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Genetic Diversity of Ecuadorian Quinoa Using Microsatellite Molecular Markers

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Abstract

Quinoa (*Chenopodium quinoa*) is a crop that is appreciated worldwide for its nutritional value and adaptive qualities, among which stands out its tolerance to drought and salinity. Although several studies have characterized the genetic diversity of this crop in the Andean region, none have extensively described Ecuadorian germplasm. To clarify this genetic diversity, 84 accessions were collected from 7 provinces of the Ecuadorian highlands. These were molecularly characterized using 15 microsatellite markers; which yielded a total of 159 alleles, with an average of 10.6 alleles per locus. The expected global heterozygosity index (He = 0.71) revealed a high level of genetic variability for the analyzed individuals. However, this value could be overestimated by the presence of rare alleles in a high percentage (~ 60%). In the analyzed samples, no population structure was found according to geographic distribution by provinces, but several analyzes (i.e., *Structure and Neighbor-Joining*) suggest the existence of 3 genetic lineages. These lineages could include cultivated ecotypes developed through artificial selection and disseminated throughout the country through informal exchange of seeds. This study provides preliminary information about the evolutionary history and the level of genetic diversity of quinoa in Ecuador.

Keywords: Genetic diversity, microsatellite markers, rare alleles, genetic lineages, artificial selection.

Resumen

La quinua (*Chenopodium quinoa*) es un cultivo apreciado en todo el mundo por su valor nutricional y cualidades de adaptación, entre los que destaca su tolerancia a la sequía y la salinidad. Aunque varios estudios han caracterizado la diversidad genética de este cultivo en la región andina, ninguno ha descrito a profundidad el germoplasma ecuatoriano. Para aclarar esta diversidad genética, se obtuvieron 84 accesiones de 7 provincias del altiplano ecuatoriano. Estos se caracterizaron molecularmente utilizando 15 marcadores microsatélites. Se obtuvo un total de 159 alelos, con un promedio de 10.6 alelos por locus. El índice de heterocigosidad global esperado (He = 0.71) reveló un alto nivel de variabilidad genética para los individuos analizados. Sin embargo, este valor podría estar sobreestimado debido a la presencia de alelos raros en un alto porcentaje (~ 60%). En las muestras analizadas, no se encontró una estructura poblacional según la distribución geográfica por provincias, pero varios análisis (i.e., *Structure y Neighbor-Joining*) sugieren la existencia de 3 linajes genéticos. Estos linajes podrían incluir ecotipos desarrollados mediante selección artificial y diseminados por todo el país mediante el intercambio informal de semillas. Este estudio proporciona información preliminar sobre la historia evolutiva y el nivel de diversidad genética de la quinua en Ecuador.

Palabras clave: Diversidad genética, marcadores microsatélite, alelos raros, linajes genéticos, selección artificial.

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1. Introduction

1.1. History, origin and distribution of quinoa

Quinoa (*Chenopodium quinoa* Willd., *2n = 4x = 36*) is a highly nutritious crop that has been cultivated for more than 7000 years in the Andean region. It is an allotetraploid species from the Amaranthaceae family that includes numerous wild and domesticated species widely distributed throughout the Americas, the subtropical regions of Asia, Europe and Africa (Jacobsen *et al.*, 2003).

Recent studies demonstrate that quinoa likely originated from a hybridization event between *C. pallidicaule* and *C. suecicum,* followed by a whole-genome duplication event which made the species sexually viable (Jarvis *et al.*, 2017). Quinoa was presumably domesticated by pre-Columbian cultures from the Andean Altiplano around Lake Titicaca (Fuentes *et al.*, 2009; Jarvis *et al.*, 2017). Today, the species has adapted to thrive in a wide range of contrasting agroecosystems and can be found throughout the Andean region, from Colombia to northern Argentina. Thus, quinoa is generally classified into 5 major ecotypes: *Altiplano* (Peru and Bolivia), *Salar* (Bolivia, Chile and Argentina), *Valle* (Colombia, Ecuador and Peru), *Costa* (Chile) and *Yunga* (Bolivia) (Bazile *et al.*, 2014).

1.2. Morphology

Quinoa is a dicotyledonous, herbaceous annual plant species (Jacobsen & Stølen, 1993). The plant can grow between 1 to 3 meters high. Its central stem can be branched or unbranched, and may be green, red or purple. Its leaves are broad, hairy and lobed, and are generally arranged alternately. The quinoa inflorescence is organized into a dense panicle which typically arises from the top of the central stem; although certain ecotypes can exhibit panicle growth from leaf axils along the stem (National Research Council, 1989). Flowers are small (they can range from 2-5 millimeters), lack petals, and typically exhibit a reddish or purple perigonium

which gives the quinoa panicle its characteristic color. The quinoa fruit is an achene of approximately 2 millimeters in diameter, which protects a lenticular seed with an abundant and starch-rich perisperm of great nutritional value (Jacobsen & Stølen, 1993).

1.3. Agronomic characteristics

Quinoa is an agronomically versatile species that has adapted to a wide range of agro-climatic conditions. Today, the crop is primarily cultivated in the high Andes, at altitudes ranging from 2,500 to 4,000 meters above sea level; although Chilean ecotypes are uniquely adapted to coastal altitudes (Zurita-Silva, 2014). In general, quinoa prefers cooler climates with high luminosity for optimal growth, but the crop can withstand temperatures ranging from -4°C to 35 °C. It is important to highlight, nevertheless, that unseasonal frosts and high-temperatures will limit crop productivity if these occur during flowering or perisperm formation (Bois *et al.*, 2006; Jacobsen *et al.*, 2006). Rainfall requirements can vary widely between quinoa ecotypes, landraces and cultivars, but are generally low (150 to 1,000 millimeters per annum) when compared to other species (Martínez *et al.*, 2015). For optimal productivity, quinoa requires abundant and well-distributed rainfall during seed germination and early development, but the crop can withstand sustained periods of drought during vegetative growth, flowering and seed maturation. For example, Martinez *et al.* (2009) have demonstrated that certain quinoa landraces can grow with as little as 50 millimeters of rainfall throughout the entire growing season.

Another important attribute of quinoa is its tolerance to salinity. Being a facultative halophytic plant species, quinoa is able to germinate and grow under salinity levels as high as those found in sea water (up to 500mM of NaCl) (Adolf *et al.*, 2013). When analyzing the germination rates of different quinoa cultivars, several studies (Gómez-Pando *et al.*, 2010; Jacobsen *et al.*, 2003; Ruiz-Carrasco *et al.*, 2011) suggest different percentages of germination at various levels of

salinity; therefore, concluding that this capability is dependent on the cultivar and probably on the substrate in which the seeds germinate (Adolf *et al.,* 2013). As found by Adolf et al., (2012) this is not necessarily correlated with the tolerance at later development stages. However, optimal plant growth has been seen at concentrations of 100-200 mM of NaCl (Hariadi et al., 2011) and some cultivars even show higher yield when grown under moderate saline conditions (10-20 dS/m) than under non-saline conditions (Jacobsen et al., 2003). This reinforces the notion that salinity tolerance varies between ecotypes and cultivars.

1.4. Nutritional quality

Quinoa is a highly nutritious crop that has recently gained international attention because its seeds are gluten-free, have a low glycemic index, and are rich in fibers, proteins and secondary metabolites (Vega-Gálvez *et al.*, 2010; Jarvis *et al.*, 2017). Its outstanding nutritional quality was historically praised by the Incas, who deemed quinoa "the mother of all grains" (Jarvis *et al.*, 2017). Above all, quinoa is mainly valued today as a high-quality protein source; its seeds contain an excellent balance of all nine essential amino acids, including a high content of lysine which is absent in the majority of whole grains (*e.g.*, rice, barley, wheat, etc.) used in human and animal diets (Koziol, 1992). Carbohydrates are also abundant in quinoa seeds, and can constitute up to 60% of total dry seed-matter. These usually come in the form of complex starches which are a readily available source of energy (Vega-Gálvez *et al.,* 2010). In terms of mineral content and profile, quinoa contains high amounts of calcium, magnesium, iron, copper and zinc; all of which are useful for human diets in their bioavailable form (Schlick & Bubenheim, 1996). Moreover, quinoa has a rich vitamin composition, which includes alfa-carotenes, niacin, vitamin A, vitamin B2 and vitamin E (Vega-Gálvez *et al.,* 2010); as well as a high content of polyunsaturated fatty acids (7% of total grain dry matter), which have positive effects on cardiovascular diseases and insulin sensitivity (James, 2009).

Overall, quinoa is considered one of the most nutritious grains in the world. However, its

seeds also contain a diverse range of anti-nutritional components, which include saponins, phytic acid, tannins, and protease inhibitors (Vega-Gálvez *et al.*, 2010). Out of these, saponins have had special attention as they confer a bitter taste to quinoa grains and have shown to cause poor food conversion in *in-vivo* models. Harvested seeds therefore undergo through intensive post-harvest processing (i.e., scarification and washing) to remove saponins, but these activities significantly increase the selling cost of quinoa, and are also a burden on the environment in terms of water usage (Gee *et al.*, 1993). In this regard, breeding efforts in quinoa are concentrated on reducing saponin content *in planta* in order to increase quinoa's availability to consumers by reducing processing costs (Spehar & Rocha, 2010). Nonetheless, it is also important to highlight that quinoa crops with high saponin content have been repurposed for industrial processes like soap, detergent, and shampoo production and cosmetic and pharmaceutical industries (Jacobsen, 2003).

