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Original Paper

Assessing the genetic diversity of *Myrsine umbellata* (Primulaceae) in Brazilian Atlantic Forest remnants – an important step towards reforestation efforts

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Abstract
The investigation of genetic diversity in natural populations of species that show potential for use in reforestation programs is a key step in making management decisions. However, reforestation programs with native species in Brazil are still rarely based on a genetic understanding of the seed matrices used for seedling production. This is also the case for *Myrsine umbellata*, a dioecious shrub within the family Primulaceae that has been used in reforestation programs in Brazil, mainly due to its high production capacity of fruits attractive to the avifauna. The goal of this study was to measure intra- and interpopulational genetic diversity in natural populations of *M. umbellata* in six forest remnants of the Atlantic Forest using ISSR markers. The results revealed that the intrapopulational genetic diversity was greater than the genetic diversity among the studied populations. For this reason, the cultivation of seedlings from seeds obtained in more than one population seems the most appropriate strategy for reforestation purposes. Even though the most isolated populations are also the ones with highest genetic structure, all populations of *M. umbellata* included in this study revealed to be an important germplasm bank conserved *in situ*.
Key words: Capororoca, ISSR markers, Myrsinoideae, tropical forests.

Resumo
Investigar a diversidade genética em populações naturais de espécies com potencial para uso em programas de reflorestamento é um passo importante para a tomada de decisões relacionadas ao planejamento e manejo dos plantios. No entanto, programas de reflorestamento com espécies nativas no Brasil raramente são realizados com base no conhecimento do genótipo das matrizes doadoras das sementes utilizadas para produção de mudas. Este é o caso de *Myrsine umbellata*, um arbusto dioico pertencente à família Primulaceae, que vem sendo utilizado em programas de reflorestamento no Brasil principalmente por apresentar elevada capacidade de produção de frutos atrativos à avifauna. O objetivo deste estudo foi mensurar a diversidade genética intra e interpopulacional em populações naturais de *M. umbellata* em seis remanescentes florestais da Floresta Atlântica, utilizando marcadores ISSR. Os resultados revelaram que a diversidade genética intrapopulacional foi maior que a diversidade genética entre as populações estudadas. Por essa razão, o cultivo de mudas a partir de sementes obtidas em mais de uma população parece ser a estratégia mais apropriada para reflorestamento. Mesmo considerando que as populações mais isoladas são aquelas mais estruturadas, todas as populações de *M. umbellata* incluídas neste estudo revelaram ser importantes bancos de germoplasma conservados *in situ*.
Palavras-chave: Capororoca, marcadores ISSR, Myrsinoideae, tropical forests.

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Introduction

Anthropogenic actions have been modifying the structure of tropical forests, particularly the Atlantic Forest domain and its associated ecosystems (Magnago *et al.* 2014). These changes lead to a reduction in population density among species affecting their reproduction, pollination and the gene flow between and within populations (De Lacerda *et al.* 2013). The consequent forest fragmentation process may lead to the reduction of genetic variability between populations due to the isolation of populations and their individuals, and the increase of self-fertilizations and correlated mattings (Young & Boyle 2000; Duminil *et al.* 2016). In this context, the use of genetic markers in population diversity studies of tree species is a powerful tool to help with decision-making on management and conservation.

The assessment of genetic diversity of a species at the intra- and interpopulational level allows inferences about the genetic structure of the population, gene flow and potential genetic bottlenecks, due to habitat fragmentation or other spatial or temporal barriers (Mondini *et al.* 2009). In addition, genetic studies at population level can be applied to identify individuals with satisfactory genetic characteristics to be used as seed matrices to be collected for recovery and restoration of degraded areas (Rodrigues *et al.* 2009). For these purposes, it is essential to increase the knowledge about genetic diversity of interesting species for reforestation.

