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Molecular characterization of *Chenopodium quinoa* Willd. using inter-simple sequence repeat (ISSR) markers

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Quinua (*Chenopodium quinoa* Willdenow) is a pseudocereal of Amaranthace family which originated from the Andes of South America. Quinoa is an interesting plant whose capacity to tolerate adverse environmental factors and exceptional nutritional qualities warrant further research in all fields of plant biology, agronomy, ecology and biotechnology. Presently, it is an underutilized crop, which has the potential become a major crop. It has increases in importance in the world due to the nutritional quality of its grains and crop adaptability to diverse climatic conditions. In Colombia, more accurately in the Department of Nariño, Cauca, Cundinamarca y Boyacá currently Quinua has had a huge boost due to their agronomic potential and different benefits derived from the production, processing and marketing of its products. The objective of this research was to characterize the genetic diversity of a collection of 82 materials of with seven microsatellite markers [inter-simple sequence repeats, (ISSRs)]. The analysis by the coefficient of Nei-Li at the level of similarity of 0.65 divided the population into four groups according to the site of origin of the materials. The value of average heterozygosity was 0.38 which is considered low compared to other studies of genetic diversity in *Chenopodium*. Molecular Analysis of Variance (AMOVA) and Fst demonstrate the existence of genetic variability at the intraspecific level that should be used in breeding programs of the species lead to obtaining new and better materials of quinua.

Key words: Genetic diversity, microsatellites, Andean cereal.

INTRODUCTION

Quinua (*Chenopodium quinoa* Willd.) is a pseudocereal of the Amaranthace family which originated from the Andes of South America where it has been cultivated since more than 5,000 (Adolf et al., 2012). Quinua is an allotetraploid (2n=4x=36)y, thus exhibits disomic inheritance for most qualitative traits (Maughan et al., 2004); their seeds, and to some extent its leaves, are traditionally used for human and livestock consumption in

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the Andean region and have exceptional nutritional qualities (Lamothe et al., 2015; Yasui et al., 2016). Moreover, the species, being adapted to the harsh climatic conditions of the Andes (De Jesus Souza et al., 2016), exhibits remarkable tolerance to several abiotic stresses such as frost (Jacobsen et al., 2005), salinity (Shabala et al., 2013) and drought (Jacobsen et al., 2012). Production of quinoa has, until now, been prevalently conducted in Bolivia and Peru and still its productions are very small in other Andean countries like Ecuador, Chile, Argentina, and Colombia. Production in Peru, Ecuador, and Bolivia has increased from 1980 to 2011 by approximately 300%, with the largest increase (from ca. 9 to 38 metric tons) in the latter country (FAOSTAT, 2015).

Cultivated quinua display a genetic diversity, mainly represented in an ample range of characters like plant coloration, flowers protein content, seeds, saponin content and leaves calcium oxalates content, which allows obtaining a wide range of adaptability to agroecological conditions (Rodríguez et al., 2009). Within the diversity centers, the center of Perú (Huancayo, Ayacucho, Cajamarca), the Ecuadorian Mountain range, the Argentine Northeast, the South of Chile and of Colombia (Pasto, Nariño and Cundinamarca) are identified (Jacobsen, 2003). The adaptation capacities of quinua are huge since we can find varieties developed from sea level up to 4,000 m above and from 40°S to 2°N of latitude (Zurita et al., 2014). The genetic bases of several quinua traits was identified several decades ago (Lescano-Rivera, 1980), but the first true genetic descriptions more recently provided the starting point for improvement of quinua. Several genetic tools have been developed, and today molecular markers are an effective way to enhance breeding efficiency (Ruíz et al., 2014).

Quinua is one of the Andean crops with little research in the area of genetics and plant breeding, although, it has a high variability in characteristics such as plant color, flowers, nutritional contents and metabolites of interest (Bazile et al., 2014). Collecting, conservation and characterization studies are necessary for the development of strategies to improve of this species. At the international level, approximately 16,263 *Chenopodium* accessions are collected worldwide, which have been preserved and characterized in part by institutions mainly from Bolivia, Perú, United States and India (Rojas et al., 2015). In Colombia, Corpoica Tibatitatá reports a germplasm bank with 28 accessions of quinua (Rojas et al., 2015), however, small collections are conserved in the main producers departments. In the country, the characterization of this plant genetic resources only morphoagronomic studies developed by Torres et al. (2008) in the Savannah of Bogotá.

