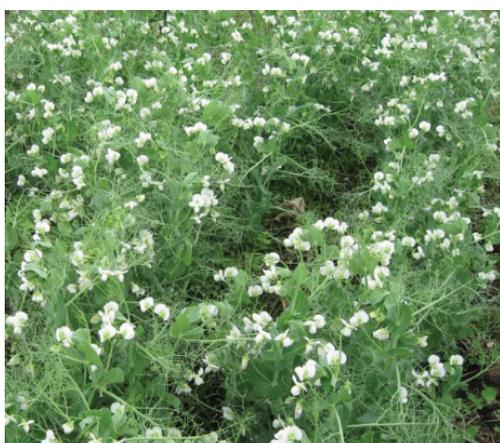


Molecular characterization using SSR markers in 50 shrub pea genotypes (*Pisum sativum* L.) from the GRICAND Collection, Colombia

Caracterización molecular con marcadores SSR para 50 genotipos de arveja arbustiva (*Pisum sativum* L.) de la Colección GRICAND, Colombia



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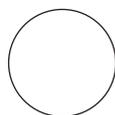
Shrubby pea crop (*Pisum sativum* L.) with Afila gene in Nariño (Colombia).

Photo: D. Herrera, GRICAND

ABSTRACT

The pea (*Pisum sativum* L.) is one of the more important legume crops produced globally. We studied the structure and genetic diversity in a collection of 50 pea accessions with 16 simple sequence repeat (SSR) markers, whose average polymorphic information content (PIC) was 0.62. The SSR markers amplified a total of 28 alleles with an average of 4 alleles per locus, with locus AB71 and D21 amplifying the largest number of alleles (6). The observed heterozygosity (H_o) was 0.09 ± 0.08 and the expected heterozygosity (H_e) was 0.42, indicating an elevated level of inbreeding ($F_{is} = 0.60$). The genetic relationships were inferred with a similarity index (DICE) and a bayesian analysis (STRUCTURE), detecting 2 clusters for the genotypes, with a high similarity of the morphological characteristics of each genotype. The results of this study will be useful for the creation of future breeding programs.

Additional key words: genetic diversity; genetic structure; pre-breeding; SSR markers; grain legumes; plant habit.



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RESUMEN

La arveja (*Pisum sativum* L.) es uno de los cultivos de leguminosas más importantes producido a nivel mundial. Estudiamos la estructura y diversidad genética en una colección de 50 accesiones de arveja con 16 marcadores de Secuencias simples repetidas (SSR), cuyo promedio del contenido de información polimórfica (PIC) fue de 0,62. Los marcadores SSR amplificaron un total de 28 alelos con un promedio de 4 alelos por locus, siendo el locus AB71 y D21 los que amplificaron el mayor número de alelos (6). La heterocigosidad observada (Ho) fue de 0,09 y la esperada (He) de 0,42, indicando un alto nivel de endogamia ($F_{is} = 0,60$). Se infirieron las relaciones genéticas por medio de un análisis de similitud (DICE) y un análisis bayesiano (STRUCTURE) detectando 2 agrupaciones para los genotipos de arveja analizados, con una alta similitud con las características agromorfológicas de cada genotipo. Los resultados del presente estudio serán útiles para la creación de futuros programas de fitomejoramiento en arveja.

Palabras clave adicionales: diversidad genética; estructura genética; marcadores SSR; pre-mejoramiento; leguminosas de grano; hábito de crecimiento.

Received for publication: 23-05-2019 Accepted for publication: 31-07-2019

INTRODUCTION

Pisum sativum, known as pea or sweet pea, is a self-pollinated legume, with a genome made up of seven chromosomes ($2n = 14$) (Smýkal *et al.*, 2008). It is one of the staple foods of the basic market basket of the Colombian family because of its high content of protein and micronutrients, such as zinc and iron (Amarakoon *et al.*, 2012; Peralta *et al.*, 2013).

