Genetic diversity of the Andean tuber-bearing species, oca (*Oxalis tuberosa* Mol.), investigated by inter-simple sequence repeats

A. Pissard, M. Ghislain, and P. Bertin

Abstract: The Andean tuber-bearing species, *Oxalis tuberos*a Mol., is a vegetatively propagated crop cultivated in the uplands of the Andes. Its genetic diversity was investigated in the present study using the inter-simple sequence repeat (ISSR) technique. Thirty-two accessions originating from South America (Argentina, Bolivia, Chile, and Peru) and maintained in vitro were chosen to represent the ecogeographic diversity of its cultivation area. Twenty-two primers were tested and 9 were selected according to fingerprinting quality and reproducibility. Genetic diversity analysis was performed with 90 markers. Jaccard's genetic distance between accessions ranged from 0 to 0.49 with an average of 0.28 ± 0.08 (mean ± SD). Dendrogram (UPGMA (unweighted pair-group method with arithmetic averaging)) and factorial correspondence analysis (FCA) showed that the genetic structure was influenced by the collection site. The two most distant clusters contained all of the Peruvian accessions, one from Bolivia, none from Argentina or Chile. Analysis by country revealed that Peru presented the greatest genetic distances from the other countries and possessed the highest intra-country genetic distance (0.30 ± 0.08). This suggests that the Peruvian oca accessions form a distinct genetic group. The relatively low level of genetic diversity in the oca species may be related to its predominating reproduction strategy, i.e., vegetative propagation. The extent and structure of the genetic diversity of the species detailed here should help the establishment of conservation strategies.

Key words: oca, *Oxalis tuberosa*, Andean tuber, genetic diversity, ISSR, vegetative propagation.

Résumé : L'espèce andine tubéreuse, *Oxalis tuberosa* Mol., est une plante cultivée dans les Andes et propagée par voie végétative. La détermination de sa diversité génétique par la technique ISSR est présentée dans cet article. Trente-deux accessions provenant d'Amérique du sud (Argentine, Bolivie, Chili et Pérou) et maintenues in vitro ont été sélectionnées pour représenter la diversité eco-géographique de son aire de culture. Vingt-deux amorces ISSR ont été testées et 9 ont été sélectionnées sur base de la qualité et de la reproductibilité des empreintes génétiques obtenues. L'analyse de diversité génétique a été réalisée avec 90 marqueurs. Les distances génétiques de Jaccard entre accessions variaient de 0 à 0,49, la moyenne étant de 0,28 ± 0,08 (moyenne ± déviation standard). Le dendrogramme (méthode UPGMA) et l'analyse factorielle des correspondances (AFC) ont montré que la structure génétique est influencée par la provenance géographique. Les deux groupes les plus distants contenaient toutes les accessions péruviennes, une seule provenant de Bolivie, aucune provenant d'Argentine ni du Chili. L'analyse par pays a montré que le Pérou présente les plus grandes distances génétiques vis-à-vis des autres pays et possède la diversité génétique la plus élevée (0.30 ± 0.08). Cela suggère que les accessions péruviennes d'oca forment un groupe génétique distinct. Le niveau de diversité génétique relativement bas chez cette espèce peut être mis en relation avec son mode de propagation prédominant c'est-à-dire la propagation végétative. Le niveau et la structure de la diversité génétique mis en évidence dans cette étude devraient pouvoir contribuer à la mise en place de stratégies de conservation de l'espèce.

Mots clés : Oca, *Oxalis tuberosa*, plante à tubercule des Andes, diversité génétique, ISSR, propagation végétative.

Introduction

Oxalis tuberosa Mol. is an Andean tuber-bearing crop species that has been cultivated since the pre-Inca times and still forms part of the staple diet of many peasant communities along the Andes. Today, oca is sporadically cultivated in all Andean countries, especially in southern Peru and Bolivia (Trognitz et al. 1998), although it is also found rang-

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ing from Venezuela to Chile. Oca is the only known octoploid ($2n = 8x = 64$) member of the *O. tuberosa* alliance group of species that comprises a polyploid series between $2x$ and $8x$ (De Azkue and Martínez 1990). The domestication of the species happened in the central region of Peru and in the north of Bolivia where the highest diversity of cultivated forms, as well as wild forms, is encountered (Arbizu and Tapia 1992). Pictograms like pottery and decorative patterns of the old highland civilization have constituted the best evidence of its original home (Hodge 1951). Two wild tuber-bearing taxa were recently identified as progenitor candidates, *Oxala picchensis* R. Knuth from southern Peru and an as yet unnamed taxon from Bolivia (Emshwiller and Doyle 2002). Cultivated forms gather together in a unique species that includes a large range of forms and colours and many indigenous cultivars exist as a result of the long history of cultivation of the species (Arbizu and Tapia 1992).

