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Full Length Research Paper

Comparative analysis of genetic and morphologic diversity among quinoa accessions (*Chenopodium quinoa* Willd.) of the South of Chile and highland accessions

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Quinoa (*Chenopodium quinoa* Willd.) is a widely consumed food crop and a primary protein source for many of the indigenous inhabitants of the Andean region in South America. Identification of quinoa cultivars has been based on phenotypic characters. In the present work, the level of polymorphism and the genetic relationship were studied by means of molecular markers using the amplified fragment length polymorphism (AFLP) technique and twenty morphological characters. Fourteen accessions of quinoa collected in the Araucania and Los Rios Regions, three Andean accessions, and one commercial cultivar were analyzed. Two wild parents were included as outgroup controls. A similarity tree-diagram was made, based on all the AFLP bands generated in the range between 70 and 300 base pairs. With these tools, it was possible to identify molecular differences and similarities that might be associated with important morphological traits such as grain color, panicle color, phenology and geographic distribution.

Key words: *Chenopodium quinoa*, cluster analysis, molecular markers.

INTRODUCTION

Chenopodium quinoa is one of the most important food crops in the Andean highland of South America. We can find it in Chile, Argentina, Ecuador, Perú and Bolivia. It belongs to the Amaranthaceae family (Kadereit et al. 2003), which traditionally includes the economically important species spinach (*Spinacea oleracea* L.) and sugarbeet (*Beta vulgaris* L.). Quinoa is an allotetraploid (2n = 4x = 36), and thus exhibits disomic inheritance for most qualitative traits (Ward 1998; Maughan et al., 2004). The small achene fruits contain an excellent balance of carbohydrates, lipids and protein, making it an excellent food source (Chauhan et al., 1999). This fruit provides an ideal balance of all 20 essential amino acids (Ruas et al., 1999). Quinoa is one of the only few crop plants adapted to the extreme conditions that characterize this region (Prado et al., 2000). There is a great interest for this crop in developing countries since it is considered as one of the most important crops involved in feed conditions improvement of this century. Although increasing quinoa productivity is a primary food-security issue in the Andean Region, limited research on quinoa genetics and plant breeding has been conducted. In general, cultivated quinoa displays 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. Within the diversity centers, the center of Peru (Huan cayo, Ayacucho, Cajamarca), the Ecuadorian Mountain range, the Argentine Northeast, the South of Chile and of Colombia (Grass, Nariño and Cudinamarca)are identified (Jacobsen, 2003). Great ignorance still exists and little investigation has been carried out regarding varieties in the South of Chile, conserved and selected by Mapuche communities and other smallholder farmers. The adaptation capacities of quinoa 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 (Jacobsen, 2003). Native Chilean varieties are adapted to other latitudes due to their absence or minor sensitivity to photoperiod during