1.5. Quinoa: a relevant crop for global food security

As described by the Food and Agricultural Organization of the United Nations: "Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life" (FAO, 2017). Meeting food security becomes harder to achieve as we consider the growing population that demands unprecedented quantities of food. In the past, agriculture would cope with this by increasing the amount of land devoted to agriculture; however, nowadays this is a costly option as land is also considered for other human activities (Godfray *et al.*, 2010). Achieving food security becomes an even more complex task when we take climate change into consideration with problems of drought and soil erosion already surfacing around the world, creating adverse ecosystems in which agriculture is no longer viable (Christiansen, 2000). Recent studies have suggested that food necessities will double by

2050, slowly shifting the modern panorama of agriculture towards more efficient, highly productive systems with the use of non-traditional crops that can feed the world population in the upcoming decades (Godfray *et al.*, 2010).

This is where quinoa's inherent ability to thrive in otherwise marginal soils provides an opportunity to produce highly nutritious food in areas which would otherwise have no competitive value for agriculture (Flowers *et al.*, 1986). These repurposed areas can therefore contribute to the production of food, helping to achieve food security. The challenge today is to expand the production of this versatile crop around the world by creating specialized cultivars which can secure sufficient grain production in both, prime agricultural and marginal soils. This has lead quinoa from being grown only in the Andean region to countries all around the globe (Zurita-Silva *et al.*, 2014).

Historically, the commercial cultivation of quinoa has been primarily established in Peru, Bolivia and Ecuador. The first two countries generate over 90% of the crop's worldwide production, with 50000 and 45000 dry tons of grain per year, respectively (Peralta, 2009; Bazile *et al.*, 2014). While these two nations remain the most important producers of the quinoa, the current scenario is slowly shifting as both, industrialized and developing nations begin to invest in the crop by producing new varieties with outstanding yield which are adapted to new latitudes and climates (Bonifacio *et al.*, 2014).

Today, quinoa production has been expanded beyond the Andean region; the crop is successfully grown in England, the Netherlands, France, Spain and North America (United States and Canada). New varieties are also being developed to match the marginal agricultural conditions (*e.g.*, drought, salinity, high temperature, etc.) of several developing nations around the world where food security is a major issue (Zurita-Silva *et al.*, 2014).

1.6. Genetic diversity: definition and importance

Genetic diversity is defined as the extent of variation of genetic characteristics (*i.e.*, in the form of alleles) that exists between organisms of the same species (UN, 1992). This variation originates from several molecular processes in the form of mutations such as insertions, deletions and recombinations. Genetic variability is also a key factor to the evolution process through natural selection as is contributes to the adaptation of organisms to changing environments (Karp, 2011).

Knowing the degree of genetic diversity of a species or population is especially important in terms of conservation as it helps to monitor the erosion of natural diversity and to identify genotypes that need specialized preservation (Fuentes *et al.*, 2009). When it comes to agriculture, it is also important for the identification of new sources of alleles and relevant agronomic traits which can be used for plant breeding purposes (Christensen *et al.*, 2007; Jarvis *et al.*, 2017). With this information, breeders can make systematic crossings between interesting individuals to obtain new varieties with desired characteristics (Patterson *et al.* 1991).

1.7. Molecular Markers

Today, molecular markers are used to assess the degree of genetic diversity of a species or population. These are considered accurate indicators of the genetic information of an individual as they are not influenced by the environment (Picó & Pérez de Castro, 2012). Molecular markers are regions of the genome that are highly polymorphic because they are frequently subject to mutations. These mutations differ from one individual to another and can therefore provide a unique fingerprint-like pattern when molecular techniques such as PCR or restriction enzymes are used (Karp, 2011). In order for a molecular marker to be informative it has to have unique characteristics such as dominant or codominant nature, they must be widely distributed along the genome, therefore targeting a broad range of loci, and most importantly

they must be highly polymorphic. Some of these markers include Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), Random Amplified Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), and most recently, Single Nucleotide Polymorphisms (SNPs). Some of the most commonly used markers are SSRs. These interrogate short tandem rehashing sequences of about 1-6 base pairs (more commonly known as microsatellite regions) which are common across the genome of eukaryotes and which exhibit high mutation rates (Karp, 2011). The amount of repetitions of these rehashing sequences can be unique between individuals; this variation is a source of polymorphisms that can be studied via standard molecular techniques (*e.g.,* PCR, electrophoresis, etc.) (Karp, 2011).

1.8. Current knowledge of the genetic diversity of quinoa

Previous studies have been undertaken to analyze the extent of genetic diversity of quinoa; with analyses of Chilean, Peruvian and Bolivian germplasm being the most well-known and broad (Christensen *et al.*, 2007). These studies (Mason *et al.*, 2005; Jarvis *et al.*, 2008; Fuentes *et al.*, 2009) have relied on the development and utilization of species-specific SSR markers for analyzing the genetic diversity of quinoa.

Overall, these studies have shown a high degree of genetic diversity for the crop (He>0.7) (Fuentes *et al.*, 2009; Costa-Tartara *et al*, 2012; Ortiz *et al.*, 1998). These have also determined the clear genetic differentiation between the two main ecotypes, Coastal (mainly present in Chile and Argentina) and Highland (mainly present in Bolivia, Peru, Ecuador and Colombia). In fact, Jarvis *et al.* (2017) have recently suggested, via sequencing of the quinoa genome, that quinoa was domesticated independently (and probably simultaneously) in highland and coastal environments. Moreover, when analyzing the extent of diversity of highland ecotypes more closely, evidence shows a further genetic divergence between northern-highland (Ecuador and Colombia) and southern-highland (Peru and Bolivia)

ecotypes.

A few of the above-mentioned studies (Christensen *et al.*, 2007; Fuentes *et al.*, 2009) have also included a limited number of Ecuadorian accessions in their surveys of genetic diversity, however, a fully descriptive study that focuses on Ecuadorian germplasm was yet to be carried out. Nonetheless, a thesis project conducted by Gonzáles-Marín (2009) did study several quinoa samples present in various Ecuadorian provinces as well as commercial INIAP Tunkahuan varieties, however this research mainly analyzed possible gene flow between various cultivars showing allelic diversity indexes and FST values. However it did not report any other genetic diversity indexes or population structures. Given the fact that the crop did not originate in Ecuador (Jarvis *et al.*, 2017), it is unknown whether the diversity of Ecuador is unique relative to other Andean regions. However, quinoa was historically used as a staple crop by local indigenous communities of the Ecuadorean high Andes and has gone through a process of intensive adaptation to the varying agro-climatic conditions of the region (Peralta, 2009). The expectation is that Ecuadorian germplasm has a unique genetic structure and that individuals could be a source of novel alleles and phenotypic traits relevant to the development of cultivars with improved agronomic and nutritional properties. Following new-era studies such as SNP analysis or gene identification, researches and plant breeders can relate phenotype characteristics that could be beneficial for producers such as faster growth, biomass production, plague resistance, to genotypes contained within the quinoa populations (Zurita-Silva *et al.*, 2014). At the same time, these studies will also provide important information regarding the state of conservation of the species, providing information that could help the conservation and controlled exploitation of these biological resources.

In this study, 15 quinoa-specific SSR makers were used to amplify microsatellite regions of quinoa (*Chenopodium quinoa*). This was done to evaluate genetic diversity and population structures of 84 quinoa accessions from 7 provinces of the Andean region of Ecuador.

2. Objectives

2.1. General objective:

- To characterize the extent of genetic diversity available in a range of cultivated Ecuadorian quinoa varieties using microsatellite molecular markers.

2.2. Specific objectives:

- Construction of a quinoa germplasm bank comprising cultivated material from across the Ecuadorian Andean region. The bank will be preserved at Universidad San Francisco de Quito (USFQ; Cumbayá, Ecuador).
- Selection and validation of 15 quinoa-specific SSR markers.
- Standardization of a fluorophore-based multiplex SSR amplification and analysis system for quinoa.
- Molecular characterization of the USFQ quinoa germplasm collection using 15 quinoa-specific SSR markers.
- Statistical analysis to determine genetic diversity estimates and elucidation of population structure of Ecuadorian quinoa germplasm.

3. Justification

Today, 95% of our global food production is provided by 5 major grain species: rice, wheat, corn, millet and sorghum (FAO, 2017). Due to an increasing demand for food, international organizations, such as the United Nations, are looking for alternatives that will provide nourishment to the world population. On July 2011, the UN declared 2013 as the "International Year of Quinoa" based on a proposal made by Bolivia acknowledging quinoa as a promising crop to ensure global food security. Quinoa's unique nutritional content as well as resilience under the growing threat of climate change make it a great candidate to achieve this objective. For quinoa to become a relevant alternative for equitable and sustainable food production, new varieties must be developed with an increase yield, resistance to marginal environments and worldwide production capabilities.

As one of the places where quinoa has expanded and probably diversified, Ecuador holds within its mountains an ancestral diversity that farmers have carried with them for generations. Because of the geographical characteristics of the region, quinoa grown by farmers has been able to thrive under harsh abiotic conditions such as saline soils and poor nutrient contents (Peralta, 2009). These ancestral varieties could provide breeders with new alleles and characteristics that would contribute to obtaining new varieties that can withstand marginal environments and therefore boost the quinoa production worldwide. Since Ecuadorian quinoa had not yet been characterized, our study is fundamental if farmers are to benefit from the diversity of the Andean region. A genetic diversity study will also allow us to understand the extent of conservation of quinoa in Ecuador and its impact of the future of this crop in agriculture.

4. Area of Study

For this project, quinoa samples were collected from 7 provinces of the Andean highlands of Ecuador (Azuay, Cañar, Carchi, Chimborazo, Cotopaxi, Imbabura and Pichincha); these currently represent the range of cultivation of the crop across the country. Collected samples were either purchased from local markets or directly from small-scale farmers growing the crop either for direct consumption or for commercial purposes.