Molecular markers are also employed for the genetic characterization of *Chenopodium* germplasm. They have been used to differentiate genotypes under environmental conditions that confounded their phenotypes (Nolasco et al., 2013). Simple sequence repeats (SSR) are one of the frequently used molecular markers for genotyping crops (Jarvis et al., 2008). A number of research studies have demonstrated the use of SSRs and ISSRs to detect polymorphism and diversity in quinua (Costa, 2014; Lu et al., 2015; Fuentes et al., 2009) related species like amaranth (Jimenez et al., 2013; Oduwaye et al., 2014) and others (Morillo et al., 2015, 2016; Dotor et al., 2016). However, inter-simple sequence repeat (ISSR) markers are simpler to use than SSR technique (Oduwaye et al., 2014; Morillo et al., 2015). The use of ISSR does not require prior knowledge of the target sequences flanking the repeat regions, is not expensive and is relatively easy to score manually compared to SSR.

In order to establish a strategy and management plant for phytogenetic resources for quinua, it is necessary to begin studies on morphological, agronomic, physiological and molecular characterizations to know the genetic diversity, to generate basic information necessary to obtain sustainable solutions for the problems of low levels of technology in production, common in quinua cultivation, such as lack of homogeneity in organoleptic characteristics in materials used in beverages and flour, genetic transformation that allows for a shorter growth cycle for production in less time and tolerance to pests and diseases through plant breeding (Zurita et al., 2014). Yazici and Bilir (2017) reported that genetic knowledge is one of the important tools used for different purposes such as gene conservation, managing of genetic resources, evolutional and genetic management of populations for plant breeding. Within this context, this research aimed to molecularly characterize quinua materials using inter-simple sequence repeat (ISSR) markers or random amplified microsatellite markers (RAM) to reveal genetic polymorphism in quinua.

MATERIALS AND METHODS

Plant

A total of 81 individuals of quinua belonging to genebank from Secretary of Agriculture of Government of Boyacá, Colombia, were evaluated in the Molecular Biology Research Laboratories, Gebimol and Bioplasma, of Pedagogical and Technological University of Colombia, Tunja located at 2,820 msnm with average temperature of 13°C (Table 1).

Molecular characterization

For DNA extraction, the Dellaporta et al. (1983), protocol was used. The total DNA was visualized with 0.8% agarose gels, stained with Z-Vision, with a Maxicell EC-340 Primo Gel Electrophoresis System chamber. In order to determine the DNA concentration of each accession, a dilution curve with DNA from bacteriophage Lambda with an initial concentration of 20 ng/µl was made. The quantified DNA was diluted in HPLC type water to a total volume of 100 µl to 10 ng/µl and stored at -20°C.

For analysis, seven ISSR primers synthesized by Technologies

Table 1. Quinua materials used for the assessment of genetic diversity with Inter-Simple Sequence Repeat (ISSR).

Origin	Quantity	Height (msnm)	Latitude	Longitude	Average temperature (°C)
Nariño	48	2,817	3°17´20" N	77°21´28"	13
Soracá	13	2,942	5°30´02" N	73°19´59"	13
Tunja	11	2,822	5° 32′ 25″ N	73° 21′ 41″	13
Perú	4	3,259	12°04´15" N	75°12´24"	12
Chocontá	2	2,689	5° 8′ 48″ N	73° 40′ 57″	13
Siachoque	2	2,753	5°30´47" N	73°14´39"	13
Cauca	1	2,750	2°27´00" N	76°37´00"	14

Table 2. Primers used in the ISSR technique.

Markers	Sequence (5´to 3´)
CCA	$DDB(CCA)_5$
CGA	$DHB(CGA)_5$
ACA	$BDB(ACA)_5$
AG	$HBH(AG)_7A$
CT	$DYD(CT)_7C$
TG	$HVH(TG)_7T$
CA	$DBDA(CA)_7$

Inc. were used (Table 2). The amplification reaction was prepared in a sterile microcentrifuge tube (1.5 ml) to a final volume of 25 µl. The reaction mixture was prepared with 1X buffer, 1.5 mM $MgCl_2$, 0.2 M dNTPs, 1U Taq Polymerase, 2 µM primer and 10 ng genomic DNA.

The following designations are used for degenerated sites: H (A/T/C); B (G/T/C); V (G/A/C) and D (G/A/T).