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the grain filling. In that sense quinoa crops at sea level are less sensitive to damage caused by the conjunction between long high-temperature days, which would explain his extreme adaptability (Bertero, 2001). In spite of the importance that has been attributed to the quinoa crop in the Region of the Andes, only few lines of investigation exist to establish applied genetics and molecular characteristics of this crop. Nevertheless, initiatives to adjust the crops and to open new markets have even made possible to generate some beginning improvement programs outside the original places. These programs aimed to prioritize the increase of grain production, resistance to diseases, tolerance to hydric stress and control of the saponines content (Ochoa et al. 1999). Molecular markers are an effective way to enhance breeding efficiency (Lande, 1991; Patterson et al., 1991; Staub et al., 1996). Up to now, only a few researchers have reported the development and use of molecular markers in quinoa. Wilson (1988a) used data of alozymes in quinoa to confirm the genetic difference between ecotypes of the plateau and valleys. Maughan et al. (2004) made a genetic map. Ruas et al. (1999) used RAPD markers to detect the degree of polymorphism between cultivated and wild species of quinoa. In order to establish genotypic differences between quinoas (Chenopodium quinoa Will.) from the North and the South of Chile, Wilckens et al. (1996) reported to use stored proteins of seed (isoenzimes). Recent studies of characterization of quinoa germplasm have been developed to create a map of microsatellite markers (Mason et al., 2005). In Chile, genetic diversity studies regarding quinoa are only focused on morphologic and biochemical comparison between Andean and local varieties. Nevertheless the molecular markers offer could enable us to obtain a greater and deeper accuracy compared to biochemical markers and therefore, to identify and characterize highly related individuals. Both in Chile and in the rest of the world, molecular markers are considered as excellent and accurate techniques to study genetic diversity and improvement based on DNA analysis and are used for a wide range of crops (Pillay and Myers 1999; Mason et al. 2005; Solano et al. 2007) : *Solanum tuberosum* (Solano et al., 2007), *Lens culinaris* (Sharma et al. 1996), *Glycine max* (Maughan et al., 1996), *Lactuca spp*. (Hill et al. 1996) and *Hordeum vulgare* (Becker et al., 1995). Among the molecular markers available with greater ability of accuracy and reproducibility, AFLP (Amplified Fragment Length Polymorphism) analysis represents the most recent technology for taining a great number of molecular markers in prokaryote and eukaryote genomes (Hartings et al., 2008). The AFLP technique (Vos et al., 1995) generally produces between 50 and 100 scorable fragments per polymerase chain reaction (PCR (Maughan et al. 1996). On the other hand, the low cost, easy use, and great quantity of polymorphism of AFLP markers make them very useful for crop analysis investigated in developing countries (Maughan et al. 2004). The use of this method is also highly relevant since it was used to make the first genetic quinoa map. This technology based on PCR involves three essential steps. First, the digestion of genomic DNA by two restriction enzymes, followed by the ligation of adapter to the extremities of restricted fragments and finally a selective amplification with two consecutive reactions of PCR. The PCR product is denatured and separated in a polyacrylamide gel in which there are usually 60 to 80 bands per DNA sample. The objectives of this study were to (i) analyze the genetic diversity of 14 coastal accessions of (C. quinoa Willd.) using AFLP markers and morphological data, (ii) determine the genetic relationships among southern accessions and (iii) to compare these data with highland accessions and commercial varieties.

MATERIALS AND METHODS

Plant material

The material used (quinoa varieties) came from small-holder farmers of diverse localities in the regions of Araucania and Los Rios. These varieties are ancestral ones, inherited from generation and generation of use and cultivation free from the diverse agricultural modernization programs (Thomet et al., 2003). During this investigation, we evaluated fourteen local varieties (Table 1), three varieties from the Tarapacá Region (Iquique-Universidad Arturo Prat), one enrolled variety (Regalona-Baer) and two outgroup controls (C. album and C. ambrosoide (Figure 1).

Morphological character analysis

Twenty characters (Table 2) of the all varieties and controls, codifying according to description assigned were described and analyzed for quinoa according to IPGRI (1981), then to be exported in a matrix of number-data. The morphologic data were subjected to cluster analysis. A standardization of the data by using Z-scores function was made. Average was applied to the technique of hierarchical conglomerate linkage using a matrix of quadratic similarity applying the Euclidean distances.

DNA isolation

Approximately 100 - 200 mg of material was freeze-dried and ground in liquid nitrogen with a mortar and pestle. Genomic DNA was isolated with Plant DNAzol® following the manufacturer's instructions. RNA was further eliminated by treatment with RNase. The quality and concentration of DNA was evaluated by agarose gel electrophoresis and spectrophotometry. Two independent extractions were performed on each accession.

AFLP analysis

AFLP reactions were carried out using the AFLP Analysis System I kit (Invitrogen Life Technologies) according to the manufacturer's instructions. Each reaction was repeated at least once to verify the AFLP patterns generated.

Approximately 500 ng of genomic DNA was digested for 2 h at 37°C using 2 µl *Eco*RI/*Mse*I restriction enzyme solution. The AFLP procedure (Vos et al. 1995) was carried out as described by Arens et al. (1998) with slight modifications. Briefly, the entire genomic DNA (400 - 500 ng) was digested with *Eco*RI and *Mse*I, followed by

Table 1. Material plant included in the analysis.