It is possible to find shrub-type peas, semi shrub-type peas and snowy-peas within *P. sativum*. Shrub peas are low-bearing, and their growth ends in flower, semi-shrubs are medium-sized peas that end in leaflets and snowy peas are high, require a tutor and end in leaflets. Two types of planting are used: with tutor and crawling planting without tutor or broadcast planting. Planting with a tutor generates high yields and a higher fresh pod quality; however, one of the limiting factors in this production system is the cost of the tutoring because the structure of the plants does not have enough tendrils to be left on the tutors without the plants falling on the ground; a high usage of polypropylene threads is necessary for their mooring (Checa-Coral and Rodriguez, 2015).

In Colombia, the pea is the second most important legume, after the bean, with a cultivated area of 35,211 ha and a green pod yield of 4.1 t ha⁻¹. Its production is concentrated in six departments: Nariño, Cundinamarca, Boyaca, Tolima, Huila, and Nariño,

the latter being the main producer with 15,816 ha (Checa-Coral and Rodriguez, 2015). This department has suitable areas for producing shrub peas, a suitable alternative for diversification in the cereal zone of Nariño because of its capacity for adaptation, high yield potential and possibility of harvesting green pods or dry-grained sheaths, depending on market conditions. However, in Nariño, the varieties of peas include snowy peas, which require tutoring systems to help the plant stay off the ground, allowing an increase in potential yield. According to Checa-Coral *et al.* (2017), employing a tutoring system represents 52% of total crop costs, which has led farmers with limited finances to not use tutored systems for the cultivation of peas, significantly increasing the incidence of foliar diseases such as mildew (*Erysiphe polygoni*) and Ascochyta (*Ascochyta pisi*), reducing yield and deteriorating quality of the final product. Because of technification difficulties in snowy pea tutoring systems, studies on shrub peas are vital, within which genotypes with the *Afila* gene (*aF*) are found, which, in the homozygote condition, transforms leaves into tendrils (Mike, 2008; Smýkal *et al.*, 2013), reducing tipping and preventing the sheaths from coming into contact with the ground, avoiding pod rotting under high relative humidity conditions (Checa-Coral *et al.*, 2017). This type of pea with a shrub growth habit tends to obtain low-bearing and early genotypes, with sheath qualities for fresh

consumption and for the agro-industry (Checa-Coral *et al.*, 2017). This material is adapted to the cereal zone and, with an adequate harvest time, could be an alternative to partially cover the national demand for dry peas and for producers to obtain good profitability because of the low cost of implementation of this crop.

Given the importance of shrub peas as a crop in the Department of Nariño-Colombia, it is necessary to initiate genetic improvement programs for genotypes with this growth habit. It is advisable to carry out molecular characterization of collections that allow breeders to identify genes of interest to associate them with agronomic characteristics and thus generate results that express genetic diversity. Knowledge on genetic diversity fosters the efficient use of germplasm, identifying and eliminating duplicates, and helps the establishment of nucleus collections (Ghafoor *et al.*, 2005). Genetic diversity is the main input for the genetic improvement of a cultivated species. Local and wild varieties are kept in germplasm banks as genetic resources; however, their use in genetic performance improvement has been limited (Ali *et al.*, 2007).

One of the most used molecular methods for characterization and genetic diversity studies are molecular markers. Microsatellites have become the most used markers because of their high polymorphism and easy handling in the laboratory; these markers have the ability to differentiate homozygous and heterozygote individuals. These markers consist of DNA fragments with a few nucleotides in length, between 2 and 6 base pairs, which are randomly repeated several times (Vieira *et al.*, 2016). In 2005, a research group established sets of specific SSR microsatellites for *P. sativum* (Loridon *et al.*, 2005), which have been used in several studies to observe the genetic diversity of the pea; for example, 21 SSR markers were used in a collection of more than 1,000 introductions. Zong *et al.* (2009) found that the genetic diversity of the Chinese collection has several differences that were detected in the global gene pool, such as the presence of rare alleles. These markers have been used in various studies over the years thanks to their high polymorphic information content, codominance and reproducibility (Smykal *et al.*, 2008; Cieslarová *et al.*, 2011; Rana *et al.*, 2017).

SSRs have also been used for studies of disease resistance; for instance, it has been found that resistance to *Aphanomyces euteiches* (cause of rotten root) is moderately heritable (Hamon *et al.*, 2011).