In the context of reforestation, *Myrsine umbellata* Mart. (Primulaceae) stands out as a facilitating species in areas of natural regeneration, which supplies fruit and shelter for the avifauna (Backes & Irgang 2002; Pascotto 2007). Also, *M. umbellata* is the second most widely distributed species of *Myrsine* in South America (Sánchez-Tapia *et al.* 2018). The members are dioecious shrubs, with ornithochoric seed dispersal (Jung-Mendançolli *et al*. 2005). These attributes made this species, and its close relatives in the genus *Myrsine*, important components in reforestation programs in Brazil. However, until now seed obtainment has been carried out without considering the genetic variability of natural populations.

Inter Simple Sequence Repeat (ISSR) is a DNA marker widely used to estimate the genetic diversity of natural plant populations (*e.g.*, Sheng *et al.* 2017; White *et al.* 2018). This marker was previously applied to study the genetic diversity of *M. coriacea* (Sw.) R. Br. *ex* Roem. & Schult. (Paschoa *et al.* 2018). Here we used the same molecular marker to measure the genetic diversity in natural populations of *M. umbellata* found in forest remnants of Atlantic Forest. Our main goal was to improve the knowledge of genetic diversity in the selected species in order to guide practices that allow the use of *M. umbellata* in reforestation programs.

Material and Methods

Populations studied and leaf samples collection

Healthy young leaves of 63 individuals of *Myrsine umbellata* were collected at six different locations in the Atlantic Forest (Fig. 1) in the state of Espírito Santo following a previous study on another species in the same genus (Paschoa *et al.* 2018). Samples were taken only from individuals which had a height of 4 to 5 m, a diameter at *breast* height (DBH) of at least 30 cm, and had clearly reached reproductive age. The leaf samples were stored individually in paper bags, sealed, labelled, and kept on silica gel until transported to the laboratory.

DNA extraction and ISSR marker analysis

The total genomic DNA was isolated and purified using the extraction methodology by Doyle & Doyle (1990), with modifications following Ferreira & Grattapaglia (1998). DNA quality and concentration were verified using a *nanoDrop* TM 2000 spectrophotometer (Thermo Scientific). Integrity was confirmed with gel electrophoresis in 0.8% agarose gel.

A screening was performed with 32 ISSR primers obtained from British Columbia University, Vancouver, Canada and, based on the highest number of polymorphic fragments presented and the quality of amplification, the ten best were selected (Tab. 1). We selected ISSR markers due to the absence of SSR (codominant) markers for this species. It is has been shown that polymorphism analysis based on ISSR generates robust and highly polymorphic data for both intraspecific and interspecific analyses within the genus. In addition, ISSRs are considered neutral genome markers, an important feature for studying patterns of genetic dispersion, diversity and genetic structure in natural populations (Paschoa *et al.* 2018).

Volumes of 20 µL containing 8.3 mM enzyme mix for PCR (Promega®) 1X, 0.5 µM primer, 1 unit of Taq polymerase and 60 ng of DNA were used for amplification. Amplification conditions were: denaturation 5 minutes at 94 ºC, followed by 40 cycles of 1 minute at 94 ºC; 1 minute at 55 ºC and 1 minute at 72 ºC, with a final extension phase of 2 minutes at 72 ºC. The fragments obtained from the PCR reactions were separated by horizontal gel electrophoresis in 1.5% agarose gel containing 0.02 µL/mL ethidium bromide and TBE running buffer (Tris-base, boric acid and EDTA), at 90 volts for about 1 hour and 15 minutes. After electrophoresis, gels were visualized with an imaging system (BioRad Gel DocTM EZ Imager) in acordance to Carvalho *et al*. 2020, with modification.

Data analysis

To analyze the molecular data, the monomorphic and polymorphic bands were coded as absent (0) or present (1), respectively. The polymorphic loci of the 10 selected primers were used to generate a matrix of binary data of individuals (rows) by bands (columns). The Sorensen-Dice coefficient (Dice 1945) was used to calculate genetic dissimilarity among individuals. Dissimilarity averages were used to run a grouping analysis of the individuals following the UPGMA method (Unweighted Pair-Group Method Average).