The breeding system added to the traditional farming system applied by Andean peasant communities for centuries must have been of primary importance for the oca genetic diversity which has never been clearly investigated. Indeed, the crop possesses an intact trimorphic system of genetic incompatibility (Gibbs 1976; Trognitz and Hermann 2001) and the extent of both fruit and seed production appears to be controlled mainly by the expression of the tristylous system of incompatibility (Trognitz et al. 1998). This autoincompatibility system and the consequent cross-pollination, in addition to the morphological selection applied by farmers, must have favoured the great diversity of colour and form of tubers. Moreover, sympatry of cultivated forms and related wild forms has been observed (Arbizu and Tapia 1992). Presently, oca is propagated essentially by tubers and botanical seed propagation by farmers has never been observed (Trognitz et al. 1998). This propagation mode is likely to decrease the genetic diversity since the genetic recombination is avoided. However, the coexistence of different cultivars in the same field or in the neighbouring fields is a common practice to develop a defensive versatility against climate changes, blights, and various environmental conditions. Local cultivars are also conserved by the cultivation of each one in a range of microclimatic conditions (Terrazas and Valdivia 1998). These agronomic practices and cultivation conditions are favourable to genetic exchanges by potential cross-pollination, which could contribute sporadically to the heterozygosity of the cultivated *Oxalis tuberosa* germplasm (Gibbs 1976).

ISSR (inter-simple sequence repeat) amplification uses anchored or non-anchored simple-sequence repeat (SSR) primers to amplify DNA sequences between two inverted SSRs made up of the same sequence. It is known to differentiate between closely related individuals. Some of the advantages of this technique are that there is no need for previous knowledge of the genome sequence and that it leads to multilocus patterns. ISSR markers are known to be more reproducible than RAPD markers because of the longer length of their primers (Bornet and Branchard 2001). They can also detect more diversity than RAPD markers (Esselman et al. 1999) and have been successfully applied to the study of genetic diversity in plants such as corn (Kantety et al. 1995), potato (Bornet et al. 2002; Provan et al. 1996; Prevost and Wilkinson 1999), and rice (Blair et al. 1999).

The impact of the reproduction mode, cultural practices, and the relative marginalisation of the oca species on its genetic diversity are not known. The determination of its genetic diversity and its evolution are necessary steps for establishing appropriate conservation strategies. The purpose of the present study is to develop the ISSR technique on the oca genome to assess its genetic diversity.

Materials and methods

Plant materials

Plant materials were obtained from the in vitro germplasm collection of CIP (International Potato Center, Lima, Peru). Thirty-two oca accessions collected from different Andean countries (9 from each Argentina, Bolivia, and Peru and 5 from Chile) were examined (Table 1). The accessions were chosen according to their collection site (country, latitude, longitude, and altitude) to represent the ecogeographic diversity of its cultivation area (Fig. 1). In each country, as many regions as possible were sampled and, within each region, accessions coming from different altitude areas were chosen. Since oca is cultivated widely in Peru, in several regions in Bolivia (Lake Titicaca; Cochabamba), and in small areas in Argentina and Chile (Altiplano region), the apparent unequal distribution of the sampled accessions between and among countries is the consequence of the geographical and agronomic reality.

DNA extraction

DNA was isolated from fresh leaflets tissue in the CIP laboratory. Several leaflets from two plants of each accession were ground with liquid nitrogen. The resulting powder was immediately transferred to a microfuge tube with 700 µL extraction buffer (100 mmol/L Tris–HCl (pH 8.0), 2 mol/L NaCl, 2% cetyltrimethylammonium bromide (CTAB), 2% polyvinyl pyrollidone (PVP), 20 mmol/L EDTA (pH 8.2), 1% β-mercaptoethanol) and mixed vigourously. After 40 min incubation at 65 °C, 600 µL of chlorophorm – isoamyl alcohol (24:1) was added. After mixing, it was centrifuged at 14 000 r/min for 10 min at room temperature. The aqueous phase was recovered in a new Eppendorf tube. The extraction step with chlorophorm – isoamyl alcohol and the centrifugation were repeated. After addition of 550 µL isopropanol at –4 °C, the DNA was left to precipitate for 15 min at –20 °C, centrifuged at 14 000 r/min for 10 min and then washed twice with 500 µL ethanol (70%). Finally, the DNA was dissolved in 150 µL TE (10 mmol/L Tris–HCl, 1 mmol/L EDTA (pH 8.0)). After addition of 1 µL RNAse, DNA concentration and quality were estimated by running 1 µL sample on 1% *w*/*v* agarose gel in TBE buffer along with the lambda DNA standards. The DNA was stored at –20 °C until use.