Having specific pea genotypes of determined growth or shrubs adapted to the region, for which an adequate cultivation technology is established, improves the income of farming families in municipalities that used to be wheat producers in Nariño and allows farmers to return to agricultural activities by generating new work opportunities (Checa-Coral *et al.*, 2017).

The aim of this work was to carry out the molecular characterization of 50 shrub pea genotypes from the collection of the GRICAND Research Group of the Universidad de Nariño, using SSR microsatellite molecular markers to serve as a starting point for the establishment of breeding programs for the species.

MATERIALS AND METHODS

Plant material

The study population was formed by 50 genotypes of *P. sativum* with a shrub-like growth habit, which are part of the *Pisum sativum* L. collection of the GRICAN Research Group on Andean Crops of the Universidad de Nariño. Sowing and germination of the plant material was carried out during the last three weeks of September, 2018 in the greenhouse of the Universidad Nacional de Colombia, Palmira Campus. Three plants of each genetic material were sown, and the sowing was carried out in germinators used for seedlings. When the plants were approximately 10 cm high, young leaflets were extracted, collecting approximately 5 g of foliar tissue which was stored in ultra-freezers at 80°C. Simultaneously and in order to renew the seed in the *Servicio Nacional de Aprendizaje* of Colombia - SENA, 50 pea genotypes were multiplied in the field, using furrows 2 m in length with a distance between furrows of 60 cm, depositing one seed per site. The lot was used to register the characteristics of leaf type and grain form based on UPOV descriptors (UPOV, 2009) (Tab. 1).

Table 1. Morphological characteristics of leaf type and grain form for 50 genotypes of *Pisum sativum* L.

Genotype	Leaf type	Grain form	Genotype	Leaf type	Grain form
ARB001	3	2	ARB026	1	1
ARB002	3	2	ARB027	1	1
ARB003	3	2	ARB028	1	1
ARB004	3	4	ARB029	1	4
ARB005	3	2	ARB030	1	1
ARB006	3	4	ARB031	1	1
ARB007	3	4	ARB032	1	4
ARB008	3	4	ARB033	1	4
ARB009	3	4	ARB034	1	4
ARB010	3	4	ARB035	1	4
ARB011	3	4	ARB036	1	4
ARB012	3	4	ARB037	1	4
ARB013	3	3	ARB038	1	4
ARB014	3	4	ARB039	1	1
ARB015	3	4	ARB040	1	2
ARB016	3	3	ARB041	1	4
ARB017	3	4	ARB042	1	1
ARB018	3	4	ARB043	1	2
ARB019	3	4	ARB044	1	4
ARB020	3	4	ARB045	1	2
ARB021	3	4	ARB046	1	4
ARB022	3	4	ARB047	1	1
ARB023	3	4	ARB048	1	4
ARB024	3	4	ARB049	1	2
ARB025	1	1	ARB050	1	2

Leaf type: 1 = normal or terminated in tendrils, 3 = without leaves, tendrils only. Grain form: 1 = round, 2 = angular round, 3 = elongated oval, 4 = several rectangular shapes.

DNA extraction

The SPIN Plant Mini Kit® (STRATEC Biomedical AG, Birkenfeld, Germany) with its respective procedure was used for the DNA extraction. The quantification of the genetic material was carried out with a Colibri Spectrophotometer® (Titertek-Berthold, Pforzheim, Germany) to obtain more precise measurements. The DNA dilutions were carried out for the normalization and homogenization of DNA concentration, with values of 30 ng μL^{-1} , for the subsequent molecular evaluations with PCR reactions.

Genotyping

The genotyping used 16 molecular microsatellite fluorescent markers (SSR), described by Lordon *et al.* (2005) (Tab. 2). These markers were subjected to a process of standardization to determine what was the best hybridization temperature of each one. Subsequently, the amplification of the samples was done with the PCR technique.

Once products of amplification were obtained, the markers were mixed in plates with 96 wells where

PCR products were combined; each well contained a maximum of three markers, which could not be the same color (PET, VIC, FAM, NED) and had to have distant molecular weights. These plates were sent to the laboratories of Macrogen (Seoul) for the analysis of the fragments of the SSR microsatellites.