From this matrix, the values of Φ_{ST} between the

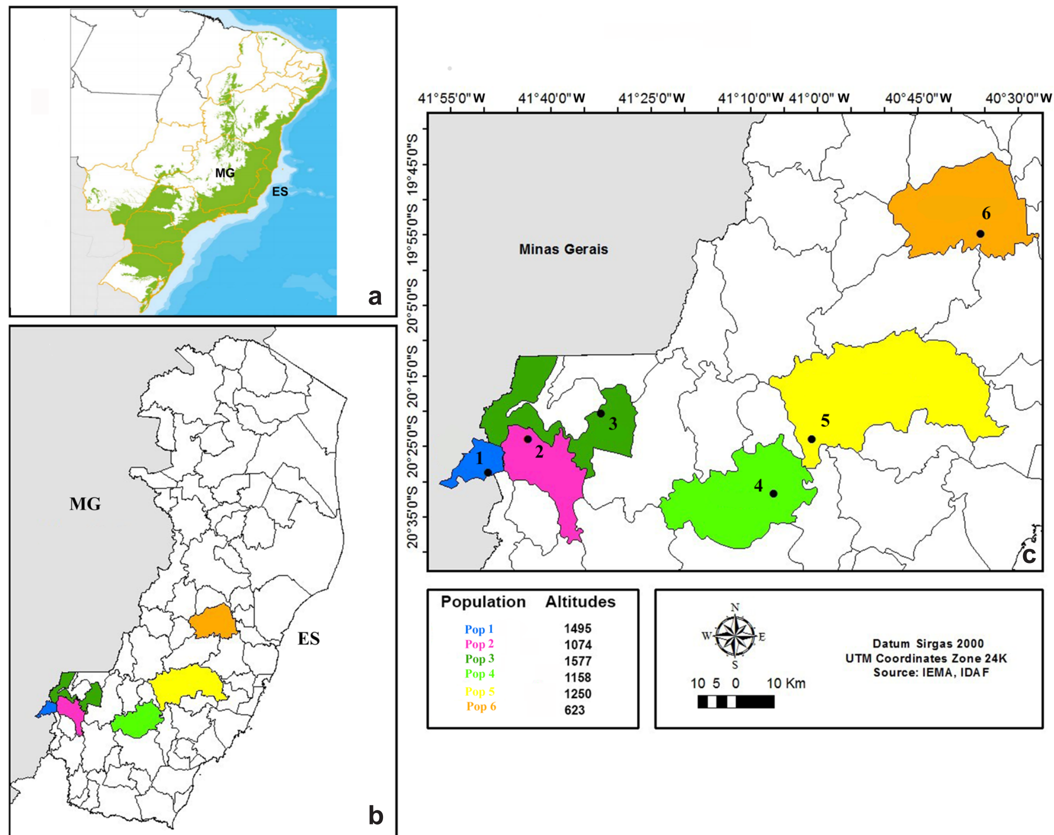

Figure 1 – a-c. Geographic locations of the six studied natural populations of *Myrsine umbellata* – a. map of Brazil and natural distribution of the Atlantic Forest (Fundação SOS Mata Atlântica 2019); b. state map of Espirito Santo; c. localities of the studied populations[blue: population 1 (municipality of Dores do Rio Preto, locality Macieira in the Caparaó National Park - 20º41'20"S, 41º50'43"W); pink: population 2 (municipality of Ibitirama, locality on rural property - 20º32'29"S, 41º40'02"W); dark green: population 3 (municipality of Iúna, locality Serra do Valentim - 20º20'45"S, 41º32'09"W); light green: population 4 (municipality of Castelo, locality Forno Grande State Park - 20º36'13"S, 41º11'05"W); yellow: population 5 (municipality of Domingos Martins, locality Pedra Azul State Park - 20º21'48"S, 40º39'33"W); orange: population 6 (municipality of Santa Teresa, locality on rural property 19º56'08"S, 40º36'01"W)].