PCR conditions

Twenty-two anchored ISSR primers (taken from the literature or previously unpublished) were tested for amplification (Table 2). To optimize the amplification reaction, several PCR parameters were tested: DNA quantity (5 ng, 10 ng, and 50 ng), primer concentration (0.4 µmol/L and 0.6 µmol/L), dNTPs concentration (80 µmol/L and 200 µmol/L), and $MgCl_2$ concentration (1.5 mmol/L and

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Table 1. Accessions of *Oxalis tuberosa* used in this study according to their country, geographical position (latitude, longitude, and altitude), and locality of the collection site.

ID No.	Original No.	Country	Latitude (°S)	Longitude (°W)	Altitude (m)	Locality
1	CIP 202052	Peru	5.18	80.61	30	Piura
2	CIP 202005	Peru	6.55	78.73	2850	Calmarca
3	CIP 202003	Peru	7.15	78.50	3100	Chamis
4	CIP 202442	Peru	7.98	78.55	3560	Motil
5	CIP 202081	Peru	9.25	77.41	4000	Poncos
6	CIP 202049	Peru	12.23	75.98	3200	Tintin
7	CIP 202028	Peru	13.37	71.81	3820	Amaru
8	CIP 202235	Peru	16.46	69.10	3850	Ch'imu Alto Ayriwa (Zepita)
9	CIP 202251	Peru	15.88	69.85	3850	Añu Callejón (Platería)
10	CIP 202180	Bolivia	15.83	69.00	3830	Quilima
11	CIP 202303	Bolivia	17.30	65.75	3200	Tiraque
12	CIP 202219	Bolivia	17.73	65.20	2800	Totora
13	CIP 202192	Bolivia	18.90	66.76	3750	Challapata
14	CIP 202199	Bolivia	19.31	65.91	3800	Huancarani
15	CIP 202206	Bolivia	19.41	65.73	3600	Manquiri
16	CIP 202216	Bolivia	19.60	65.40	3650	Lagunillas
17	CIP 202211	Bolivia	19.60	65.66	4070	Potosi (Manquiri)
18	CIP 202203	Bolivia	19.81	66.10	3850	Sacasaca
19	CIP 202228	Chile	18.38	69.53	3300	Chapiquina
20	CIP 202229	Chile	18.46	69.51	3550	Belén
21	CIP 202224	Chile	22.33	68.21	2550	Caspana
22	CIP 202225	Chile	22.33	68.21	2250	Caspana
24	CIP 202160	Argentina	22.25	64.93	2500	San Felipe
25	CIP 202153	Argentina	22.20	64.93	2850	El Puesto
26	CIP 202155	Argentina	22.11	64.86	2900	Mecoyita
27	CIP 202146	Argentina	22.28	65.35	3900	Chalguamayoc
28	CIP 202164	Argentina	22.33	65.08	3800	Campo La Paz
29	CIP 202149	Argentina	22.26	64.96	2500	Acoite
30	CIP 202167	Argentina	22.21	64.98	2650	La Huerta
31	CIP 202173	Argentina	22.98	65.20	3800	Colanzuli
32	CIP 202136	Argentina	23.60	65.33	3300	Estancia Grande

2.5 mmol/L). The annealing temperature was also optimized by testing several temperatures including the theoretical annealing temperature (i.e., 5 °C below the melting temperature). The best conditions were retained according to the intensity and sharpness of the bands.