SSR microsatellites statistical analysis

The size of the alleles in base pairs, was estimated using standard lane size ROX-500 (Applied Biosystems, Foster City, CA) with GENEIOUS Software® v 11.1 (<http://www.geneious.com>) (Kearse *et al.*, 2012). The number of polymorphic alleles observed, expected heterozygosity, and genetic diversity of Nei (1973), were observed using Arlequin Program v 3.5.2.2 (Excoffier and Lischer, 2010). Likewise, the Polymorphic Information Content was calculated (Botstein *et al.*, 1980).

For a distance or similarity analysis, two methodologies may be used: a Principal Coordinates Analysis (PCoA) or a dendrogram (tree diagrams). A PCoA generates a scattering graph that can have two or three dimensions, where the distances between the samples shown in the graph reflect the

genetic distances between them. On the other hand, a dendrogram performs a grouping of the samples that are genetically similar (Nisar *et al.*, 2017). The PCoA was carried out using GenAlex v 6.5 program (Peakall and Smouse, 2012). On the other hand, the dendrogram was performed considering the created matrix and the similarity method of UPGMA/DICE using the Simqual programs of the NTSYS-PC package (Rohlf, 2006).

To assess the genetic structure of the genotypes, Structure v 2.3.4 was used (Falush *et al.*, 2007). The criteria recommended by Porras-Hurtado *et al.* (2013) were used: the number of subpopulations (K-value) was 1-10, with 10 runs, for a *Burn-in* period of 100,000 steps and 200,000 interactions of the Monte Carlo Markov Chain (MCMC). To determine the best K, the method of Evanno *et al.* (2005) was considered. The Structure Selector Program was used (<http://lmme.qdio.ac.cn/StructureSelector/>). These steps were repeated the necessary times until the structural behavior of the samples was clearly defined. In order to determine a significant genetic differentiation between the possible groups created in Structure, an analysis of molecular variance was carried out (AMOVA), along with determination of using Arlequin v 3.5.2.2 (Excoffier and Lischer, 2010).

Table 2. SSR markers employed in research with their sequence forward and backward, hybridization temperature and molecular weight.

Marker	Forward sequence 5'-3'	Reverse sequence 5'-3'	Tm ^a	Molecular size
AA5	TGCCAATCCTGAGGTATTAACACC	CATTTTTGCAGTTGCAATTTTCGT	61	235
AA355	AGAAAAATTCTAGCATGATACTG	GGAAATATAACCTCAATAACACA	51	180
AB53	CGTCGTTGTTGCCGGTAG	AAACACGTCATCTCGACCTGC	51	120
AD147	AGCCCAAGTTTCTTCTGAATCC	AAATTCGCAGAGCGTTTGTAC	61	330
D21	TATTCTCCTCCAAAATTCCTT	GTCAAAATTAGCCAAATTCCTC	51	200
AA122	GGGTCTGCATAAGTAGAAGCCA	AAGGTGTTCCCTAGACATCA	61	190
AA135	CCGTACACATCATTAAAGATG	TCCATATCCAGATTAGTCAGA	51	291
AD146	TGCTCAAGTCAATATATGAAGA	CAAGCAAATAGTTGTTTTGTTA	51	390
AD148	GAAACATCATTGTGTCTTCTTG	TTCCATCACTTGATTGATAAAC	54	190
AA238	TATCATCAAGGTCCAATTTAGT	AGCTAAATCGTACCTAATCTGT	51	190
D23	ATGGTTGTCCAGGATAGATAA	GAAAACATTGGAGAGTGGAGTA	51	170
AB71	CCAACCATTTGTGAGTCCCTT	TTCGTGCAACCACGAGAATAGA	61	145
AD60	CTGAAGCACTTTTGACAACACTAC	ATCATATAGCGACGAATACACC	51	216
AC58	TCCGCAATTTGGTAACACTG	CGTCCATTTCTTTATGCTGAG	61	205
AD175	CTTGTGCAGAAGCATTTGATTA	AGAGACAATGGATGCTCATAGT	55	405

Tm^a: hybridization temperature.