Sample Nº	ID Accession	Common name	Species	Localities	Altitud masl	Material
1	KM 01	Yellow	*Chenopodium quinoa*	Lautaro	243	Cotiledon
2	KM 02	Red	*Chenopodium quinoa*	Nueva Imperial	51	Cotiledon
3	KM 03	Red	*Chenopodium quinoa*	Ercilla	286	Cotiledon
4	KM 04	Red	*Chenopodium quinoa*	Liquiñe	631	Cotiledon
5	KM 05	Red	*Chenopodium quinoa*	Melipeuco	549	Cotiledon
6	KM 06	Mixture	*Chenopodium quinoa*	Panguipulli	420	Cotiledon
7	KM 07	Red	*Chenopodium quinoa*	Ercilla	290	Cotiledon
8	KM 08	Red	*Chenopodium quinoa*	Vilcún	355	Cotiledon
9	KM 09	Red	*Chenopodium quinoa*	Vilcún	338	Cotiledon
10	KM 10	Yellow	*Chenopodium quinoa*	Vilcún	347	Cotiledon
11	KM 11	Red	*Chenopodium quinoa*	Lautaro	233	Cotiledon
12	KM 12	Yellow	*Chenopodium quinoa*	Temuco	171	Cotiledon
13	KM 13	Red	*Chenopodium quinoa*	Temuco	165	Cotiledon
14	KM 14	Red	*Chenopodium quinoa*	Ercilla	279	Cotiledon
15	R-B	Regalona - Baer	*Chenopodium quinoa*	Temuco	161	Cotiledon
16	R01	Red I	*Chenopodium quinoa*	Iquique- Sector Plomo Loma	3.795	Cotiledon
17	A01	Yellow I	*Chenopodium quinoa*	Iquique- Sector Plomo Loma	3.743	Cotiledon
18	A02	Yellow II	*Chenopodium quinoa*	Iquique- Sector Plomo Loma	3.740	Cotiledon
19	Control I	Quinguilla	*Chenopodium album*	Temuco	161	Cotiledon
20	Control II	Paico	*Chenopodium ambrosoide*	Temuco	161	Cotiledon

ligation of the adapters. Pre-amplification was performed using a single adenine (A) selective nucleotide for each primer. For sele-ctive amplification, an *Eco*RI primer, with three selective nucleo-tides, was used in combination with *Mse*I primer with three selective nucleotides. For both pre-amplification and selective amplification, the following amplification profile was used: an initial cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, followed by 12 touchdown cycles in which the annealing temperature was reduced of 0.7°C per cycle. The annealing temperature was then kept constant at 56°C for the other 23 cycles. Amplification products were separated on a 6% polyacrylamide gel, and made visible with silver staining. Ten primer combinations were tested for their ability to generate reproducible AFLP profiles that could be scored unambiguously. Reproducibility of the primer combinations was tested by comparing the AFLP profiles of two DNA samples collected from the same individual. Three combinations were specifically chosen for their ability to generate a large number of bands in order to increase accuracy for the identification of possible identical plants.

Data analysis

For each primer combination, the presence or absence of a band in each sample was visually scored. Data were analysis in a binary matrix (Paul et al., 1997; Yee et al., 1999). Genetic similarities were calculated using the Simple Matching coefficient and tree-diagrams obtained by clustering according to the unweighted pair group method with arithmetic average (UPGMA), using the NTSYSpc 2.0.1 program (Applied Biostatistics Inc., NY, USA). The correspondence between the morphological and AFLP similarity coefficient matrices was tested on the basis of correlation analysis for Mantel's test using the MxComp procedure of NTSYS.

RESULTS

Morphological analysis

The grouping analysis of the morphological data made it possible to form three groups (Figure 2). In Group I, two sub-groups were identified. The characteristics which are gathered by the Sub-group (A) accessions are: intense yellow for grain

