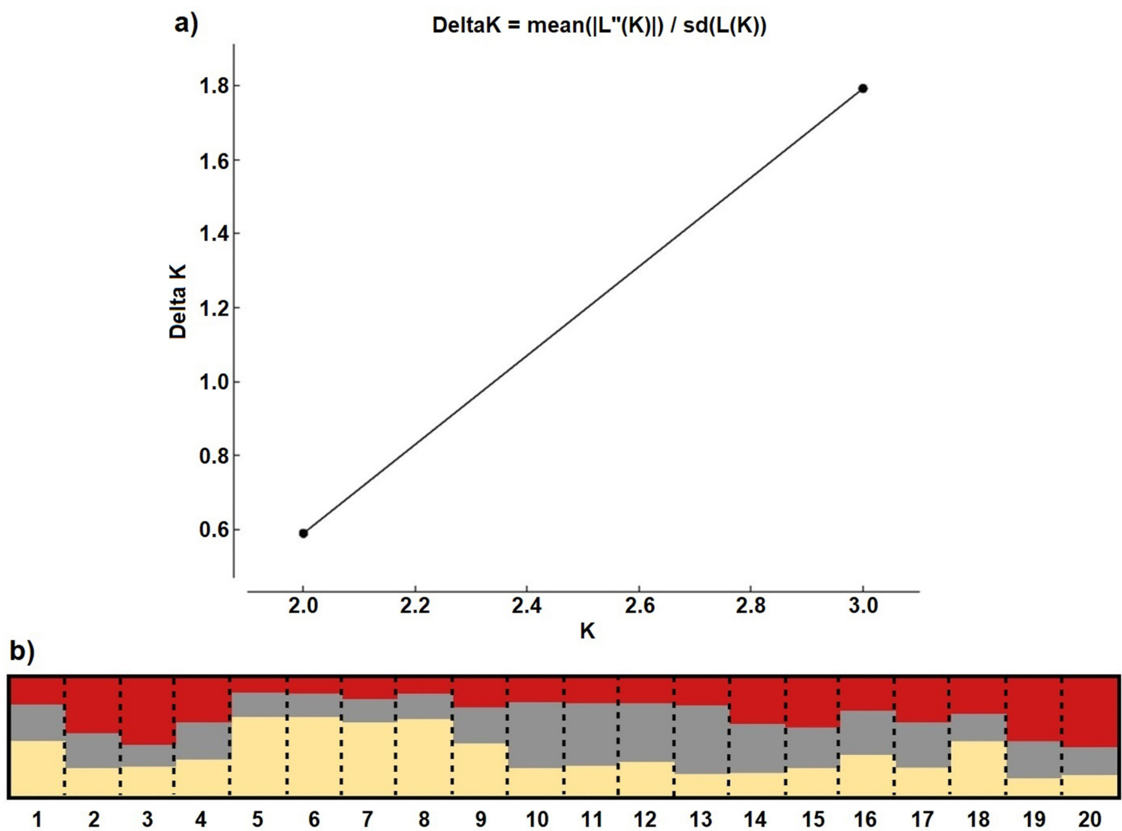

Figure 4. Bayesian approach performed for a population with 20 individuals of the species *Paratecoma peroba*. (a) Most likely number of K clusters in three ($K = 3$). (b) Distribution of genetic groups in each sampled individual.

alleles influencing trait expression in a population, so it is correct to say that higher levels of genetic diversity represent a greater probability of maintaining the population.

The formation of a majority group in the dendrogram (Fig. 3 (a)) corroborates the low genetic diversity evaluated in the population. However, this analysis allows the development of another strategic action to be used to expand the genetic base, as individuals located in different groups can be used in the production of seedlings from controlled pollination, being later implanted in the place.

Although controlled pollination can be used to expand the genetic base for population conservation, its main use is in forest improvement, due to the possibility of gene introgression, hybrid formation and increased genetic gains (Ortega-Ramirez *et al*., 2021). Thus, it is noteworthy that the evaluation of genetic dissimilarity in the population of *P. peroba* also allows the use of individuals as possible matrices in breeding programmes for the species.

To assess the structure and dispersion of individuals, PCoA has the advantage of allowing the use of matrices of genetic distances obtained from qualitative data, such as those generated for ISSR markers (Prado *et al*., 2002). Therefore, it was observed that PCoA (Fig. 3(b)) corroborated the results of the dendrogram (Fig. 3(a)), demonstrating pairs of individuals that were genetically closer, while others were more distant.

The pairs of individuals 3×4 and 19×20 remained genetically related for the two analyses, being also geographically close (~50 m) in the forest fragment where they were selected. This observation between genetic and geographic relationship has already been confirmed by several genetic-population studies involving forest species (Silva *et al*., 2004; Silva Júnior *et al*., 2022; Vieira *et al*., 2022) and are directly associated with ecological factors of the species such as reproductive system, mechanisms of dispersion and pollination, population size, among others (Santo-Silva *et al*., 2016). In *P. peroba*, insect pollination and anemochoric fruit dispersal (Lins and Nascimento, 2010) allow related individuals to establish themselves geographically close. According to Motta *et al*. (2004), tree species tend to form groups with homogeneous allele frequencies, due to seed dispersal close to the parent tree.

Regarding the analysis performed by the Bayesian approach, the number of genetic groups found indicates the non-structuring of the population (Fig. 4(a)), however, the distribution of the three colours in all individuals infers (Fig. 4(b)), once again, on the occurrence of *loci* in homozygosity. Therefore, the vulnerability of the population with the advancement of generations is highlighted, mainly due to factors such as genetic drift and inbreeding depression.

Conclusion

The selected ISSR primers were efficient in revealing polymorphisms between individuals, being able to be applied in strategic actions of conservation and improvement programmes that demand the knowledge of the variability, diversity and genetic structure of the *P. peroba* species or its populations. However, the evaluated population is vulnerable, confirming the presence of homozygous *loci* and low genetic diversity. To reverse this

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situation of vulnerability and expand the genetic base of the population, an increase in the connectivity of neighbouring forest fragments and the establishment of genetically divergent individuals originating from other populations are indicated.

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