RESULTS AND DISCUSSION

Analysis of SSR microsatellites

Seven out of the 16 markers showed polymorphisms, and 27 alleles were found within the 50 genotypes (Tab. 3). Loci with a greater allelic richness included AB71 and AD21, with 6 alleles each. The average number of alleles was 4, with a range of 2 to 6 alleles per locus (Tab. 3); this result is greater than that reported by Ahmad *et al.* (2012) but similar in other studies on the *Pisum* genus (Cupic *et al.*, 2009; Ponnaiah *et al.*, 2011) and less than that reported by Hagenblad *et al.* (2014), where a range of 5 to 12 alleles was found, as was recently reported by Rana *et al.* (2017). The size of the alleles had a range of 50-493 base pairs. The polymorphic information content (PIC) had an average of 0.62; according to Botstein *et al.* (1980), this value indicated that the markers were highly informative since they were above 0.50 and indicated that there could be an allelic variation within the samples. Similar values were reported in other pea research (Nasiri *et al.*, 2009; Ahmad *et al.*, 2012). Studies that have evaluated a larger number of *Pisum* accessions have observed a larger allele, indicating that some markers may become more polymorphic depending on the number of accessions that have been implemented (Ahmad *et al.*, 2012). The inbreeding coefficient (F_{is}) was 0.60 which indicated that there was a level of inbreeding, which explained that the observed heterozygosity (H_o) was less than the expected heterozygosity (H_e) (0.09 and 0.42, respectively). This indicated that there was a high homozygosity normal behavior in the *P. sativum* individuals because of its pollinated nature (self-fertilization).

The low observed heterozygosity has been reported in other research on *P. sativum*: Handerson *et al.* (2014) obtained a H_o of 0.031, Smýkal *et al.* (2008) obtained a H_o of 0.069 with a collection from the Czech Republic and Teshome *et al.* (2015) used a collection of *P. sativum* from Ethiopia, finding a H_o of 0.05, which confirms that *P. sativum* is a species that tends towards homozygosity (Teshome *et al.*, 2015). On the other hand, according to Vallejo and Estrada (2002), in autogamous plants (such as *Pisum sativum*), inbreeding is expected to give a value equal or very close to one (1). Likewise, loci AB71 and AA135 had H_o values of 0.20 and 0.16, respectively. The inbreeding values and H_o values could be explained by the fact that the seeds were obtained through open field sowing, which increased the probability that pollinating insects visited the flowers. Some research has suggested that the main pollinators of *Pisum* belong to the order of diptera, hymenopteros and lepidoptera (Naeem *et al.*, 2018). In addition, in some cases, *Pisum* pistil may remain in a suitable state after anthesis (Kosterin and Bogdanova, 2014). These factors could suggest that the inbreeding and H_o resulted from the existence of some cases of allogamy or cross-pollination in the collection.

Genetic diversity

In the dendrogram (Fig. 1A), two main groups were observed with a similarity coefficient (DICE) of approximately 0.74 (Group I and Group II). With an approximate coefficient of similarity of 0.93, most of the sub-groups (15 subgroupings) were formed, which were highly related to the groups formed with the PcoA (Fig. 1B). In Group 1 of the dendrogram, sub-groups of genotypes with a high coefficient

Table 3. SSR markers with annealing temperature (Ta), number of alleles (Na), effective number of alleles (ENA), allele size range, polymorphic information content (PIC), observed heterozygosity (Ho) and expected heterozygosity (He), inbreeding coefficient (F_{is}).