Amplification reactions were performed in a 10 µL volume containing 10 ng genomic DNA, 80 µmol/L of each dNTP (Amersham Bioscience Corp., Piscataway, N.J.), 0.4 µmol/L primer (Eurogentec SA, Seraing, Belgium), 1U *Taq* polymerase (Amersham Bioscience Corp.), and 1× *Taq* DNA Poly buffer (Amersham Bioscience Corp.). The PCR amplifications were performed using a PTC 100 thermal cycler (MJ Research, Waltham, Mass.) according to the reaction conditions outlined below. For the primers Nos. 1–5, a denaturation step of 10 min at 95 °C; 35 cycles of 30 s at 95 °C, 45 s at T_a (annealing temperature, empirically determined; Table 2), and 2 min at 72 °C; and a final elongation step of 5 min at 72 °C were applied. For the other primers, a denaturation step of 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 45 s at T_a, and 2 min at 72 °C; and a final elongation step of 4 min at 72 °C were applied.

Electrophoresis and data analysis

PCR products were analysed by electrophoresis using 1.5% *w/v* agarose gel carried out at a constant voltage of 90 V for 210 min. A DNA ladder (Smartladder, Eurogentec SA) was used in each electrophoresis gel as a molecular mass marker. Gels stained with ethidium bromide were visualized under UV light and recorded with a video image analyser (Biocapt, Vilbert–Lourmat, Marne-La-Vallée, France). The patterns were analysed using the Gene Profiler software (Scanalytics, Inc.) and only clearly scorable and reproducible bands on multiple independent runs were considered. The intensity of the bands was not taken into account for the general scoring. They were scored qualitatively as present (1) or absent (0) and both polymorphic and monomorphic bands were included in the final data sets. All patterns obtained from the same primer were compiled in the database manager of Gene Profiler. However, the band positions had to be adjusted manually by modifying the match tolerance applied or band positions on the analysed gel images, since the compilation of many patterns changed the relative positions of bands and consequently the different bins created.

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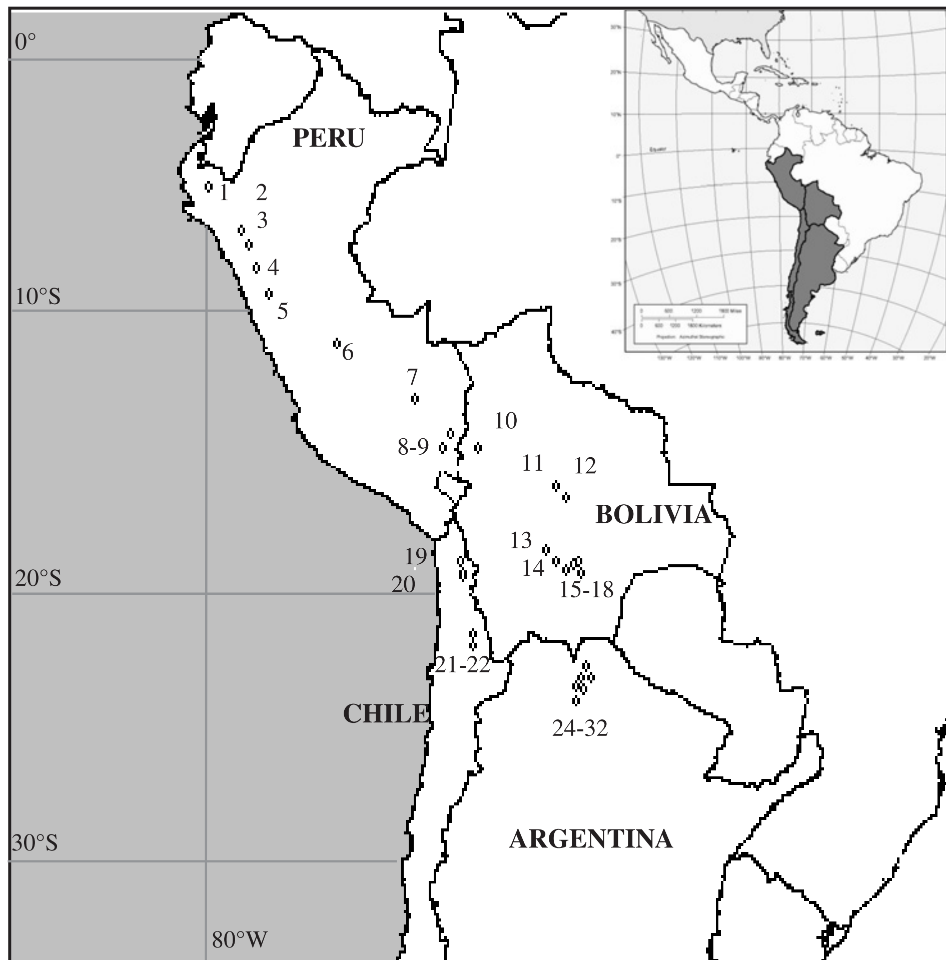
Fig. 1. Distribution of the 32 oca accessions (represented by points) used for the analysis of genetic diversity. Accessions Nos. 1 to 9 are Peruvian; Nos. 10 to 18, Bolivian; Nos. 19 to 23, Chilean; and Nos. 24 to 32 are Argentinian.