Seed germination and leaf collection (for genomic DNA extraction) was performed at the greenhouse of the Laboratory of Plant Biotechnology of Universidad San Francisco de Quito (USFQ), Cumbayá, Ecuador. DNA extraction and molecular analyses (including PCR and electrophoresis) and statistical analyses were conducted at the Laboratory of Plant Biotechnology USFQ, Cumbayá, Ecuador. Marker genotyping and scoring via capillary electrophoresis was performed at Macrogen, Seoul, South Korea.

5. Materials

5.1. Plant Material

- Quinoa leaves collected from 96 individuals from 7 provinces of the Ecuadorian Andes (Figure 1; Appendix 1).

5.2. Seed Germination

- Black potting soil
- Plastic seedling growing beds

5.3.DNA Extraction

- Multi-Blok Dry Heater (Thermo Scientific)
- Microcentrifuge (Eppendorf)
- Chloroform/Isoamyl Alcohol (24:1)
- 2X CTAB Extraction Buffer
- Isopropanol MARCA O DETALLES
- Liquid Nitrogen
- TE Buffer (Tris Base 10mM, EDTA 1mM, pH 8.0)
- 1.5 mL Eppendorf Tubes
- 2, ß-mercaptoethanol
- 70% Ethanol

5.4. DNA Quantification

- UltraPure^{TM} Distilled Water (GIBCO)
- TE Buffer (Tris Base 10mM, EDTA 1mM, pH 8.0)
- NANODROP 1000 Spectrophotometer (Thermo Scientific)

5.5. SSR Marker Amplification and Electrophoresis

- 30 Quinoa-specific Primer Pairs (Table 1)
- 4 Universal Type-A Primer Adaptor (described in Blacket *et al.*, 2012) marked with the following fluorophores: NED, PET, VIC and FAM
- Taq Platinum DNA polymerase 5U/mL (Invitrogen)

- PCR Buffer 10X (Invitrogen)
- $MgCl_2$ 50mM (Invitrogen)
- UltraPure™ Distilled Water (GIBCO)
- dNTPs 10mM (Invitrogen)
- T-Personal Thermocycler (Biometra)
- T100 Thermal Cycler (Bio-Rad)
- UltraPure Agarose (Invitrogen)
- BioRad Gel Doc XR Photo-documenter
- SYBR Safe DNA Gel Stain (Invitrogen)
- TBE 1X Buffer (Tris-Boric acid, EDTA)
- Blue Juice 10X Loading Buffer (Invitrogen)

5.6. Capillary Electrophoresis

- 96 Well-plates (Applied Biosystems)
- Plastic Strip Caps (Applied Biosystems)

5.7. Statistical Analysis

- GenMarker (Softgenetics)
- *Adegenet 2.0.0* (R-Statistics)
- DARWin 5.0 software
- STRUCTURE software
- Coancestry software
- Bottleneck 1-2-02

6. Methods

6.1.Germplasm sampling and collection

In this study, a total of 96 *Chenopodium quinoa* accessions were collected from 7 different provinces (Azuay, Cañar, Carchi, Chimborazo, Cotopaxi, Imbabura & Pichincha) across the Ecuadorian Andes. For each accession, approximately 500 g of sowing seed were purchased directly from farmers or from local markets. A detailed description of collection site localities, including geographical coordinates, is provided in Appendix 1. Sampling was conducted at localities which are known for the production of quinoa (Figure 1); therefore, collected accessions homogeneously represent *(in so far as possible)* the geographic distribution of Ecuadorian cultivated quinoa.

For all accessions, seeds were planted in the greenhouse of the Plant Biotechnology Laboratory of Universidad San Francisco de Quito (Cumbayá, Ecuador). Five seeds per accession were planted in a seed nursery with regular black potting soil. Plants were watered every other day and after approximately 2 months, approximately 20g of young leaf samples were collected for DNA extraction and further analysis.

6.2. DNA extraction

Total genomic DNA was isolated from young leaves using the CTAB extraction protocol described by Cota-Sánchez *et al.* (2006). For this protocol, approximately 20g of young leaf samples were macerated using liquid nitrogen in a ceramic mortar. This was followed by the addition of 800 ul of CTAB detergent that helps break nuclear membranes. After this, 8 ul of B-mercaptoethanol were added to further denature the protein structures present in the sample. The samples were then incubated at 62^{o}C for 1 hour with agitation every 10 minutes. Afterwards, 500 ul of chloroform-isoamilic alcohol 24:1 was added. The tubes were then left to sit for 20 minutes at room temperature followed by a 20-minute centrifugation process at

5000 rpm and the supernatant was discarded. After, 70% ethanol was added to separate the pellet that was formed in the previous step. Alcohol was discarded and then let to dry and with a final addition of 100 ul of TE buffer for resuspension. DNA concentration and quality were measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). Additionally, DNA samples were visualized in 1.5% agarose gel electrophoresis.

6.3. Molecular Characterization via SSR analysis

Thirty quinoa-specific SSR markers were surveyed for their suitability to explore the genetic diversity of Ecuadorian germplasm. These markers were selected from a set of approximately 400 SSR markers previously reported in the literature by Mason *et al.* (2005), Jarvis *et al.* (2008) and Fuentes *et al.* (2009). The selection was based on primers showing both, a high degree of heterozygosity ($H_e > 0.7$) and a high number of alleles (>8) as reported in the literature. For all selected markers (30 in total), Table 1 shows a concise description of their sequences, SSR motifs, annealing temperatures and expected allele size ranges. It is important to highlight that primer design included the addition of 15 extra nucleotides (Universal Tail A) to the original sequence of forward primers to enable analysis by capillary electrophoresis as described by Blacket *et al.*, 2012. This methodology uses an auxiliary round of PCR cycling to re-amplify target amplicons; however, the original forward primer is replaced with a fluorophore-marked primer sequence complementary to the Universal Tail A sequence. The resulting amplicons therefore carry a fluorescent tag which enables their analysis via capillary electrophoresis.

A set of 14 samples were amplified using the initial set of 30 SSR markers, following the aforementioned methodology of Blacket *et al.*, 2012. These samples were chosen to include individuals from all sampled provinces in an attempt to obtain the most polymorphic information from this initial screening. The master mix reagent concentrations were as follows:

PCR buffer (1X), MgCl2 (0.2µM), dNTPs (0.2µM), forward primer (0.15µM), reverse primer (0.5µM), fluorescent universal primer (0.5µM), Platinum Taq polymerase (1U) (Schuelke, 2000; Appendix 2). Cycling conditions were as follows: initial denaturing (95ºC/15 min), 35 cycles of denaturing (94 ºC/30sec), annealing (59-63 ºC/1.5min) and extension (72 ºC/1min) and a final extension (72 ºC/5min) (Appendix 3). Samples that showed a clear amplification pattern, as visualized by horizontal electrophoresis on 1.5% agarose gels, were sent to Macrogen (Seoul, Korea) for analysis by capillary electrophoresis on a 3730XL ABI sequencer (ABI Systems, California).

After genotyping analysis of the initial sub-set of 14 samples, 15 SSR markers were selected based on polymorphism information such as highest number of alleles and heterozygosity values. These 15 markers were used to amplify the whole quinoa germplasm collection using the previously described amplification protocols.

6.4. Analysis of North-European quinoa germplasm

To investigate the genetic relations of Ecuadorian germplasm relative to ecotypes from other regions of the Andes, we also characterized 5 Northern-European quinoa cultivars with the 15 SSR markers selected for this study using the same methodology described earlier in this section. These cultivars were developed by the Laboratory of Plant Breeding at Wageningen University (Wageningen, The Netherlands) in two phases. First, quinoa accessions from the Dutch Center for Genetic Resources (Wageningen, The Netherlands), most of which were of Peruvian, Bolivian and Chilean origin, underwent a process of mass selection for a period of 10 years, in order to accumulate genotypes with the capacity to grow in Northern-European latitudes. Focus of this breeding process concentrated on earliness, photoperiod sensitivity and grain yield. Subsequently, outstanding genotypes derived from the mass selection process were systematically crossed to create highly segregating base

populations with distinct characteristics. All 5 cultivars have been developed via pedigree selection starting from these base populations.

6.5. Data analyses

A total of 89 samples were used for downstream data analyses. Allele size determination and SSR marker scoring were performed using the GeneMarker software package (Softgenetics, Pennsylvania). Standard genetic diversity indices, including expected (H_e) and observed (H_o) heterozygosity, allelic frequencies, fixation index (*FST*) values and Mantel test were calculated using the R-based *adegent 2.0.0* statistical genetic package (Jombart, 2008). Population structure analysis were done using STRUCTURE (Pritchard, J., 1998). This software uses the Bayes Theorem to calculate statistical probability of an occurring event taking into consideration previous known parameters (priors) for the analysis (Mesa *et al.*, 2011). In this case, seven provinces were used as geographical location priors for the initial analysis. Parameters were set to 30000 for burning and 100000 for MCMC (Markov Chain Monte Carlo) with 5 iterations. Principal coordinate analysis (PCoA) was performed using DARWin 5.0 software (Perrier, X. *et al.*, 2006). In order to estimate inbreeding coefficients Coancestry software (Wang, 2011) was used. In order to analyze our samples a threat value of 1 was given to calculate Wang coefficients for each population. Finally, Bottleneck software V 1.2.02 (Cornuet & Luikart, 1999) was used to calculate possible bottlenecks in our quinoa populations.

7. Results

7.1. Genetic diversity in Ecuadorian quinoa germplasm

Total genomic DNA was successfully extracted for 84 quinoa accessions collected from 7 provinces across the Ecuadorian Andes. DNA isolations showed good quality (*260/280 Index = 2.3*) and quantity (75-5500 ng/µL) (Appendix 4).