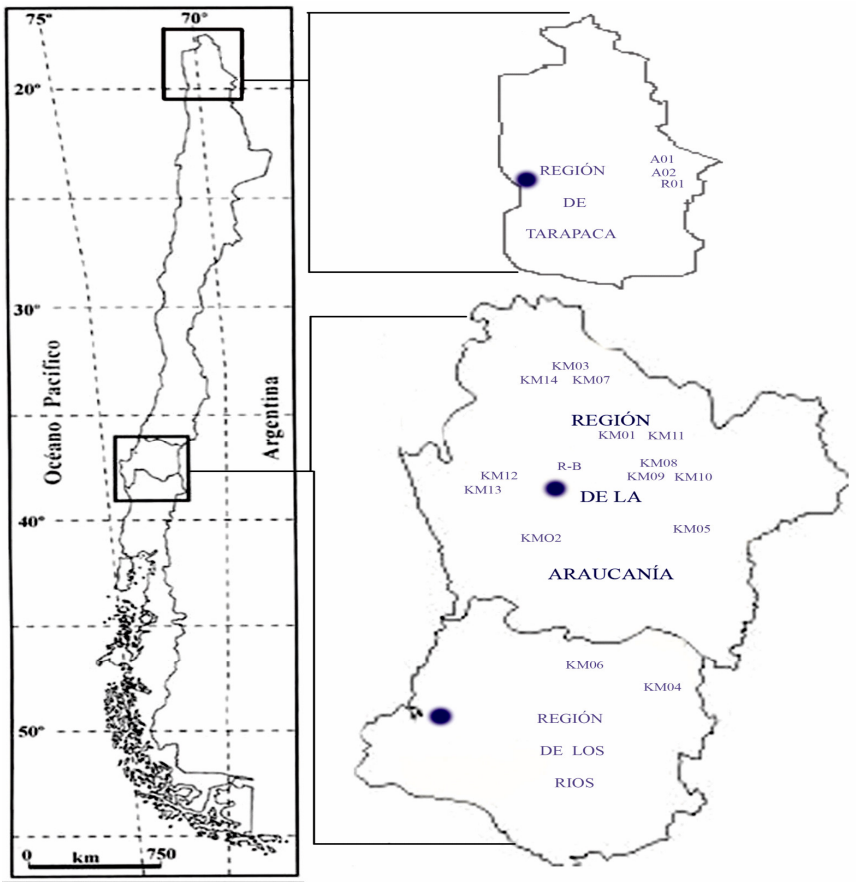

Figure 1. Localities quinoa collections.

Table 2. Descriptive names of the 20 morphological characters examined.

Descriptor list	Morphological characters
Grain	Colour of pericarp Shape of fruit edge
Inflorescence	Colour of panicle Intensity color of panicle Shape of panicle Kind of panicle Density of panicle
Stem	Colour of stem Intensity of colour Formation of stem Presence of lines Colour of lines in stem
Type of growth	Growth habit type Number of the primary stem Plant height Kind of stems
Leaf	Teeth of basal leaf Edge of basal leaf Colour of basal leaf
Phenological (days)	Emergence Flower buds Initiation of flowering 50% flowering Physiological maturity

colour, agglomerated form and yellow colour for the panicle, compact panicle, and precocity. In this group we can find accessions KM01, KM10, KM12 and the registered variety Regalona-Baer. Sub-group (B) included accessions which presented variations in grain colour from intense yellow to brown, red colour, agglomerated form and intermediate density of the panicle. These presented a precocity ranging from early to semi-early. In this group we find accessions KM07 and the accessions which exhibit the same co-efficient of morphological similarity, including accessions KM03, KM04, KM05; KM02, KM08, KM09 and KM11, KM13, KM14 respectively even though they come from different localities. Accession KM06 may be set apart within the sub-group given though it has retained its mixed condition. Group II corresponds to highland accessions of later phenology, yellow to red colour of grain, panicle for are amarantiform and com-pact. The accessions included in this group were A0I, A02 and R01. Group III consisted of two different species *C. album* (CH19) and *C. ambro-sioides* (CH20) representing outgroup controls.