Finally, binary data from different primers were gathered to perform the analysis.

A polymorphism index was calculated for each primer to evaluate the efficiency of primers to distinguish the genotypes. The PI (polymorphism index) value is defined as

$$\sum[1-(p_i^2+a_i^2)]$$

where p_i and a_i are, respectively, the presence and absence frequencies of the ith allele (band).

A factorial correspondence analysis (FCA) based on the ISSR markers was performed using the Genetix software package. The genetic distances among accessions (Jaccard's coefficient) were calculated and a dendrogram was generated using the UPGMA (unweighted pair-group method with arithmetic averaging) cluster analysis with Treecon software (Van de Peer and De Wachter 1994). Different primer data sets were tested and bootstrap analysis was performed to test the reliability of the trees obtained. Additionally, the "goodness of fit" of the clustering of the data matrix was determined by calculating the cophenetic correlation coefficient between the dissimilarity matrix and the cophenetic matrix. Its significance was evaluated by a Mantel test (Mantel 1967).

Geographical data of the accessions collection site (latitude, longitude, and altitude) were compared with the genetic data to highlight some correspondence. Two geographical distances matrices were designed based on the latitude–longitude position and on the altitude level. Mantel tests were performed to determine the correlation between geographical and genetic distances matrices.

Results

Optimization of PCR conditions

PCR amplification was optimal with primer concentration of 0.4 µmol/L and dNTPs concentration of 80 µmol/L. The quantities of 5 ng or 10 ng DNA gave similar results and were better than the results provided by 50 ng of DNA. The annealing temperature was the most important parameter on the quality pattern and had to be determined empirically for each primer (Table 2). In some cases, the optimal annealing temperature appeared to differ from the theoretical annealing temperature.

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Table 2. ISSR primers tested, melting temperature (T_m), annealing temperature (T_a) determined empirically, total number of bands, number of polymorphic bands, and primer polymorphic index (PI).

Primer No.	Sequence (5′→3′)	Reference	T_m (°C)	T_a (°C)	Total no. of bands	No. of Polymorphic bands	PI
1	BDB-(ACA)5	McGregor et al. 2000	50	—	—	—	—
2	DD-(CCA)5	" "	56	52	—	—	—
3	DHB-(CGA)5	" "	58	53	8	6	1.84
4	VHV-(GT)7-G	" "	56	51	14	8	2.38
5	DBD-(AC)7	" "	50	—	—	—	—
6	BDB-(CAC)5	Prevost and Wilkinson 1998	60	55	10	0	0
7	(AG)8-YT	" "	52	47	9	6	1.77
8	(GA)8-YC	" "	56	51	6	4	1.18
9	(AC)8-G	" "	52	47	7	0	0
10	(AC)8-YG	" "	56	51	—	—	—
11	(AG)8-T	" "	50	50	10	7	2.22
12	(GA)8-A	" "	50	50	9	8	1.78
13	(GA)8-C	Joshi et al. 2000	52	50	11	11	3.18
14	(GATG)4-C	" "	52	—	—	—	—
15	VHV-(GTG)5	" "	60	—	—	—	—
16	DDC-(CAC)5	" "	60	55	8	0	0
17	(GT)8-C	Previously unpublished	52	—	—	—	—
18	(GT)8-YC	" "	56	47	10	2	0.7
19	(TG)8-A	" "	50	—	—	—	—
20	(CA)8-G	Pasqualone et al. 2000	52	—	—	—	—
21	(CCA)8-YA	" "	52	49	13	10	3.25
22	(GACA)4	" "	48	49	—	—	—

Note: Degenerate primers are mixed PCR primers where B = G, T, or C; D = G, A, or T; H = A, T, or C; and V = G, A, or C.