Genetic diversity was assessed using 15 quinoa-specific SSR markers previously described in literature (Fuentes *et al.*, 2009; Jarvis *et al.*,2008; Mason *et al.*, 2005). These 15 loci were highly polymorphic and produced a total of 159 alleles across the evaluated sample set, with a range of 5 to 28 alleles per SSR marker (Table 2). For all loci, observed alleles fell within the expected size-range reported in literature (Fuentes *et al.*, 2009; Christensen *et al.*, 2007).

Overall, mean expected heterozygosity was high (H_e = 0.71) for the collection and demonstrates a high degree of genetic diversity for cultivated quinoa in Ecuador. This value may be overestimated, nevertheless, as our data shows (Table 2) a high proportion of rare alleles (~60%) with a representation frequency of less than 5% across the entire dataset. In accordance with this finding, global observed heterozygosity was significantly low (H_o = 0.18) for the collection; which is in clear contradiction with the aforementioned indication of a high degree of genetic diversity for Ecuadorian quinoa.

When comparing diversity indices between provinces (Table 3), expected heterozygosity values were highest for Imbabura (H_e= 0.67) and Chimborazo (H_e=0.67); both provinces currently lead the commercial production of quinoa in Ecuador (Monteros, 2016). The lowest expected heterozygosity was found for Azuay (H_e= 0.50), although it is important to highlight that this province also had a lower number of representative individuals (n=4) when compared to other provinces (*e.g.*, Imbabura [n=24]; Chimborazo [n=30]).

7.2. Population structure of Ecuadorian quinoa germplasm

A PCoA cluster analysis was performed to study the genetic structure of Ecuadorian quinoa germplasm. This resulted in a multivariate biplot showing 3 reasonably delimited groups (Figure 2): cluster A, located in the top-right quadrant; cluster B, located at bottom-right quadrant; and cluster C, located on the left half of the PCoA biplot. These 3 clusters were highly heterogeneous in their composition, with each group containing individuals from all sampled provinces. This preliminary analysis therefore discarded the possibility to structure Ecuadorian quinoa diversity based on a geographical pattern. It is important to highlight, nevertheless, that the 5 Northern-European cultivars (Cluster D) analyzed in this study, were positioned inside Cluster C (Figure 2).

To further understand the genetic structure of Ecuadorian quinoa germplasm, a Bayesian clustering analysis was conducted. This analysis showed that the 89 evaluated genotypes could be structured into 3 main groups (K = 3) (Figure 3), as determined via standard analyses using Structure Harvester (Earl, 2012). Remarkably, these results were highly consistent with the clustering patterns revealed by PCoA analyses. In other words, evaluated samples (including the 5 Northern-European cultivars) grouped similarly using both clustering approaches (Figure 4), with only a few discordant genotypes showing no overlap between analyses. Once again, these results showed no geographical patterning of the genetic diversity of Ecuadorian cultivated quinoa. This pattern was further confirmed by a Mantel test which did not show a correlation between genetic distances and geographical distances (R^2=0.0004). It was therefore speculated that the consistent clustering of genotypes into 3 groups could correspond to 3 distinct genetic lineages which have resulted from historical selection and breeding processes of the crop.

7.3. Genetic nature of 3 distinct lineages in Ecuadorian quinoa germplasm

Genetic diversity values found for the 3 main groups identified by PCoA and Structure clustering analyses are presented in Table 4. Overall, the 3 proposed lineages showed high expected heterozygosity values with lineage C showing the highest degree of genetic diversity (H_e = 0.72). By contrast, lineage A, which included the widely disseminated commercial cultivar, INIAP Tunkahuan, showed the lowest degree of genetic variability (H_e = 0.53). Here we also noted that both lineages A and B showed a high percentage of rare alleles (46% of total number of alleles), while lineage C showed a lower percentage (13%). Furthermore, Nei genetic distances and FST values showed that the 3 proposed lineages significantly diverged from each other, albeit at different levels (Tables 5 and 6). Thus, while lineages A and C exhibited a high degree of divergence (D_S = 0.84), lineages A and B appeared to be more closely related (D_S = 0.31).

Finally, co-ancestry analyses showed a negative Wang index (W_I = -0.186) for the collection, which indicates that on average all analyzed genotypes show a low degree of relatedness amongst each other. Notwithstanding, when single lineages were tested, only lineage A showed a positive Wang coefficient value (W_I = 0.066), which would demonstrate that there is a high degree of genetic relatedness between individuals conforming this proposed lineage. These results were further confirmed by a Wilcoxon test which showed a positive bottleneck effect on lineage A with a *p-value* of 0.00061 for *He* deficiency, 0.99957 for *He* excess and 0.00122 for *He* excess or deficiency. None of the other lineages presented positive indexes suggesting that these lineages have not gone through breeding processes and are therefore more diverse.

8. Discussion

The primary objective of this project was to assess the degree of genetic diversity of quinoa accessions representing the cultivated range of the crop in the Andean region of Ecuador. According to Peralta (2009), quinoa had been historically grown in Ecuador as an orphan crop, meaning that farmers used to grow seeds that were passed down from generations and that were informally traded between them. It was not until the 1980's when government institutions such as MAGAP and INIAP started promoting the industrialization of quinoa production and made efforts to release and promote the use of newly developed varieties. Our study obtained samples from 7 out of 9 provinces that are historically known to produce quinoa in hopes that they could represent both the historical diversity of quinoa while also including the new varieties that are currently grown by farmers.

Previous quinoa studies have shown that there are 5 important ecotypes that describe the history of quinoa in the region, with the two most important being Altiplano, present mainly in Bolivia and Peru, and Costal, present in Chile (Christensen *et al.*, 2007; Fuentes *et al.*, 2009). These studies have included few Ecuadorian accessions in an attempt to determine the origin of Ecuadorian quinoa diversity (*i.e.*, with respect to the 5 original ecotypes) suggesting that Ecuadorian diversity derived from the Bolivian-Peruvian ecotype *Altiplano*, followed by a subsequent adaptation to local conditions (Christensen *et al.*, 2007). These studies have since found over 2700 and 1000 landraces in Bolivia and Peru respectively (Ortiz *et al.*, 1998).

Taking into consideration that the diversity of Ecuadorian germplasm had not yet been previously described, this study provides a good idea of the crop's germplasm diversity corresponding to the northern Andean region.

8.1. Allelic information and genetic diversity indices

To analyze the genetic diversity of Ecuadorian germplasm, SSR molecular markers were chosen due to their high polymorphic content, ease for genotyping, and codominant nature (Karp, 2011). Species-specific markers were selected from a pool of over 400 SSR markers, taking special consideration to those already used to characterize quinoa in in Chilean, Bolivian, Argentinian and Peruvian germplasm collections (Fuentes *et al.*, 2009; Ortiz *et al.*, 1998; Christensen *et al.*, 2007). All 15 markers used were highly informative, producing between 5 (QAAT51) to 28 (QGAA001) alleles per locus (Table 2). On average, Ecuadorian quinoa germplasm showed a similar number of alleles per locus (n = 10.5) when compared to the USDA-CIP quinoa collection (11.7) (Christensen *et al.* 2007), and a higher number of polymorphic alleles when compared to Chilean germplasm (n = 7.5) (Fuentes *et al.* 2009). However, Ecuadorian germplasm showed a lower number of alleles per locus when compared to Argentinian germplasm (n = 16) (Tártara *et al.* 2012); although it is important to highlight that in the aforementioned study, the authors established that the high number of alleles identified could have been overestimated due to the methodology used.

Overall, the expected heterozygosity index found for the entire collection (H_e = 0.71) shows that the Ecuadorian germplasm has a high degree of genetic diversity. Similar results were obtained from the analyses of the USDA-CIP (*He*=0.75) (Christensen *et al.* 2007), Chilean (*He*=0.65) (Fuentes *et al.* 2009) and Argentinian (*He*=0.82) (Tártara *et al.* 2012) germplasm collections. A recent study (Jarvis *et al.*, 2017) using different molecular markers (SNPs) similarly showed that quinoa is a highly variable crop with 2.7 million single nucleotide polymorphisms found when sequencing 15 coastal and highland ecotypes to a reference genome, finding at least 1 single nucleotide variant per every 3000 sequenced base pairs.

Observed heterozygosity was calculated and expectedly a much lower value was

obtained (H_o=0.18). In the case of quinoa, a relatively lower heterozygosity is expected due to the obligated self-pollinating nature of quinoa allowing for a higher homozygous proportion among individuals (Zurita-Silva *et al.*, 2014).

An additional assessment of rare alleles was performed to further explain the previously mentioned genetic diversity indices and results showed that out of the 159 alleles found across all 15 markers, around 60% of the alleles found of rare frequency (<5% representation in the total allele pool). A similar finding was previously reported by Christensen *et al.* (2007). In this study, marker QAAT50 yielded the highest number of polymorphic alleles for the USDA-CIP germplasm collection; with approximately 60% of these being rare alleles. Similar results were obtained in our study for the same marker with a slightly higher percentage of rare alleles (~70%). All markers used in our study showed over 40% of rare alleles when analyzed. The only exception to this was maker QAAT51 which only showed 20% of rare alleles; however, this marker was also the least polymorphic of all evaluated markers. The high incidence of rare alleles in quinoa seems comprehensible. Although the species is naturally autogamous, quinoa exhibits a facultative, yet limited capacity for outcrossing (up to 10%) which opens the possibility for reduced gene-flow between and within natural standing populations (Zurita-Silva *et al.*, 2014).

Even though diversity indexes such as expected heterozygosity might be overestimated due to this high proportion of rare alleles, it is important to state that high diversity in quinoa might be related to these rare alleles and therefore they must be considered in any type of analysis.