Marker	TA (°C)	Na	ENA	Allele size BP range	PIC	Ho	He	F_{is}
AA122	62	5.0	2.12	155-195	0.70	0.05	0.53	0.91
AA5	59	4.0	3.07	229-241	0.69	0.07	0.67	0.89
D21	51	6.0	3.46	185-278	0.74	0.16	0.71	0.77
AB71	59	6.0	4.65	304-334	0.81	0.20	0.79	0.75
AA135	54	2.0	1.17	114-119	0.40	0.16	0.15	-0.09
AD60	52	2.0	1.08	50-58	0.50	0.00	0.08	1.00
AA355	56	2.0	1.02	483-493	0.50	0.02	0.02	-0.01
Average		4.0	2.00	-	0.62	0.09	0.42	0.60

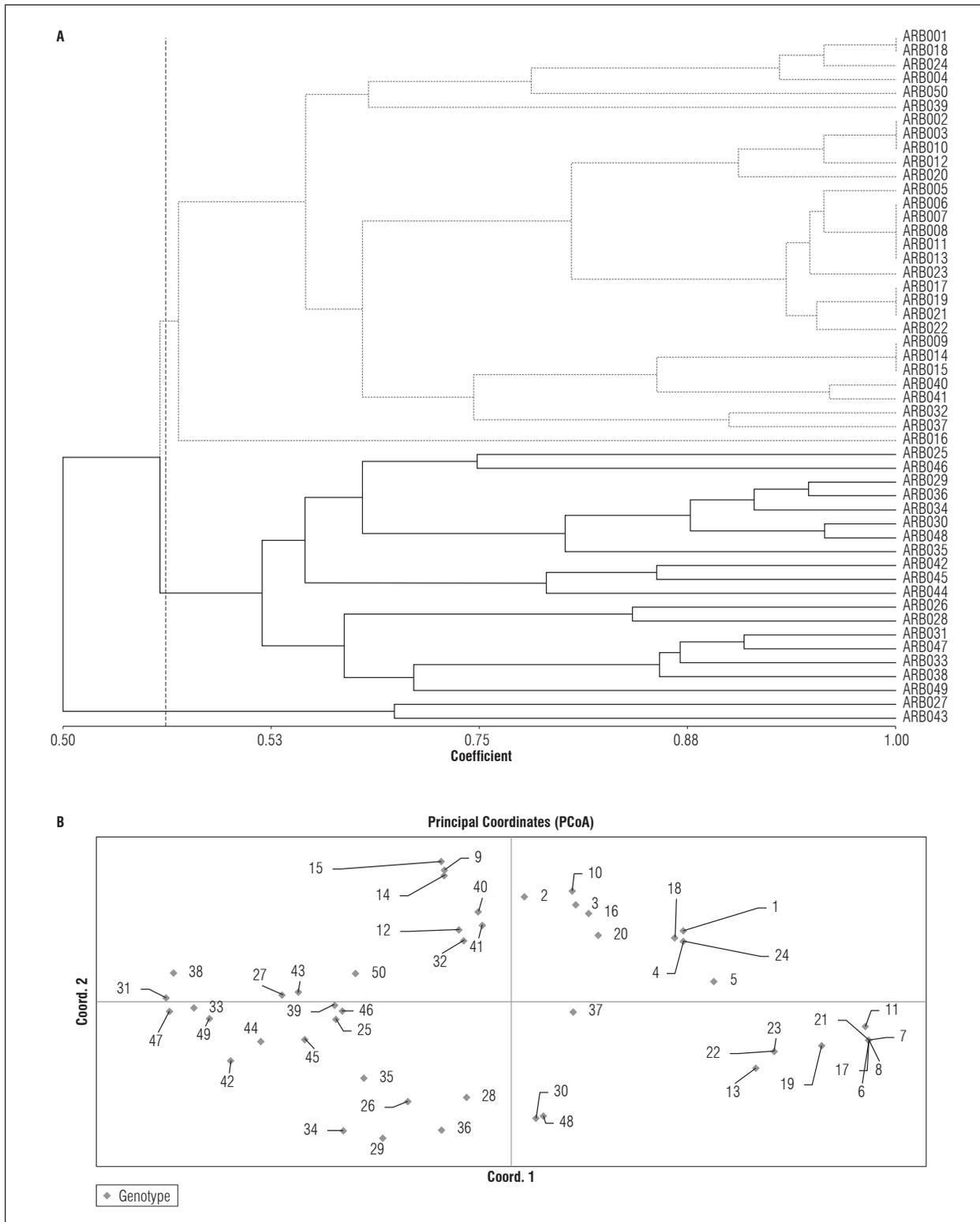


Figure 1. Similarity analysis for 50 genotypes of *P. sativum* with SSR markers: A) Dendrogram using the DICE/UPGMA method. B) Analysis of main coordinates (PCoA).

of similarity (<0.98) are observed, such as ARB 004 with 024; ARB 006 with 007, 008, 017 and 021; and ARB 022 with 023, among others. These high similarities could have been the product of the genotypes used in this study, with a very small population, a shrub growth and a particular area: Department of Nariño, along with sharing the same morphological characteristics.