Among the 22 primers screened, 8 did not produce fragments or gave only faint bands or smears (Nos. 1, 2, 5, 14, 15, 17, 19, and 20). Three primers (Nos. 6, 9, and 16) did not produce polymorphic patterns and two primers (Nos. 10 and 22) did not give reproducible bands. These primers were discarded (Table 2). All primers containing the AG or GA repeat produced clear and polymorphic fingerprints. Conversely, primers with the CAC or AC motif were more problematic: they produced patterns with numerous bands of weak intensity or patterns with no polymorphic bands and were thus unsuitable for the analysis.

Anchor position did not seem to influence the banding patterns. The electrophoresis gels obtained with 5′- or 3′-anchored primers did not present any difference in terms of reproducibility and band interpretation. Only the nucleotidic sequence of the primer and anchor seemed to have an influence. Patterns obtained with GT, TG, and GTG repeats differed according to the anchor used. A difference of one nucleotide in the anchor sequence could result in completely different patterns. That was the case with primer $(GT)_8C$ and $(GT)_8YC$. The first produced only smears and weak bands unlike the second, which resulted in a clear and multiloci pattern. Likewise, some double-anchored primers produced a smaller number of fragments than single-anchored ones (primers Nos. 7 and 8 compared with primers Nos. 11 and 13) (Table 2).

Polymorphism revealed with ISSR–PCR

The bands amplified by each primer differed in number and in intensity, with amplified fragment size ranging from 200 to 1500 bp. Amplifications using the 9 primers selected generated a total of 90 reliable and reproducible bands, of which 62 (68.8%) were polymorphic. Polymorphic band numbers varied from 2 to 11 per primer and the total number of bands scored ranged from 5 to 14 per primer (Table 2). These markers allowed one to distinguish all accessions from each other except two, accessions Nos. 19 and 20 (Figs. 2 and 3).

The informative quality of the primers in terms of polymorphism revealed by the PI values was variable, ranging from 0.7 to 3.25. The total number of bands was neither significantly correlated to the number of polymorphic bands nor to the PI values (r = 0.56 and 0.63, respectively; P < 0.001). In contrast, the number of polymorphic bands and the PI values were highly and significantly correlated (r = 0.96, P < 0.001).

Genetic diversity

The FCA realized with the ISSR markers revealed some genetic relationships between the accessions used in this study (Fig. 2). The first three axes accounted for 40% of the variation observed (respectively 17.23%, 12.95%, and 9.70% of the variation) and the first axis separated the accessions in two groups. The first group was composed of the Peruvian accessions (group A), which were distributed widely along the axis. A Bolivian accession (accession No. 10) clustered with the Peruvian ones and wa situated close to the second group composed of the Argentinian, Bolivian, and Chilean accessions ranging along the second axis (group B). The

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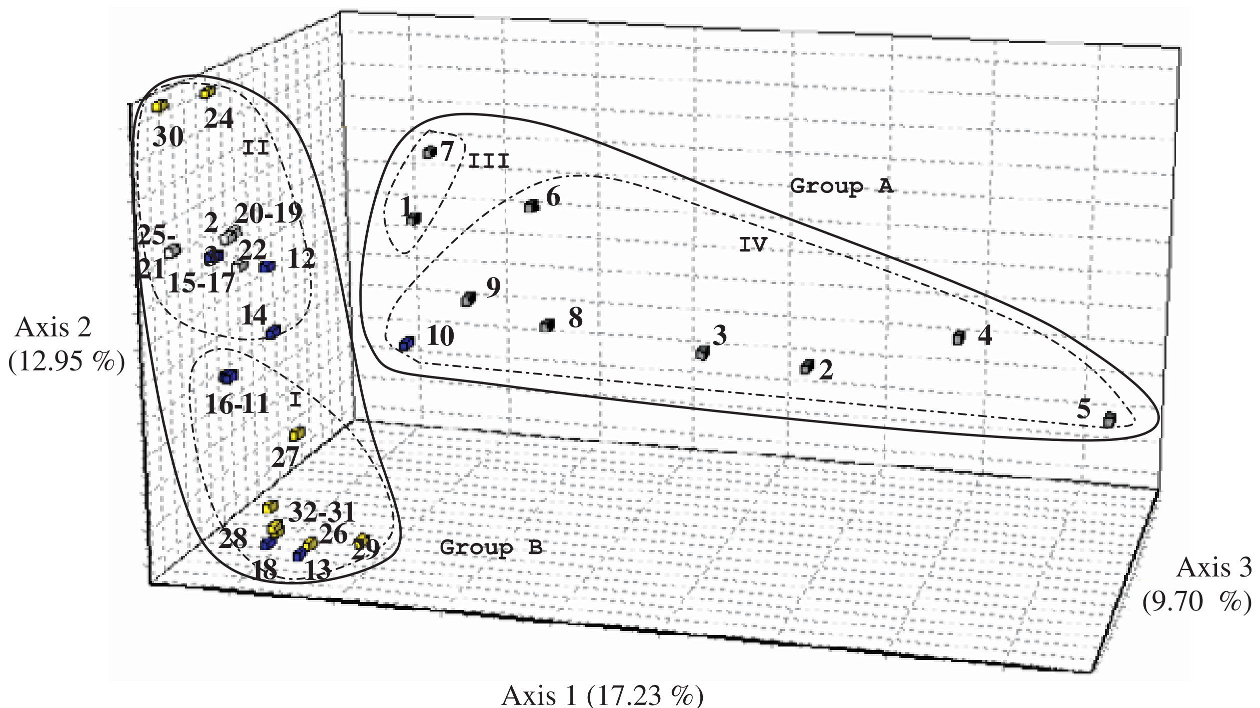
Fig. 2. Factorial correspondence analysis plot of the 32 oca accessions. Accessions Nos. 1–9 are Peruvian; Nos. 10–18, Bolivian; Nos. 19–23, Chilean; and Nos. 24–32 are Argentinian. The dotted circles (I–IV) refer to the corresponding clusters in the UPGMA dendogram (see also Fig. 3), whereas the full circles show the two main groups gathered from the axes of the factorial correspondence analysis.