The amplification was carried out in a thermocycler PTC 100 Programmable Thermal Controller (MJ. Research, Inc). Initial denaturation was at 95°C for 5 min; denaturation at 95°C for 30 s, annealing temperature of 50°C (AG and CA primers), 55°C (CCA, TG and CT primers) and 58°C (CGA primers) for 45 s, an extension of 72°C for 2 min, 37 cycles of denaturation, and finally, extension at 72°C for 7 min.

Amplified products were separated by electrophoresis in polyacrylamide gels 37:1 (acrylamide: bisacrylamide) at 7% and 150 V for 1 h in a small DNA Sequencing System chamber (Fisher Biotech FB-SEQ-3545). The staining was carried out using silver salts.

Statistical analysis

An absence (zero) and present (one) binary matrix was generated. The genetic similarity between individuals was calculated using the similarity coefficient of Nei and Li (1979). The cluster analysis was conducted by the UPGMA method and a dendrogram was generated using the statistical package NTSYS (Numerical Taxonomy System for Personal Computer, PC version 2.02). To evaluate the genetic diversity, unbiased heterozygosity and percentage of polymorphic loci were estimated using the statistical package TFPGA (Tools For Population Genetic analysis, version 1.3, 1997). Unbiased statistical f with a confidence interval of 95% was determined.

RESULTS AND DISCUSSION

The main objective of this study was to analyze the molecular diversity of 81 materials of quinua using ISSR markers. In the analysis with the Nei-Li coefficient, at a similarity level 0.65, the population was distinguished into four groups based mainly on geographical origin of materials (Figure 1). The first group contains quinua materials collected in different municipalities from Department of Nariño, it presented genetic distances ranging from 0.80 to 0.92; they are highly homogeneous materials, which can be attributed to domestication processes, constant exchange of seeds between farmers of the producer areas, mating system and bottleneck events through which this species has passed; it has led to the loss of genetic diversity (Zurita et al., 2014).

At 0.60 of similarity, group II was found with materials collected in the producing areas of Department of Nariño, to genetic distance of 0.91 with respect to group I, which showed high degree of relationship among the quinua materials in the same region. In groups III and IV, a much laxa distribution of individuals from the different evaluation sites was observed, revealing the genetic flow between them, with genetic distances within groups more than that 0.80; it could be beneficial for breeding programs that implement hybridization strategies (Bhargava et al., 2016). On the other hand, it is also possible to observe the degree of consanguinity between quinua (*C. quinoa*) and other related species such as kiwicha (*Amaranthus* species) and even with Peruvian materials, reaffirming the existence of a continuous seed exchange between farmers and researchers.

In general terms, the clusters corresponded to the geographic site where quinua materials were collected; this had already been reported in other studies of genetic diversity using different types of markers (Maughan et al., 2012; Oduwaye et al., 2014; Rajkumari et al., 2015). Studies carried out in other Andean countries had also reported low variability in local varieties of quinua; it was to be expected from the selection processes carried out by breeders. Thus, genetic diversity declined after systematic selection from farmers or breeders (Bazile et