The graph of main coordinates analysis (Fig. 1B) was carried out with the first two coordinates, which explained 51.20% of the total variation within the samples. Coordinate one explained 29.32%, and coordinate two explained 21.88%. By observing the groupings formed in the dendrogram (Fig. 1A) and in the PCoA analysis (Fig. 1B), it was observed that they were highly associated.

The analysis of the structure of the 50 *P. sativum* accessions showed a $K = 2$ with the higher hierarchical level of the genetic structure, which was supported by the Bayesian analysis (Fig. 2A). These two groupings (Group I and Group II) formed by the STRUCTURE analysis were highly associated with the groups found in the dendrogram. The accessions separated in two groups by STRUCTURE, in the same way as groups I and II of the dendrogram. On the other hand, the AMOVA analysis showed a F_{ST} value of 21.92% ($P < 0.00001$), indicating that there was significant genetic differentiation between the two large groupings. Because the structure analysis was performed for each of the two groups separately, each group threw a hierarchy level $K = 2$, which indicated that each group has two subgroups (Fig. 2B and 2C).

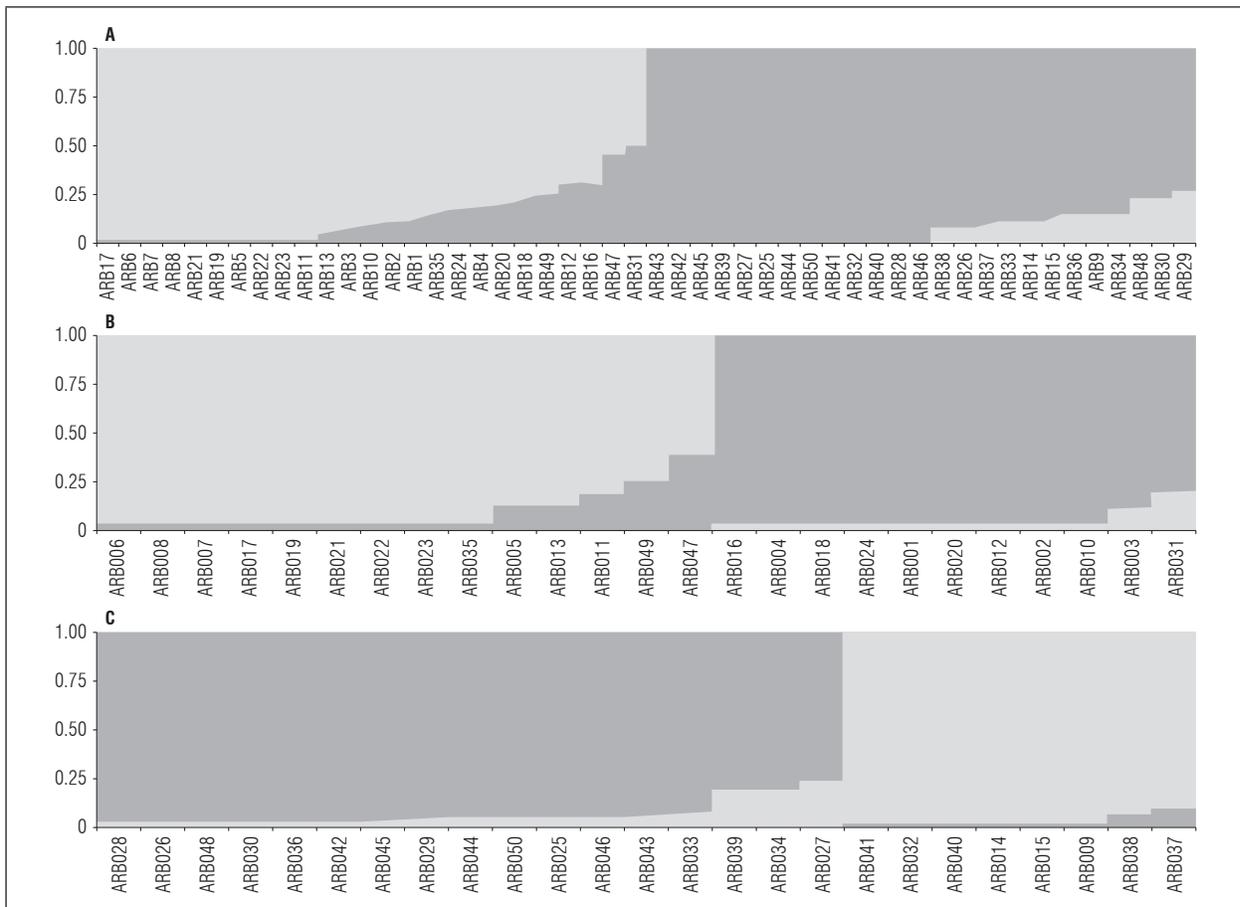


Figure 2. A) Bayesian analysis of the genetic structure ($K = 2$) in the 50 shrub pea genotypes based on seven microsatellite loci. B) Bayesian analysis of the genetic structure ($K = 2$) in group I (Group I) of the shrub pea genotypes. C) Bayesian analysis of the genetic structure ($K = 2$) in group II (Group II) of the shrub pea genotypes. Each bar equals an individual genotype.

These K values have been demonstrated by Nasiri *et al.* (2009) and Ahmad *et al.* (2012), where a K = 2 were obtained. Other authors have reported some not very distant groups with K = 3 (Jing *et al.*, 2012; Rana *et al.*, 2017). The high similarity between the groups found in both the dendrogram, PCoA and STRUCTURE may have been due to the fact that the genotypes in the same group may share similar morphological features. According to the morphological traits (Tab. 1), it was observed that most genotypes belonging to group I had a sharp blade form (without leaves), with tendrils only and a rectangular grain form in several forms. It was observed that the genotypes of grouping II had a normal leaf form with lateral leaflets that finished in tendrils and, like Group I, a rectangular grain form in various forms. Morphological level differences were not found for the subgroups.