Chilean accessions were gathered among the Argentinian and Bolivian ones, which formed mixed clusters.

The same genetic relationships between accessions were observed with the UPGMA clustering method. The dendrogram divided the 32 accessions into 4 clusters (Fig. 3). The first cluster showed the lowest pairwise genetic distances and exclusively contained accessions from Argentina and Bolivia (cluster I). The second cluster contained all the Chilean accessions analyzed and all the remaining accessions from Argentina and Bolivia except a Bolivian one (accession No. 10). The two remaining clusters were the most distant and showed the highest pairwise genetic distances (cluster III and IV). They exclusively contained all the accessions from Peru, except one from Bolivia (accession No. 10). However, this last accession originated from the lake Titicaca region, bordering Peru. The observation of the genetic structure inside each cluster did not permit the revealing of any geographical organization by region source (department or locality) (Figs. 1 and 3). Genetic relationships revealed by clustering and FCA were very concordant: group A of the FCA contained clusters III and IV and group B contained clusters I and II (Fig. 2).

To check the reliability of these results, the dendrogram was compared with trees obtained through the stepwise elimination of primers. The random removal of 1–4 primers did not affect the general structure of the tree and only slight modifications appeared gradually with the increasing take off of primers. A few primers (5) were sufficient to initiate the clustering by country of the collection site but the use of more primers permitted the elucidation of the structure and the acquisition of bootstrap values reaching 99% with the set of 9 primers. Cophenetic coefficient indicated a significant correlation of the clustering with the primary genetic distance matrix (r = 0.85, P < 0.001). The consistency of the generated dendrograms, the increasing bootstraps values, and the cophenetic coefficient value strongly suggested that our final markers system was reliable.

The Mantel test revealed that the geographical distance matrix based on the latitude and longitude data was significantly correlated to the genetic distance matrix (r = 0.516, P < 0.001), but no correlation was found between the geographical distance matrix based on the altitude level and genetic distance matrix (r = 0.098, P = 0.307).

The analysis performed with the 9 primers generated pairwise genetic distances ranging from 0.01 to 0.49. The average genetic distance between all accessions was 0.28 ± 0.006 (mean ± SD). To test the relationship between the genetic distance and the country of the collection site, the mean genetic distance was calculated between and among countries. The same 62 markers were used to analyze separately the Argentinian, Bolivian, Chilean, and Peruvian accessions. Pairwise genetic distances were calculated between accessions of the same country (intracountry distance) and between accessions of different countries (intercountry distance) (Table 3). Among the four countries, Peru presented the greatest intracountry average genetic distance (0.30) compared with Argentina, Bolivia, and Chile (0.23, 0.24, and 0.23 respectively). The greatest pairwise genetic distances reached 0.32, 0.35, and 0.39 for the Chilean, Bolivian, and Argentinian accessions (between accessions Nos. 20 and 21, 10 and 17, 24 and 29, respectively), whereas it reached 0.49 for the Peruvian accessions (between acces-