8.2. Population structure

In our study, we found that Ecuadorian quinoa germplasm could be structured into three clusters which showed no obvious geographical patterning which was further confirmed by the

lack of correlation shown through a Mantel test. Therefore, our initial approach was to consider these 3 clusters as distinct genetic lineages instead of populations based on the assumption that modern germplasm comprises both, ancestral diversity and commercial cultivars that could present a distinct genetic background. To help us characterize the lineages found in our study, information about the genetic identity and breeding history of several samples was used. The information of these known genotypes helped us speculate about the nature of the individuals present in each lineage. It is important to notice that the dissemination of the 3 distinct lineages throughout the country might correspond to a process of informal seed exchange between farmers which also contributes to the crop's high diversity (Fuentes *et al.*, 2012).

One of the samples of known identity corresponded to the widely disseminated local variety, INIAP Tunkahuan. This particular genotype was grouped inside Lineage A (Figure 4) and it is very likely that the other members of this subgroup are genetically similar to this variety. This assumption could be confirmed by the low degree of genetic diversity of the subgroup, along with the results of co-ancestry and bottleneck tests which show that the individuals of Lineage A are genetically homogenous and have possibly gone through a process of selection and allele fixation.

Before the decade of the 90s, quinoa was relegated as an orphan, marginal crop in Ecuador which was primarily produced by low-income Andean farmers for own-consumption. Around this period, newly developed varieties (derived from programs sponsored by MAGAP and INIAP) entered the Ecuadorian market; first came lines like INIAP Cochasquí and INIAP Imbayá, which were bitter, followed by INIAP Tunkahuan and INIAP Ingapirca which represent improved sweet varieties that also provided benefits for farmers in terms of post-harvest processing. Today, INIAP Tunkahuan is the only variety that is still promoted for industrial production by governmental agencies (Peralta, 2009). The fact that collected samples present in Lineage A come from all 7 sampled provinces shows the success of governmental

efforts to disseminate this variety. It also shows the high-performance of the variety itself, as it has been widely adopted by farmers nationwide.

Even though lineage B does not have an individual of known genotype which could facilitate understanding its origin, it still shows moderate diversity and a relatively high incidence of rare alleles. Statistical indicators such as FST values and Nei genetic distances suggest that there is possible gene flow between this lineage and both the commercially-related and the ancestral lineages found in this study, though it is more closely related to lineage A than it is to lineage C.

Five other genotypes with known origin included in this study comprised to Northern-European quinoa varieties derived from Bolivian, Chilean and Peruvian germplasm. These varieties were clustered inside lineage C and it is therefore suggested that the individuals present in this lineage could be related to the ancestral lineages which first arrived in our country. This is further supported by recent studies showing that quinoa in Ecuador would have originated from the southern Andean region corresponding to Peru and Bolivia (Jarvis et al., 2017). This suggests that some of our Ecuadorian samples might be closely related to these ancestral genotypes and that these individuals are, though lesser in number, widely distributed due to their long presence in the Andean region.

Ever since modern local quinoa varieties were released into the market, the benefits of having an improved variety became obvious, and with it, the ancestral genetic diversity of Ecuadorian quinoa was threatened as farmers started to prefer these over ancestral varieties. Our results show that the genetic diversity that is currently present in Ecuadorian quinoa still holds a high degree of diversity and that probably both ancestral and modern varieties are contributing to this diversity.

If we are to continue exploring quinoa's genetic diversity, an assessment of the germplasm present before commercial varieties were introduced must be conducted. With

government agencies like INIAP having an unreported germplasm bank with almost 600 accessions of quinoa at their disposal (Peralta, 2009), there is still much to be done if we are to take full advantage of the ancient diversity that has been present in Ecuadorian soil for centuries. These non-studied varieties may be key to developing new varieties once their diversity and phenotypical traits are properly characterized.

9. Conclusions

- A dedicated quinoa germplasm bank was constructed and it now has 97 accessions of quinoa collected from 7 provinces across the Andean region of Ecuador.
- A fluorophore-based multiplex SSR amplification and analysis system was developed for characterization of the genetic diversity of quinoa.
- All 15 analyzed loci showed a high degree of polymorphism and were highly informative to describe genetic diversity of the crop.
- Results obtained with this study show an initial assessment of the genetic diversity of quinoa in the Andean region of Ecuador.
- Population analysis did not show any structure based on geographical locations but more of a random distribution of samples across all 7 provinces explained by a free exchange of seeds occurring constantly within the region.
- Three possible lineages were described in this study with the inclusion of a commercially-derived lineage and possible an ancestral lineage.

10. Recommendations

- Use a broader set of samples in provinces where a low number of samples were used.
- Include more foreign samples from countries like Peru, Bolivia or Chile in order to compare de Ecuadorian diversity with different lineages and ecotypes.
- Take note of morphological characteristics of the collected samples in order to further corroborate the possible lineages of quinoa samples.
- Use other types of markers like SNPs to have a better resolution of quinoa polymorphism now that there is a genome database of the species.

11. References

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12. Tables

Table 1: List of 30 specific quinoa primers.

Locus SSR	Forward primer (5'—>3')	Reverse primer (5'—>3')	Motif	Expected allelic size
KAAT 007*	aggtacaggcgcaaggatac	cggtagcatagcacagaacg	(AAT)30	197
KAAT 037*	tcaacctccgaatcctatcaa	ggatgctgattggtggataaa	(TAA)19	284
KAAT 047*	tctcggttccctactaatttcttg	tttatgcagcaagggttgtaaa	(AAT)26	-
KCAA029	cagactgcaggcaccaca	gttgttgtggttgttgttattggt	(CAA)2CAT(CAA)5	-
KCAA068	cagcaactgaaaccagcaa	gcagctgctgttgctaaatac	(ACA)7	-
KCAA106	atatggaagtcggccaacag	gcatgctcatcatttgttgc	(CAA)20	-
KCAA117	ccgtggttcctctagagtcg	cctccaacaacctttctctcc	(GTT)9	-
KGA003	attgccgacaatgaacgaat	gcttctatgtaaatggcatgtcccaac	(GA)16	140-182
KGA20	tcacctacctcggtaaaggaaa	ggagcagatgatgaacatgg	-	155-185
KGA27*	ttgtacagaggaagtggcaaga	catcttacagctctggctttcc	-	-
QAAT 001*	atattgcatgtcgagcacca	Tgggacttccataaggcaac	-	182
QAAT 06	cacaaacaataattcaaccgaaga	cgctgacgcttaacattcg	-	193 – 226
QAAT 12	tcaagtgtgggatgcttgaa	Ccgacagacgaggagacaa	(AAT)10	188
QAAT 22*	tggtcgatatagatgaaccaaa	ggagcccagattgtatctca	-	153 – 235
QAAT 24*	gcttctaccataacagcacccaccttt	agggatcaatcttgttcattca	(AAT)10	201-254

QAAT 50*	ggcacgtgctgctactcata	gcttctatggcgaatggttaatttgc	(ATT)17	192-214
QAAT 51*	ccttcgacaaggtcccatta	cgtccatagtggaggcattt	(AAT)14	173
QAAT 69	gtttcctttgaggcttggac	ggatttgtacgaatagttgggatt	-	193 – 266
QAAT 70*	tgaacaggatcgtcatagtcaa	gcttctcgttcatcatctgacccaat	(ATT)15	173-210
QAAT 71	catcacccgctgaatagacac	taccctaatgccacgattcc	-	122 – 200
QAAT 74*	gcttctatggaacacccatccgataa	atgcctatcctcatcctcca	(ATT)14	172-199
QAAT 76	gcttcatgtgttataaaatgccaat	gcttcttctcggcttcccactaatttt	(ATT)30	152-224
QAAT 78	agcgaaggaaatttggaact	gcttcttaacgatacgctccaagga a	(ATT)22	186-214
QAAT 88*	tcctaacttcttgtgacatttcctt	ccacgatcccagaacaattt	(ATT)30	151
QAAT 84*	gtggatgtaaaggtggttt	acaacttatttgttagctagattatt	(AAT)12	163
QAAT112*	cccgatccaccataagagaa	tgaagtgtaagattggagaatgaca	(ATT)13	-
QCA 71	aacaacgaaattacgagaatgtca	tctcacgagagtcttccccta	-	140 – 177
QCA57	gcttcttgcaaggaaaccatctttgg	tgcctcacagtcacacctaca	(CA)22(TA)5	168-193
QGA 021	cacgaaaccaactcctctca	caccacaatcaccacctttg	(CT)21	153
QGAA 001*	ttgtatctcggcttcccact	aaccagagagatgaagaacatgc	-	279

(Fuentes *et al.*, 2009) (Jarvis *et al.*,2008) (Mason *et al.*, 2005)

*These markers were selected for the complete analysis of the whole Ecuadorian quinoa germplasm collection.

Table 2: Genetic diversity parameters for 15 SSR quinoa markers. Na: Number of alleles, *Ho*: observed heterozygosity, *He:* expected heterozygosity.

# Primer	Marker name	Na	Number of rare alleles	*Ho*	*He*
1	KAAT007				
2	KAAT037	6	3	0.22	0.59
3	QAAT24	9	4	0.26	0.81
5	KGA27	8	5	0.1	0.57
6	QAAT001	7	4	0.14	0.62
7	QAAT022	13	9	0.24	0.69
10	KAAT047	19	8	0.16	0.88
16	QAAT84	9	6	0.16	0.4
17	QGAA001	28	19	0.22	0.93
18	QAAT70	18	9	0.2	0.83
20	QAAT50	13	7	0.19	0.86
22	QAAT51	5	1	0.12	0.45
23	QAAT774	15	10	0.22	0.84
26	QAAT112	9	4	0.11	0.76
30	QAAT88				
Mean		10.5	6.84	0.18	0.71

Table 3: Expected and observed heterozygosity values for each province where samples were collected.