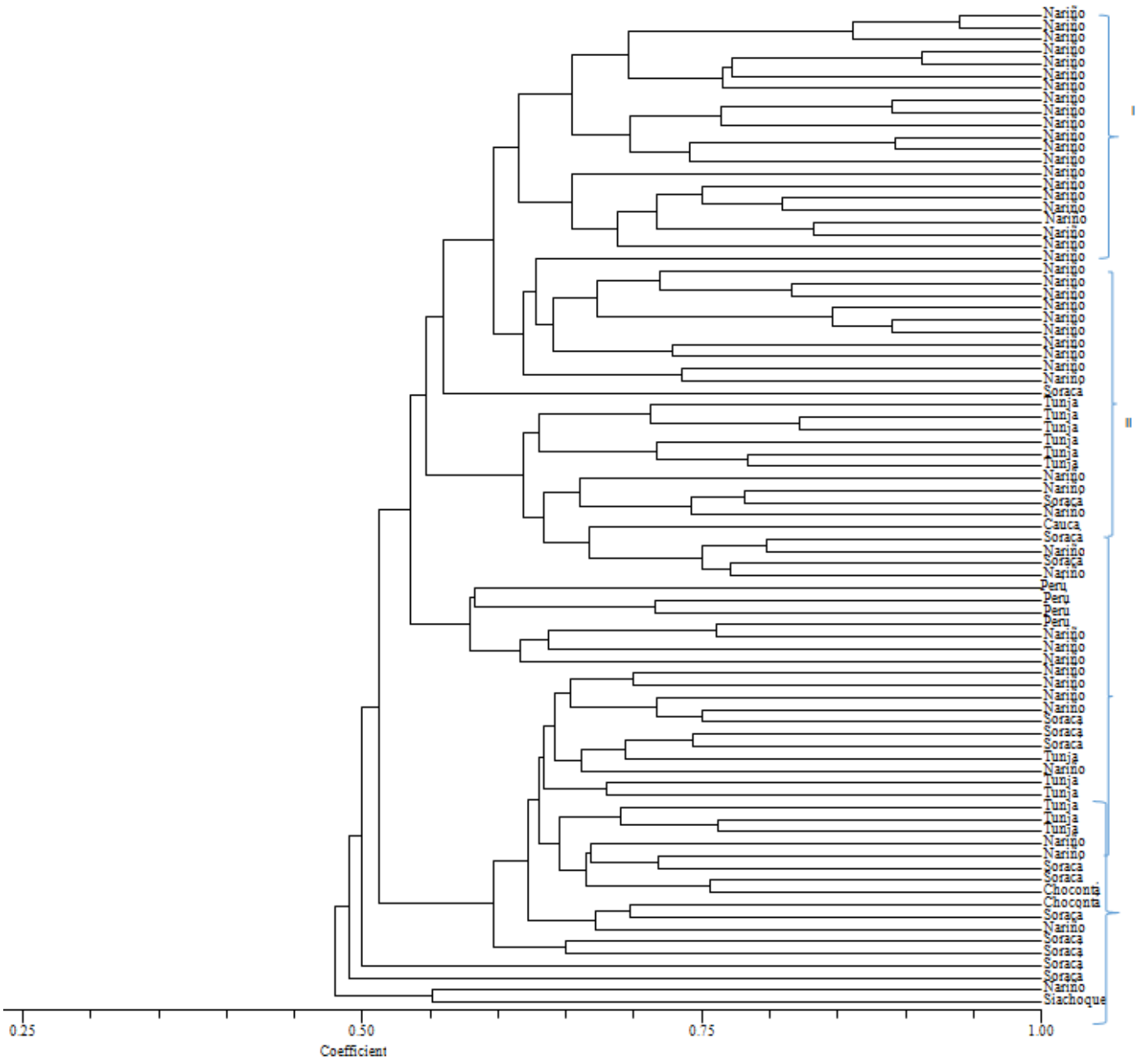

Figure 1. Dendrogram of quinua materials based on the Nei-Li similarity coefficient and calculated with seven ISSR markers with UPGMA classification method, SAHN and TREE of NTSYS-pc version 1.8 (Exeter Software, NY, USA).

al., 2014). Considering both the conditions under which quinoa is cultivated and its genetic variability, the plant has a remarkable adaptability to different agro-ecological zones. This adaptability is of great importance for the diversification of future agricultural systems; however, there is an urgent need to strengthen the breeding programs in quinoa (conventional as well as biotechnological) for its genetic improvement and conservation. The high nutritional quality and multiple uses in food products make quinoa ideal also for utilization by the food industry. Other potential uses are the medicinal and nutraceutical properties, due to its high phenolic acid content (Tuisima and Fernández, 2014).

In this study, a total of 178 bands were generated, with 99% polymorphism. The number of bands varied from 20 (CGA) to 35 (ACA), with molecular weights between 350 and 2700 pb and polymorphic loci percentages between 95 and 100% (Table 2). The number of bands and the

Table 3. Parameters of genetic diversity estimated in quinua materials evaluated.

Primer	Number of loci	Estimated He	% Polymorphic loci (95%)	Fst	SD
ACA	35	0.38	100.00	0.27	0.04
AG	30	0.38	100.00	0.09	0.03
TG	22	0.37	100.00	0.33	0.05
CT	24	0.35	100.00	0.21	0.03
CA	25	0.41	100.00	0.27	0.03
CCA	22	0.38	95.45	0.18	0.06
CGA	20	0.42	100.00	0.26	0.04
Total	178	0.38	99.44	0.23	0.02

Table 4. Analysis of molecular variance (AMOVA) for the formed groups.