Finally, this is the first study that employed SSR microsatellite markers for the study of genetic diversity in the *Pisum sativum* Collection of the Department of Nariño (Colombia). Understanding the collection of *P. sativum* with the *Afila* gene is of great importance for the horticultural sector since it prevents the deterioration of product quality by reducing foliar diseases.

Although the use of SSR can be an effective tool for characterizing germplasm, for future research, it is recommended to have information at the geographic level on the origin of the genotypes, which will allow a much more complete system for the analysis of diversity and the possible development of new growths.

CONCLUSION

This study demonstrated the usefulness of molecular markers for molecularly characterizing *P. sativum* pea accessions. Understanding the richness of alleles is a priority and a significant help in different breeding and replanting programs since they are a pillar for better development of this crop. The morphological traits used in this study, along with the molecular points, explained the differences found within and between the accessions. The results of the diversity and genetic structure are useful for identifying contrasting groups to guide the selection of parents. The genetic variability between the accessions was sufficiently high, suggesting that the genetic diversity of

the pea is sufficient for the possible combination of favorable genes.

Using these results as a first step, a self-pollination process of several generations shall follow in order to obtain pure lines. Hence, it is necessary to integrate genetic, morphological, disease resistance and production results to establish a possible relationship with the data obtained and, thus, be able to propose improvement programs. Research is currently underway to adjust planting distances, densities and economic analyses to exploit the productive potential of these genotypes.

In the future, the release of the first volatile variety with the *Afila* gene for the country is expected. These new varieties reduce the costs of tutoring (52%) by half or more.

Conflict of interests: The manuscript was prepared and reviewed with the participation of the authors, who declare that there exists no conflict of interest that puts at risk the validity of the presented results.

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