Province	**Expected Heterozygosity**	**Observed Heterozygosity**
Azuay	0.496	0.192
Cañar	0.568	0.173
Carchi	0.627	0.224
Chimborazo	0.671	0.199
Cotopaxi	0.568	0.180
Imbabura	0.671	0.155
Pichincha	0.704	0.108

Table 4: Heterozygosity parameters of 3 possible lineages.

Lineage	Expected heterozygosity
A	0.53
B	0.62
C	0.72

Table 5: Nei Genetic Distances between 3 possible lineages.

	A	B
B	0.31	
C	0.83	0.62

Table 6: Pairwise FTS value comparison between 3 possible lineages

Lineage	A	B
B	0.09	
C	0.15	0.11

13. Figures

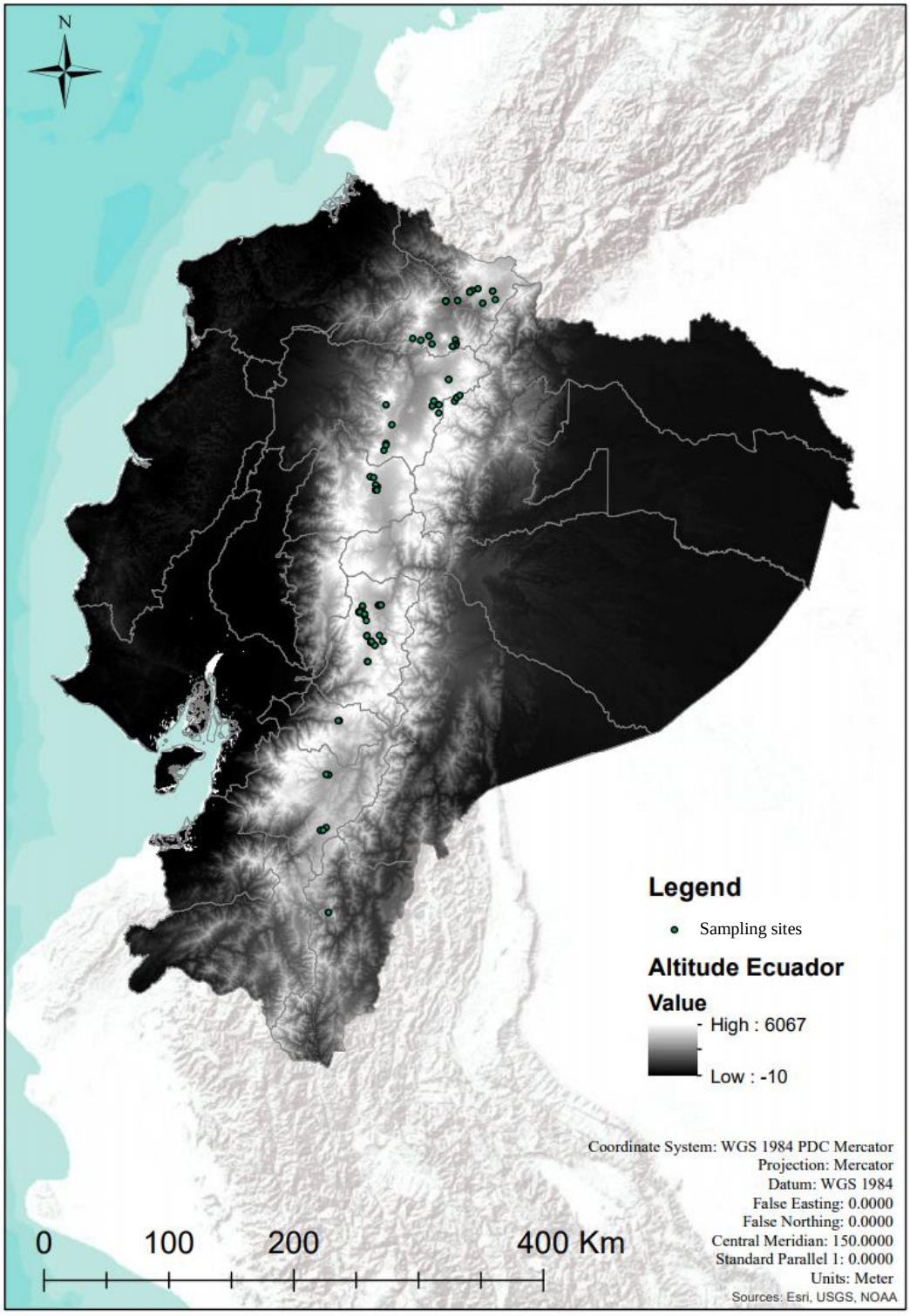

Figure 1: Map of Ecuador depicting an altitude variation with sampling sites marked with black dots along 7 provinces of the Andean region.
Elaborated by Izan Chalen.

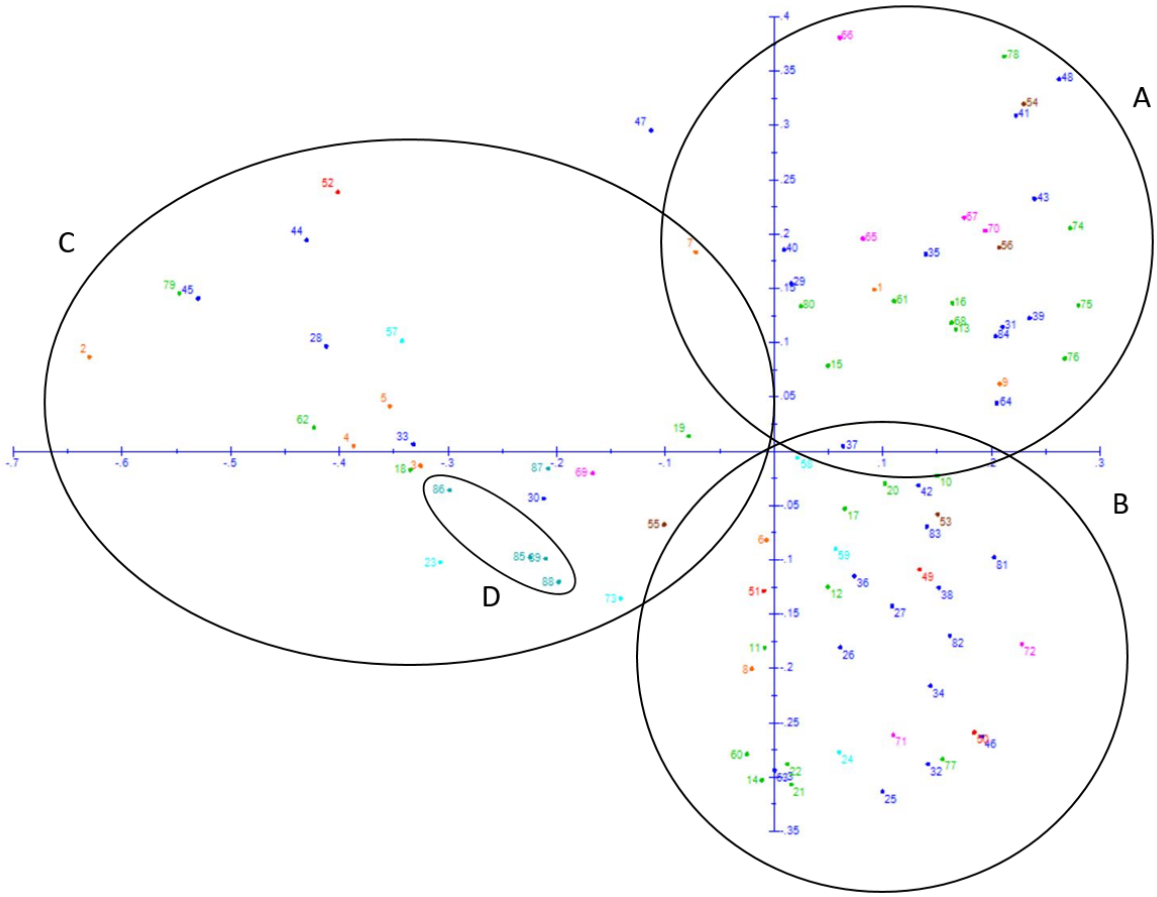

Figure 2: Two-dimensional scaling resulting from PCoA analysis of 84 accessions of *Chenopodium quinoa* using genetic diversity data from 15 microsatelite markers. Graph shows three possible clusters comprised of samples from all 7 provinces. Additional samples from Northern-European germplasm are depicted within cluster D.

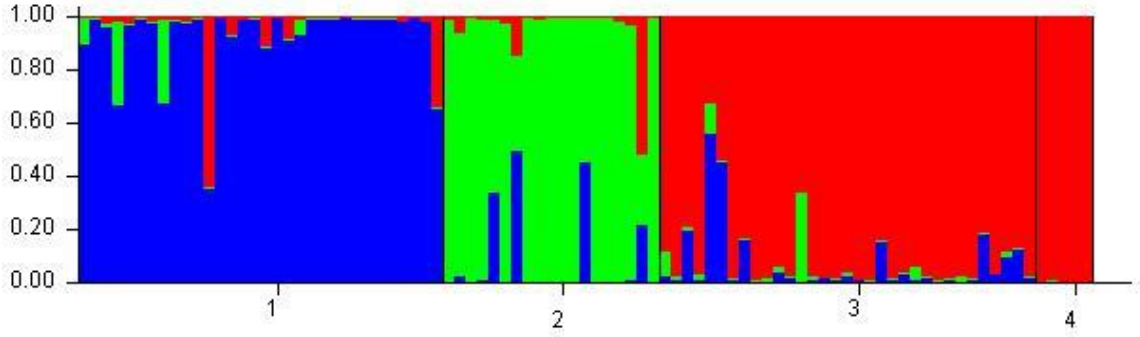

Figure 3: Structure analysis with K=3 (Q order) of 84 accessions of *Chenopodium quinoa* using genetic diversity data from 15 microsatelite markers. Different colors represent proposed lineage contribution for each sample.