Table 1 – The ten ISSR *primers* selected for amplification of DNA fragments from the six studied *Myrsine umbellata* populations. Descriptive analysis of the primers with the respective sequences, total number of bands, total number of polymorphic bands and percentage of polymorphism.

Primer sUBC	Sequence (5'-3')	Total number of bands	Polymorphic bands	% of polymorphism
849	GTG TGT GTG TGT GTG TYA	5	5	100
807	AGA GAG AGA GAG AGA GT	16	16	100
834	AGAGAG AGA GAG AGA GYT	18	18	100
810	GAG AGA GAG AGA GAG AT	16	16	100
808	AGA GAG AGA GAG AGA GC	11	11	100
880	GGA GAG GAG AGG AGA	10	10	100
842	GAGAGAGAG AGA GAG AYG	16	14	87.5
840	GAG AGA GAG AGA GAG	15	15	100
878	GGA TGG ATG GAT GGA T	12	12	100
859	TGT GTG TGT GTG TGT GRC	10	10	100
Total		**129**	**127**	**98.7**

pairs of populations were estimated in the program GENES (Cruz 2013). The values of Φ are analogous to the traditional F statistic, so that increasing positive values of Φ_{ST} (between 0 and 1) indicate increasing genetic differentiation between populations.

The consistency and stability of the formed clusters were calculated using the bootstrap reliability index, generating a dendrogram from 1,000 permutations. The analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was performed to estimate variance within and between populations. All analyses were carried out in the program GENES (Cruz 2013).

The inference of genetic groups of individuals from the *M. umbellata* populations was performed using a Bayesian Monte Carlo Markov Chain (MCMC) approach (Excoffier & Heckel 2006) using the multilocus genotypes of individuals to detect probable genetic (K) groups, assuming a mixed populations model. The analyses were later carried out in Structure version 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2007). To find the best K, we used the ΔK method of Evanno *et al.* (2005), as implemented in Structure Harvester (Earl & VonHoldt 2011).

Results

The combination of the 10 used primers revealed a total of 127 polymorphic loci, reaching 98.7% of the amplified loops. Five to 18 bands were detected (Tab. 1), and high percentages of polymorphism were observed within each sample (Tab. 2).

The genetic differentiation value of the populations (Φ_{ST}) was 0.2801. The AMOVA showed that intrapopulational genetic diversity is larger (71.92%) than interpopulational diversity (28.07%). Estimates of Φ_{ST} between pairs of populations (Tab. 3) showed that populations 5 and 6 were the most divergent from the others. Populations 1, 2, 3, 4 and 5 presented the lowest averages of Φ_{ST}, even though population 1 was the most structured one of them in relation to the others; populations 2, 3 and 4 were the closest.

Despite the high structuring the results verified, it was possible to detect two groups of populations through the dendogram analysis based on the UPGMA method. Populations 1 and 2 formed a large group with populations 3 and 4, being denominated Group 1, while populations 5 and 6, which are geographically farther from the remaining ones, fell into a second group, Group 2 (Fig. 2).

The Bayesian structure analysis confirmed Group 1 and Group 2 based on the change rate in Ln (K), statistic ΔK, indicating a convergence for 2 Bayesian groups, K = 2 (Fig. 3a,c). The two-dimensional graphic representation of genetic distances among individuals of all populations (Fig. 3b) corroborated the results of population clustering

Table 2 – Intrapopulation polymorphism for ISSR. N = Number of individuals; N.T.L. = Total number of loci; N.l.M. = Number of monomorphic loci.