Source	Dl.	SS	MS	Est. Var.	Percentage
Among Pops	5	749,154	149,831	9,091	22
Within Pops	76	2432,200	32,003	32,003	78
Total	81	3181,354	-	41,094	100

percentages of polymorphism found in this study are suitable for estimating genetic parameters when compared with others species that used ISSR markers (Rodríguez and Isla, 2009; Nolasco et al., 2013; Suresh et al., 2014). Heterozygosity values ranged between 0.35 (CT) and 0.42 (CGA). Taking into account the definition of Ott (1992), that consider marker like polymorphic, if H is greater than or equal to 0.1 and highly polymorphic if it is greater than or equal to 0.7, because ISSR are polymorphic markers that are useful for the discrimination of closely related quinua individuals (Oduwaye et al., 2014; Suresh et al., 2014; Lu et al., 2015). The TG marker made the greatest contribution to the observed variation with a 0.33 Fst which means it can be useful for the differentiation of materials of the genus *Chenopodium* in intra - interspecific genetic diversity studies (Table 3).

It was also identified that the CA, CGA and TG repeats are the most frequent repeated sequences in the quinoa genome compared to CCA and AG. Results are similar to those found by Jarvis et al. (2008), who developed a linkage map using SSR markers, AFLPs, the protein storage region in seed (11S) and the nucleolar organizing region (NOR) and in other related species such as amaranth (Jimenez et al., 2013; Oduwaye et al., 2014).

The average estimated heterozygosity value and percentage of polymorphic loci for the total population was 0.38 and 99%, respectively. The coefficient of genetic differentiation (Fst) obtained in evaluating 82 quinua materials with seven ISSR markers was 0.23 with a standard deviation of 0.02 (Table 3). According to Wright (1978), values of 0.25 show high genetic differentiation, which may be reflected in the high degree of domestication that these materials have suffered, since most of them are commercial varieties.

Genetic diversity studies using microsatellite markers in different *Chenopodium* species have shown a higher heterozygosity than this study, which may have been due to the nature of markers, genome coverage and reproductive factors (Self-pollination, cross pollination, bee pollination, seed dispersal, exchange of genetic information at intra and interspecific level between wild and ancestral relatives), which subject these species to their natural environment (Costa et al., 2012; Suresh et al., 2014; Vía and Fernández, 2015). With these molecular tools, it has been possible to identify differences at the genome level and similarities that are associated with morphological characteristics such as grain and panicle color, phenology and geographic distribution (Ruíz et al., 2014). In contrast, genetic diversity parameters found in this study show that quinua materials evaluated are very homogeneous which corroborates the results obtained in the dendrogram and genetic distances estimation and coincides with researches carried out by the Chilean and Peruvian breeding programs (Bazile et al., 2014; Vía and Fernández, 2015).

The molecular variance analysis AMOVA showed that the genetic variation observed in the evaluated quinua materials was mainly within groups, with 78% (Table 4). This high variation could indicate the presence of higher levels of subdivision and hierarchy. The remaining 22% was due to the component of genetic variance between the groups, which was significant ($P \leq 0.001$); such genetic variation between groups might be used for conservation and breeding of this species. Similar results have been reported in other studies of genetic diversity in the genus *Chenopodium* using microsatellite markers (Rodríguez and Isla, 2009; Suresh et al., 2014; Oduwaye et al.,

2014).

Considering both the conditions under which quinoa is cultivated and its genetic variability, the plant has a remarkable adaptability to different agro-ecological zones. This adaptability is of great importance for the diversification of future agricultural systems; however, there is an urgent need to strengthen the breeding programs in quinoa (conventional as well as biotechnological) for its genetic improvement and conservation. The high nutritional quality and multiple uses in food products make quinoa ideal also for utilization by the food industry. Other potential uses are the medicinal and nutraceutical activity due to its high phenolic acid content. With management strategies of quinoa cultivation among the farmers as well as the consumers, proper marketing and efficient post-harvest technologies, quinoa has the potential to become an important industrial and food crop of the 21st century (Tuisima and Fernández, 2014).

Conclusions

ISSR markers allowed the determination of the genetic variability in quinoa materials by grouping them according to the geographical location of origin.

It determined the existence of a moderate genetic variability due to the reproduction processes of the species as well as the spatial-temporal dynamics to which these materials are subjected in their natural environment and the constant exchange of seed between the Andean farmers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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