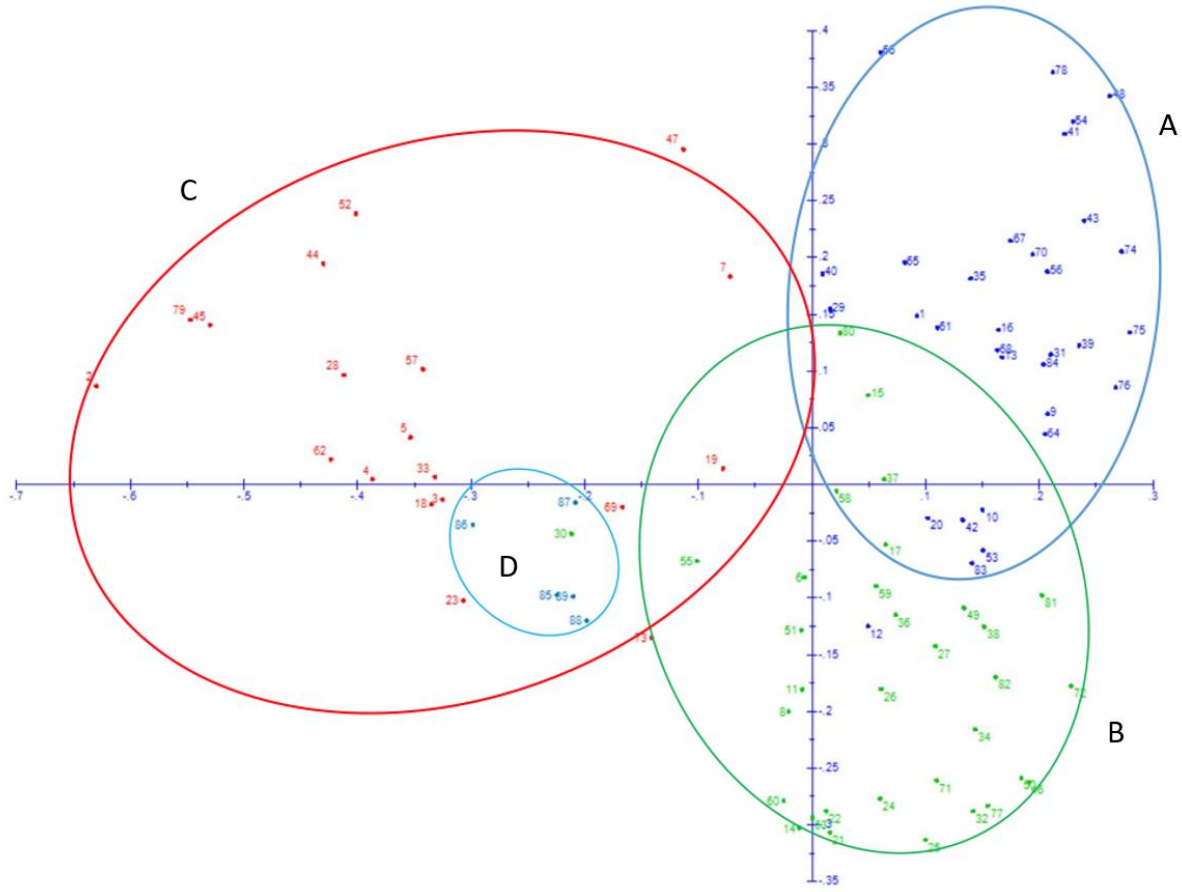

Figure 4: Two-dimensional scaling resulting from PCoA analysis of 84 accessions of *Chenopodium quinoa* using genetic diversity data from 15 microsatelite markers. Three possible lineages are depicted in colors blue (A), red (B) and green (C). Additional Northern-European germplasm samples are depicted within cluster D.

14. Appendixes

Appendix 1: Sample collection site referential information.

Sample #	USFQ Bank Code	Province of Origin	"Cantón"	Location	Coordinates	Elevation (m.a.s.l)
1	USFQ-QUI-001	Carchi	Mira	Pisquer	N 00° 31.885', W 078° 04.747'	2604
2	USFQ-QUI-002	Carchi	Mira	Pisquer	N 00° 31.885', W 078° 04.876'	2602
3	USFQ-QUI-003	Carchi	Espejo	San Isidro	N 00° 35.671', W 077° 59.512'	3012
4	USFQ-QUI-004	Carchi	Espejo	San Isidro	N 00° 35.628', W 077° 59.567'	3011
5	USFQ-QUI-005	Carchi	Espejo	San Isidro	N 00° 35.558', W 077° 59.589'	3012
6	USFQ-QUI-006	Carchi	Espejo	San Isidro	N 00° 35.703', W 077° 59.526'	3024
7	USFQ-QUI-007	Carchi	Espejo	San Isidro	N 00° 36.009', W 077° 59.269'	3021
8	USFQ-QUI-008	Carchi	Espejo	San Isidro	N 00° 36.130', W 077° 58.604'	3026
9	USFQ-QUI-009	Carchi	Montufar	El Angel	N 00° 36.942', W 077° 56.002'	3014
10	USFQ-QUI-010	Carchi	Montufar	Canchaguano	N 00° 32.281', W 077° 48.621'	2841
11	USFQ-QUI-011	Carchi	Bolivar	Cuesaca	N 00° 30.723', W 077° 54.053'	2658
12	USFQ-QUI-012	Carchi	Espejo	San Isidro	N 00° 35.558', W 077° 59.589'	3012
13	USFQ-QUI-013	Imbabura	Angochagua		N 00° 35.953', W 077° 49.704'	2865
14	USFQ-QUI-014	Imbabura	Angochagua		N 00° 35.953', W 077° 49.704'	2865
15	USFQ-QUI-015	Imbabura	Angochagua	La Magdalena	N 00°	2831

					14.759', W 078° 05.823'	
16	USFQ-QUI-016	Imbabura	Angochagua	Zuleta	N 00° 13.130', W 078° 05.356'	2877
17	USFQ-QUI-017	Imbabura	Angochagua	Zuleta	N 00° 12.401', W 078° 05.847'	2934
18	USFQ-QUI-018	Imbabura	San Pablo	Angla	N 00° 12.000', W 078° 07.060'	3039
19	USFQ-QUI-019	Imbabura	San Pablo	Angla	N 00° 12.000', W 078° 07.060'	3039
20	USFQ-QUI-020	Imbabura	Otavalo		N 00° 13.000', W 078° 15.984'	2665
21	USFQ-QUI-021	Imbabura	Quichinche	Tangali	N 00° 14.696', W 078° 20.720'	2901
22	USFQ-QUI-022	Imbabura	Quichinche	Cambugan	N 00° 15.460', W 078° 24.250'	3208
23	USFQ-QUI-023	Imbabura		San Martin	N 00° 16.388', W 078° 17.140'	2563
24	USFQ-QUI-024	Imbabura		San Martin	N 00° 16.388', W 078° 17.140'	2563
25	USFQ-QUI-025	Imbabura	La Esperanza		N 00° 31.600', W 078° 10.000'	2708
26	USFQ-QUI-026	Imbabura	La Esperanza		N 00° 31.600', W 078° 10.000'	2708
27	USFQ-QUI-027	Imbabura	La Esperanza		N 00° 31.600', W 078° 10.000'	2708
28	USFQ-QUI-028	Pichincha	Mejía	Machachi	S 00° 33.202', W 078° 36.741'	3265
29	USFQ-QUI-029	Pichincha	Mejía	Machachi	S 00° 30.205', W 078° 35.826'	3059
30	USFQ-QUI-030	Chimborazo	Colta	Comunidad Bellavista	S 01° 41.261', W 078° 45.944'	3165
31	USFQ-QUI-031	Chimborazo	Colta	Comunidad	S 01°	3210

				Bellavista	40.716', W 078° 45.970'	
32	USFQ-QUI-032	Chimborazo	Colta	Comunidad Guacona- El Belén	S 01° 43.182', W 078° 47.172'	3374
33	USFQ-QUI-033	Chimborazo	Colta	Comunidad Guacona- El Belén	S 01° 43.182', W 078° 47.172'	3374
34	USFQ-QUI-034	Chimborazo	Colta	Comunidad Guacona- El Belén	S 01° 43.182', W 078° 47.172'	3374
35	USFQ-QUI-035	Chimborazo	Colta	Guacona Grande	S 01° 43.550', W 078° 47.332'	3420
36	USFQ-QUI-036	Chimborazo	Colta	Guacona Grande	S 01° 43.550', W 078° 47.332'	3420
37	USFQ-QUI-037	Chimborazo	Colta	Guacona Grande	S 01° 43.550', W 078° 47.332'	3420
38	USFQ-QUI-038	Chimborazo	Colta	Guacona Grande	S 01° 43.362', W 078° 47.049'	3511
39	USFQ-QUI-039	Chimborazo	Colta	Guacona Grande	S 01° 43.362', W 078° 47.049'	3511
40	USFQ-QUI-040	Chimborazo	Colta	Capilla	S 01° 44.957', W 078° 45.052'	3322
41	USFQ-QUI-041	Chimborazo	Colta	Santiago de Quito	S 01° 44.586', W 078° 44.941'	3325
42	USFQ-QUI-042	Chimborazo	Colta	Comunidad San José	S 01° 44.308', W 078° 45.007'	3319
43	USFQ-QUI-043	Chimborazo	Colta	Comunidad San José	S 01° 44.308', W 078° 45.007'	3319
44	USFQ-QUI-044	Chimborazo	Guamote	San Pedro de Ayacón	S 01° 56.342', W 078° 42.332'	3080
45	USFQ-QUI-045	Chimborazo	Guamote	San Pedro de Ayacón	S 01° 56.342', W 078° 42.332'	3080
46	USFQ-QUI-046	Chimborazo	Guamote	Palmira	S 02° 04.862', W 078° 43.843'	3298
47	USFQ-QUI-047	Chimborazo	Guamote	Palmira	S 02°	3305