AFLP analysis

All of the samples showed a genetic diversity, with a simi-

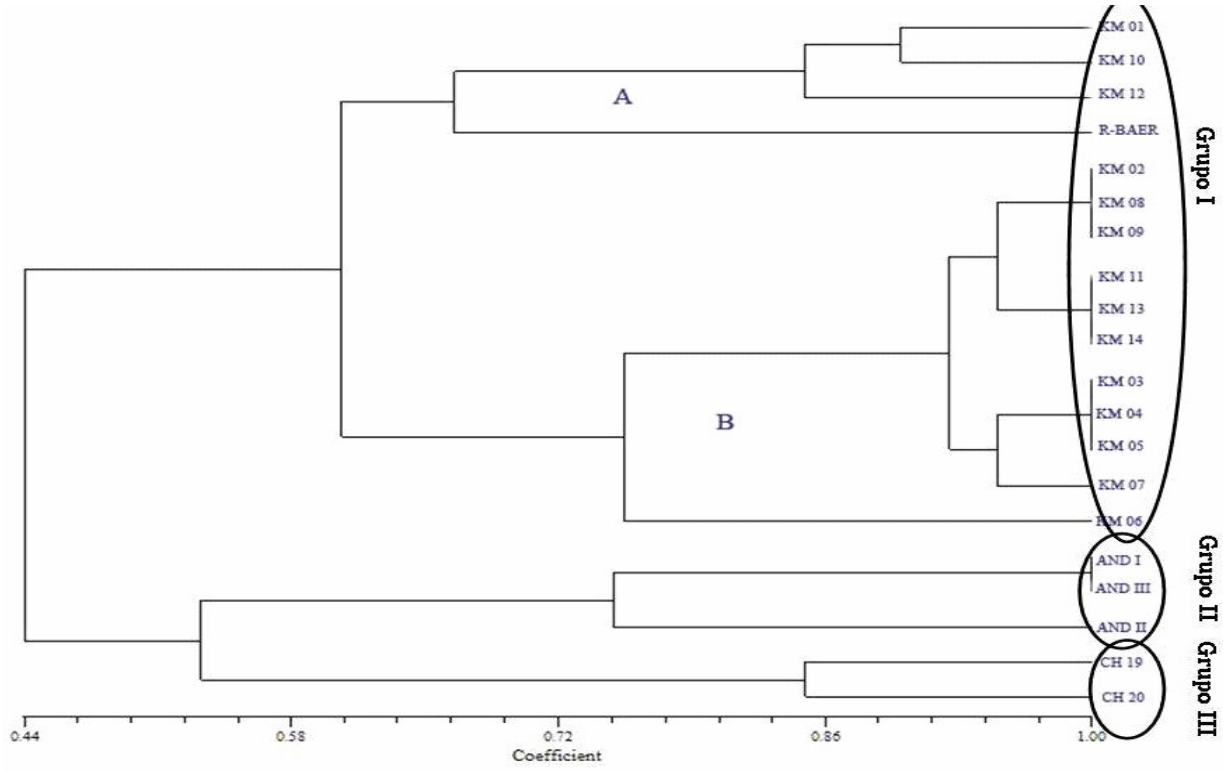

Figure 2. Cluster analysis dendrogram based on morphologic characteristics of the 20 accessions studied

Table 3. Primer combinations used and polymorphic bands generated.

Combination of Primer	Number of Bands	N° of Monomorphic bands	N° of Polimorphic bands	% of polimorphic bands
*Eco*AAG/*Mse*CAC	64	0	64	100
*Eco*ACA/*Mse*CAA	49	9	40	81.6
*Eco*ACT/*Mse*CAG	37	11	26	70.2
TOTAL	150	20	130	86.6
		13.3 %	86.6%	

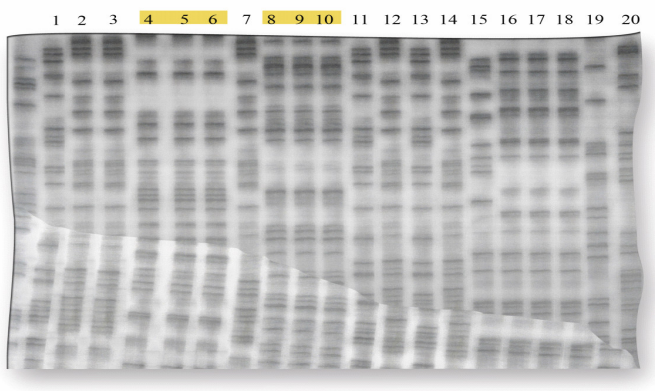

Figure 3. Polymorphisms obtained by AFLP primers EcoAAG/*Mse*CAC.

larity range between 0.54 and 0.97. The tree diagram also throws up a differentiation between the genotypes allocated to Group I, which includes the accessions from the north of Chile, with a coefficient of 0.68; and the Group II accessions, which are samples from the precordillera sector with a coefficient of 0.77. The similarity coefficient between the two groups (I and II) is 0.62. The DNA samples were amplified with10 combinations of primers, using the best three for analysis. Their products generated 150 AFLP bands, of which 130 (86.6%) were polymorphic (Table 3). The bands which presented the same electrophoretic mobility were treated as monomerphic fragments of DNA. The primer pair *Eco*AAG / *Mse*CAC contributed the highest number of bands, obtaining 100% polymorphic bands that can observe on Figure 3. These results agree with those obtained by Maughan et al. (2004), which indicate that a high level of polymorphism is obtained through analysis using AFLP markers. Moreover, in studies done on other species, AFLP markers are a powerful tool for the analysis of ge-

netic diversity and to ensure a high level of polymerphism (Kim et al., 1998). Three groups can be identified in the tree diagram (Figure 4). Group I includes eight local accessions, the commercial variety Regalona-characteristics of this group are defined by grain colour, panicle colour, panicle type differentiated and terminal, panicle density and herbaceous growth. Through DNA analysis it could be established that accessions KM13 and KM14 possess the same similarity coefficient (1.0) even though they come from different localities. Group II (Yellow shadow) consists of accessions KM04, KM08, KM09 and KM10 in addition to KM05 and KM6 which present the same similarity coefficient even though they come from different localities.