Population studied	N	N.T.L	N.L.M.	% of polymorphism
Pop 1	10	95	12	86.3
Pop 2	10	63	11	82.5
Pop 3	9	75	10	86.6
Pop 4	12	75	6	92
Pop 5	10	92	19	79.3
Pop 6	14	83	15	81.9

(Fig. 2). Population 1 presented the largest relative distance from the other populations within its group, while population 5, belonging to Group 2, was the closest to the populations of Group 1. On the other hand, individuals from population 6 were the most isolated.

Discussion

To adopt strategies of conservation or management of any species in situ, it is necessary to know the structure and genetic diversity contained in target populations. The use of the ISSR markers to access the genetic diversity of *Myrsine umbellata* has proven to be satisfactory in the present study, since it was possible to measure the level of polymorphism in the studied populations (98.7%) with the use of the 10 selected ISSR markers. Studies on different tree species have demonstrated the efficiency of ISSR in the initial assessment of genetic diversity for natural populations, such as those developed for *Larix melinii* Rupr. (Pinaceae; 98.83%, Zhang *et al*. 2013), *Erythrina velutina* Willd. (Fabaceae; 98.0%, Gonçalves *et al*. 2014) or *Myrsine coriacea* (93%, Paschoa *et al*. 2018).

The greatest diversity was found within the populations (71.92%) in relation to the diversity among them (28.07%), indicating historical gene flow between them. This high intra-population genetic variability of *M. umbellata* was already expected, since dioecious species have a high rate of recombination through obligatory cross-fertilization (Hamrick *et al*. 1992). For species conservation purposes, the existence of genetic variability in the population allows the evolution of new genetic combinations, conferring greater capacity for evolution and adaptation to environmental changes (Shihari *et al*. 2013). Moreover, this genetic variability is of fundamental importance not only for the species conservation, but also to guarantee vigor and resistance in the progeny. This factor becomes important when selecting matrices for reforestation purposes (Paschoa *et al.* 2018).

The statistic ΔK indicated two groups that represent contrasting regions in relation to the

Table 3 – Genetic differentiation values (Φ_{ST}) calculated for pairs of populations (upper diagonal) and geographic distances between populations of *Myrsine umbellata* (lower diagonal) estimated in km.

Population studied	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6	Total
Pop 1	0	0.28**	0.23**	0.26**	0.29**	0.29**	0.27
Pop 2	66.6	0	0.11ns	0.18**	0.40**	0.31**	0.24
Pop 3	94	28.7	0	0.17**	0.30**	0.26**	0.21
Pop 4	169	130	107	0	0.28**	0.32**	0.22
Pop 5	188	119	96.6	54.9	0	0.32**	0.31
Pop 6	280	212	189	147	111	0	0.30

altitude: we found a higher polymorphism in the populations of Group 1, which occur in higher altitudes (Pop 3 and Pop 1), while the populations of Group 2, located in lower regions (Pop 5 and Pop 6), presented somewhat lower polymorphic percentages. We also observed that population 6 was the most isolated; besides being located below 1,000 meters of altitude, this population is also geographically located farthest from the others. Another relevant observation is that, although most sampled individuals were grouped according to their origin, the individuals P2.1 and P4.1 were grouped with population 3 (Fig. 2).

As long distance wind pollination is unlikely, we can infer that dispersion is the agent responsible for the observed level of diversity among populations. In addition, the present study suggests that fruit dispersion is impaired not only by geographic distance, but also by the variance in altitude between populations since birds have a metabolic cost to maintain body temperature as the ambient temperature increases or decreases (Chatelain *et al*. 2013). A similar result was found in a study on *Cedrela fissilis* Vell. (Meliaceae), where long-distance dispersion was considered the most important process that influences the current distribution of the species (Mangaravite *et al*. 2016).