					04.902', W 078° 43.703'	
48	USFQ-QUI-048	Chimborazo	Guamote	Tres Aguas	S 01° 53.568', W 078° 38.540'	2974
49	USFQ-QUI-049	Chimborazo	Guamote	Tres Aguas	S 01° 56.000', W 078° 37.087'	3243
50	USFQ-QUI-050	Chimborazo	Guamote	Tres Aguas	S 01° 56.000', W 078° 37.087'	3243
51	USFQ-QUI-051	Chimborazo	Guamote		S 01° 55.947', W 078° 42.339'	3024
52	USFQ-QUI-052	Chimborazo	Colta	Cachabamba	S 01° 47.148', W 078° 44.298'	3279
53	USFQ-QUI-053	Chimborazo	Colta	Jatumpamba	S 01° 57.759', W 078° 40.617'	3602
54	USFQ-QUI-054	Chimborazo	Colta	Jatumpamba	S 01° 57.759', W 078° 40.617'	3602
55	USFQ-QUI-055	Cañar	Azogues	Cachi	S 02° 30.509', W 078° 55.927'	2960
56	USFQ-QUI-056	Cañar	Azogues	Cachi	S 02° 30.581', W 078° 56.502'	2902
57	USFQ-QUI-057	Cañar	Desconocido			
58	USFQ-QUI-058	Cañar	Desconocido			
59	USFQ-QUI-059	Azuay	Oña	Chonazona	S 03° 17.997', W 079° 04.224'	2167
60	USFQ-QUI-060	Azuay	Oña	Shiña	S 03° 16.891', W 079° 01.628'	2704
61	USFQ-QUI-061	Azuay	Oña	Santa Lucía	S 03° 18.015', W 079° 02.880'	2670
62	USFQ-QUI-062	Azuay	Cuenca	Mercado 10 de Agosto	S 02° 53.984', W 079° 00.451'	2561
63	USFQ-QUI-063	Azuay	Cuenca	Feria Libre	S 02° 53.811', W 079° 01.609'	2590
64	USFQ-QUI-064	Azuay	Cuenca	Mercado 3 Noviembre	S 03° 53.607', W	2617

					079° 00.637'	
65	USFQ-QUI-066	Pichincha		Estación Santa Catalina	S 00° 22.135', W 078° 33.307'	3055
66	USFQ-QUI-066	Pichincha			N 00° 02.400', W 078° 08.678'	2815
67	USFQ-QUI-067	Pichincha			N 00° 02.400', W 078° 08.678'	2815
68	USFQ-QUI-068	Imbabura	Otavalo		N 00° 13.945', W 078° 15.818'	2530
69	USFQ-QUI-069	Imbabura	Otavalo		N 00° 13.945', W 078° 15.818'	2530
70	USFQ-QUI-070	Imbabura	Otavalo		N 00° 13.945', W 078° 15.818'	2530
71	USFQ-QUI-071	Chimborazo			N 01° 40.457', W 078° 39.005'	2756
72	USFQ-QUI-072	Chimborazo			N 01° 40.457', W 078° 39.005'	2756
73	USFQ-QUI-073	Cotopaxi	Guaitacama		S 00° 49.004', W 078° 39.445'	2972
83	USFQ-QUI-073	Pichincha	Aloasi		S 00° 31.017', W 078° 35.742'	3063
74	USFQ-QUI-074	Cotopaxi	Toacaso	Vía Sigchos	S 00° 44.691', W 078° 42.702'	3311
84	USFQ-QUI-074	Imbabura	San Pablo		N 00° 13.292', W 078° 13.084'	2749
75	USFQ-QUI-075	Cotopaxi	Toacaso		S 00° 45.039', W 078° 41.096'	3208
85	USFQ-QUI-075	Imbabura	San Pablo		N 00° 13.347', W 078° 35.742'	2726
76	USFQ-QUI-076	Imbabura	San Roque-Los Ovalos		N 00° 17.017', W 078° 13.000'	2458
77	USFQ-QUI-077	Cotopaxi	Cuicuno		S 00° 48.187', W	3092

					078° 40.237'	
78	USFQ-QUI-078	Cotopaxi			S 00° 50.348', W 078° 40.140'	2924
79	USFQ-QUI-079	Cotopaxi			S 00° 50.348', W 078° 40.140'	2924
80	USFQ-QUI-080	Cotopaxi			S 00° 50.348', W 078° 40.140'	2924
81	USFQ-QUI-081	Cotopaxi			S 00° 50.348', W 078° 40.140'	2924
82	USFQ-QUI-082	Cotopaxi		Chatilin	S 00° 50.483', W 078° 39.697'	2916
86	USFQ-QUI-086	Imbabura	San Pablo		N 00° 13.395', W 078° 12.887'	2750
87	USFQ-QUI-087	Imbabura		Zuleta	N 00° 11.821', W 078° 06.135'	2909
88	USFQ-QUI-088	Imbabura	Eugenio Espejo		N 00° 11.720', W 078° 15.180'	3202
89	USFQ-QUI-089	Imbabura		Zuleta	N 00° 10.286', W 078° 05.321'	3104
90	USFQ-QUI-090	Imbabura		Zuleta	N 00° 10.286', W 078° 05.321'	3104
91	USFQ-QUI-091	Imbabura		Zuleta	N 00° 09.271', W 078° 03.927'	3148
92	USFQ-QUI-092	Chimborazo		Columbe	S 01° 56' 12", W 078° 42'40"	NA
93	USFQ-QUI-093	Chimborazo		Columbe	S 01° 53' 42", W 078° 44'57"	3610
94	USFQ-QUI-094	Chimborazo		Columbe	S 01° 53'42", W 078° 44'57"	3610
95	USFQ-QUI-095	Chimborazo		Columbe	S 01° 53' 41", W 078° 44'57"	3590
96	USFQ-QUI-096	Chimborazo		Columbe	S 01° 40' 20", W 078°	2860

					38'52"	
97	USFQ-QUI-097	Chimborazo		Columbe	S 01° 40' 20", W 078° 38'52"	2860

Appendix 2: Master Mix concentrations for PCR amplification

Reagent	Final concentration	Reaction volume (µl)
PCR water	-	31.05
Buffer	1x	5
Mg2Cl	0.4µM	2
Forward primer	0.15µM	0.75
Reverse primer	0.5µM	2.5
Fluorophore	0.5µM	2.5
dNTPs	0.2µM	1
Platinum Taq	1U	0.2
DNA	250ng/µl	5
Total	-	50

Appendix 3: Thermocycler program

Step	Temperature ^{o}C	Time	
Initial denaturing	95	15 min	
Denaturing	94	30 secs	35 cycles
Annealing	59-63	1.5 min	
Extension	72	1 min	
Final extension	72	5 min	

Appendix 4: DNA samples quantity and quality indexes

Sample	Concentration	260/280	Sample	Concentration	260/280	Sample	Concentration	260/280
1	389.7	2.48	35	239.4	2.51	72	486.9	2.16
2	101.2	2.23	36	208.2	2.5	73	649.3	2.52
3	2273.3	2.21	37	265.8	2.62	74	424.8	2.44
4	1934.7	2.15	38	3677.3	2.14	75	468	2.65
5	2177.4	2.13	39	5581.6	2.14	76	425.8	2.42
6	75	2.45	40	1269.4	2.27	78	450	2.33
7	3482.1	2.14	41	4035.3	2.12	79	776.8	2.52
8	182.1	2.02	42	3242.5	2.17	80	400.1	2.28
9	4116.2	2.18	43	3997.4	2.1	81	199	3.17
10	4168.2	2.17	44	3561	2.14	82	404.4	2.77
11	2408.4	2.15	45	2407.3	2.12	83	2836.6	2.21
12	422.4	2.25	46	4066.8	2.11	84	376.2	2.67
13	218.1	2.23	47	3064	2.18	85	455.9	2.46
14	192.3	2.26	48	2525.4	2.11	86	213.7	2.37
15	2866.2	2.22	49	2291.5	2.15	87	279.9	2.3
16	254.7	2.34	50	2225.8	2.26	88	254.6	2.52
17	2004.6	2.17	51	2811	2.21	89	1810.2	2.12
18	3067.6	2.19	52	3814.4	2.19	91	442.4	2.29
19	256.3	2.24	53	2472.1	2.2	93	286.3	3.12
20	119.1	2.31	55	2628	2.09	94	501.8	2.5
21	441.8	2.19	56	3019	2.17	95	398.9	2.68
22	2739	2.1	57	2754.7	2.17	97	345.5	2.42
23	2696.2	2.15	58	2737.2	2.15			
24	2558.9	2.06	59	1086.8	2.39			
25	282.7	2.21	60	517.7	2.12			
26	2188.6	2.18	61	371.7	2.44			
27	1340.9	2.14	64	469.6	2.5			
28	2180.6	2.14	65	860.2	2.59			
29	3339.2	2.15	66	4643.9	2.08			
30	1968.4	2.21	67	1218.7	2.18			
31	3080.8	2.16	68	4299.9	2.19			
32	2605.2	2.12	69	274.3	2.64			
33	3417.3	2.16	70	2812.3	2.13			
34	2836.4	2.16	71	1630.1	2.23			