The principal characteristic of this group is that it is mainly composed of accessions with brown grain, red panicle and geographical location of samples collection at an altitude between 338 m.a.s.l. and 631 m.a.s.l., in the Baer and the Andean accessions A01, A02 and R01. The pre-cordillera zone of the Araucania (IX) and Los Rios (XIV) Regions of Chile. Group III includes the species *C. album* and *C. ambrosioides* which were used to confirm the validity of the analysis, being denominated as outgroup controls.

DISCUSSION

The first objective of this study was to analyze the molecular diversity of 14 coastal accessions of (*C. quinoa* Willd.) using AFLP markers. The characterization of this diversity has improved knowledge relative to the origin of different quinoa accessions conserved by farmers and breeders.The sub group pre-cordillera accessions are differentiated from the rest of the accessions, both from coastal and from the highland accessions, since no intimate genetic relationship exists. The results coincide with previous morphological and iso-enzyme studies, which separate quinoa into two types: a coastal type (Chile) and an Andean plateau type (Wilson 1988b; Risi and Galway 1989). This study represents a complement to the work of Ruas et al. (1999) differentiating the groups of *C. quinoa*, *C. album* and *C. ambrosioides*.

Comparative analysis between AFLP and morphological data

The morphological analysis of the Andean group places it in an independent Group (Group II), however in the AFLP analysis it appears to be integrated into Group I, indicating the existence of a similarity to the genetic material of the accessions collected in the Region. This result is consistent with the existence of common ancestral genes in the crop. The commercial variety Regalona-Baer included in the study is close to Andean eco-types from the molecular point of view, confirming the existence of parental genes originating in Andean material. The Mantel's Test was used to compare the matrices generated from the AFLP and the morphological data. This test shows a correlation between the morphological and molecular (AFLP) tree diagrams with values of $r = 0.09$, $p = 0.7962$, obtained by the MxComp method. Although positive, the concordance value is low, as observed in various other studies regarding varieties of grapes, rice, rye grass and potatoes (Xu et al., 2000; Federici et al., 2001; Roldan-Ruiz et al., 2001; Solano et al., 2007). In addition to this, Spooner et al. (2005), report that DNA digital fingerprinting techniques are a better discriminator than morphological data in the analysis of genetic similarity. Our results confirm that DNA analysis is an efficient method for the exploration of genetic diversity in quinoa populations. Although the different genotypes share a common base, the pre-cordillera group differs from the rest of the accessions from the South of Chile and from the varieties coming from the North of the country. Wilson (1988b) hypothesized that ancestral colonization of quinoa in the southern zone of Chile, followed by long periods of genetic drift, was the reason for this observed lack of genetic diversity in the Chilean highland populations. Wilson (1988a) also hypothesized that Chilean popula-tions have their origin in the southern Altiplano. This was supported by the data of Christensen et al. (2007), which showed that southern Chilean populations are more similar to Bolivian populations than other quinoas from the Andean highlands. However, the present study indicates that Chilean lowland germplasm is much more genetically diverse than previously believed. Although this observation may potentially shake Wilson's (1988a) Chilean quinoa origin hypothesis, the most observed diversity at the molecular level found in this study could alternatively be explained by promiscuous outcrossing in the lowland quinoa fields involving abundant weed populations of *C. album* and C. *hircinum*. This natural process combine with the ancestral seed exchange sys-tem, anthropic pressures on selection due to edaphocli-matic and photoperiod factors, would have generated a genetic differentiation in the varieties of the pre-cordillera sector. Molecular analysis using AFLP made it possible to establish the differences and similarities between the materials collected in the South of Chile. So far as we know, this is the first study done in Chile using AFLP markers to analyse genetic diversity in the quinoa germoplasm in the southern zone and in the country.

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