Despite the high observed diversity within populations, our analyses indicated the existence of genetic diversity among populations, presenting high values of Φ_{ST}. The lowest value of genetic differentiation among pairs of populations was found between populations 2 and 3 (0.11), which are also the closest geographically, located only 28.7 km apart from each other. The greatest differentiation was in turn between geographically more distant populations (2 and 5, genetic differentiation of 0.40, geographic distance of 119 km). The degree of genetic differentiation may provide an estimate of the gene flow among the studied populations, which is a determinant of their genetic structure. The low differentiation between the populations of *M. umbellata* indicates high gene flow between them, which suggests that genetic drift is neutralized. These values also show a relationship between genetic and geographic distances in populations. This is expected, since the more distant the populations, the more different they are in allelic frequencies and phenotypic traits of genetic origin, although there is often no strict correlation.

Although the similarity of the genetic diversity values (Φ_{ST}) between the population pairs is remarkable (Tab. 2), populations 5, 6 and 1 are the most genetically diverse when compared

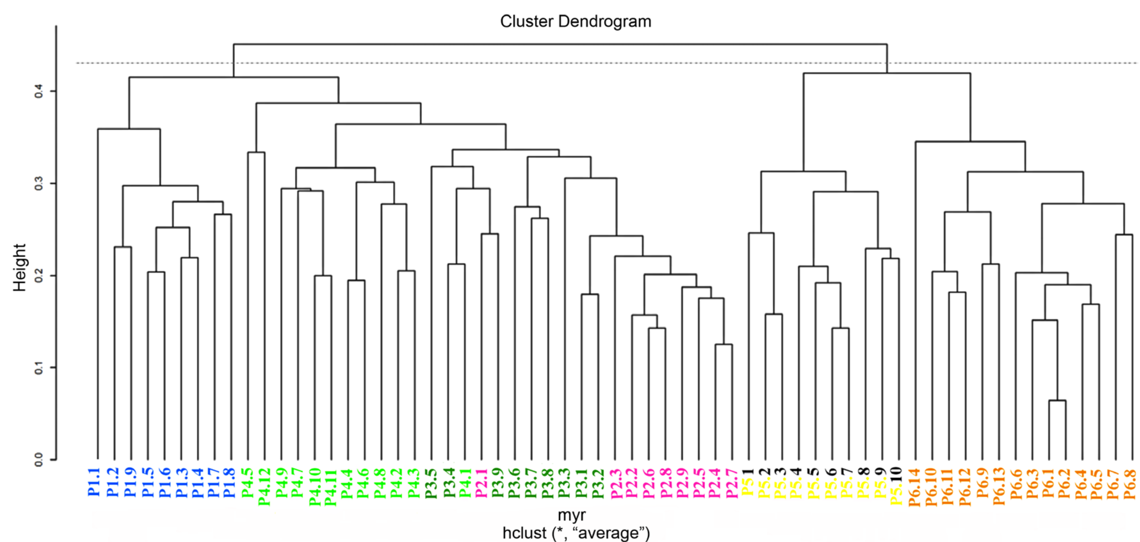

Figure 2 – Dendogram obtained by the UPGMA method based on the genetic distances expressed by the Sorensen-Dice coefficient for six populations of *Myrsine umbelatta*. P1.1 to P1.8 = individuals 1 to 8 of the population 1; P2.1 to P2.9 = individuals 1 to 9 of the population 2; P3.1 to P3.9 = individuals 1 to 9 of the population 3; P4.1 to P4.12 = individuals 1 to 12 of the population 4; P5.1 to P5.10 = individuals 1 to 10 of the population 5; P6.1 to P6.14 = individuals 1 to 14 of the population 6.

to the others. This is confirmed by the graphical representation of the genetic distances between the trees of the sampled populations (Fig. 3b), where individuals 21, 49 and 62 are the most genetically distant. These estimates indicate that seed collection should be performed in these matrices, since the more divergent the selected parents are, the greater the variability of their progenies (Manfio *et al*. 2012). This is of great importance when the seeds are destined for forest restoration (Sebbenn 2002). Taking into account that the presence of genetic structuring is a considerable prerequisite for the efficient management of plant species, our results suggest that the studied populations could be considerable seed banks for reforestation projects.

Conclusion

Our study revealed that intrapopulational genetic diversity is higher than interpopulational genetic diversity in the sampled populations of *Myrsine umbellata.* The high intrapopulational genetic variability recorded for the species can cause high morphological heterogeneity among seedlings obtained from the same population. In this scenario, it seems appropriate to obtain seeds in more than one studied population thinking of reforestation purposes. Considering the interpopulational genetic diversity, the geographically more isolated populations are more genetically structured, but all populations analyzed are important germplasm banks of *M. umbellata* conserved in situ.

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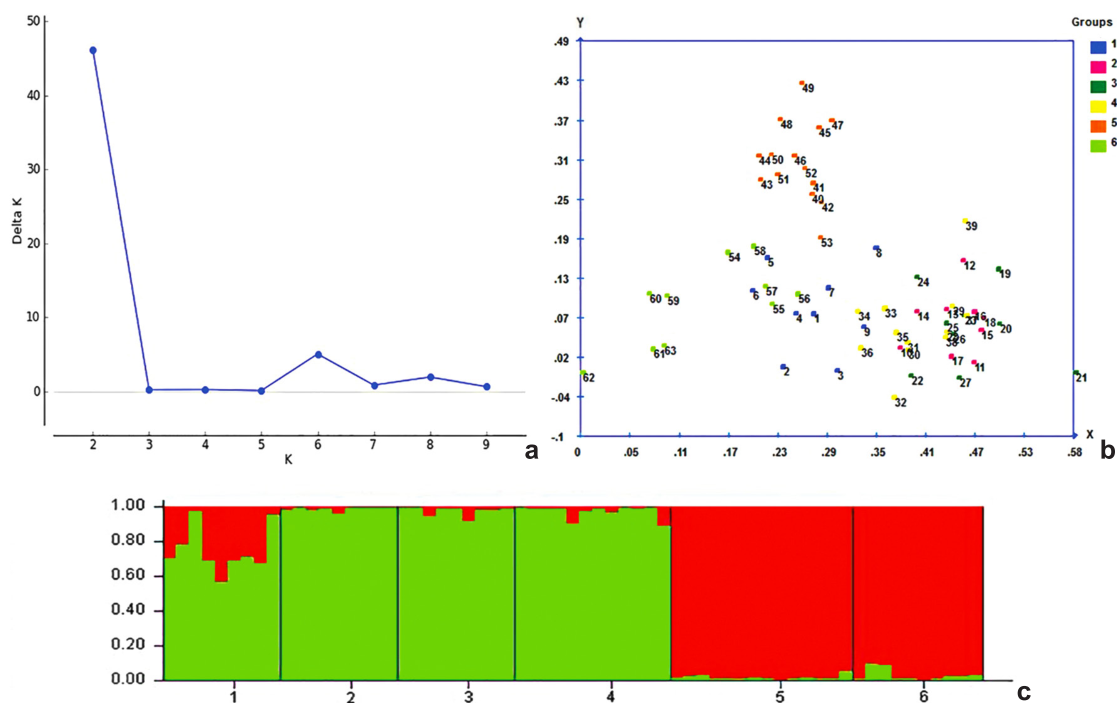

Figure 3 – a-c. Grouping analyses of the six populations of *Myrsine umbellata* in Structure – a. graph showing K(2), following the criteria proposed by Evanno *et al.* (2005) to define the correct number of groups, based on the rate of change in Ln(k). The vertical bar along the X-axis represents the sample and along the Y-axis the association coefficient of a sample for a subgroup; b; two-dimensional graphical representation of the genetic distances between the individuals of the sampled populations; c. graphical representation of Bayesian analysis in the software Structure. K = 2 shows two groups: group 1 - green (population 1, population 2, population 3 and population 4); group 2 - red (population 5 and population 6).

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