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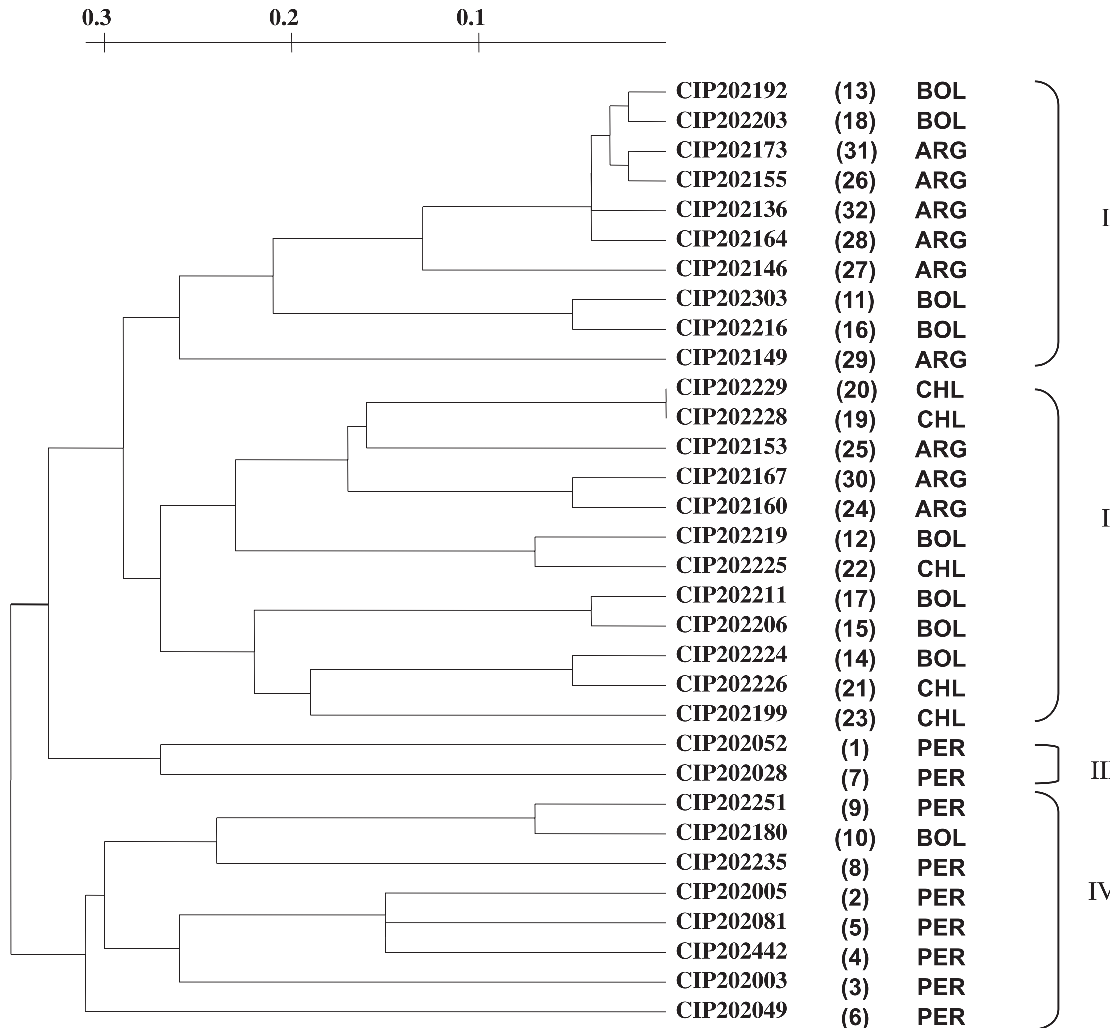
Fig. 3. Dendrogram (UPGMA method) of 32 oca accessions grouped according to collection site. The scale bar on the top of the figure represents the pairwise genetic distances between accessions (Jaccard's distance). See Table 3 for abbreviations of countries. Numbers between brackets refer to the accession description (Table 1) and localization on the map (Fig. 1).



sions Nos. 5 and 7). The intercountry genetic distances between Argentina, Bolivia, and Chile ranged from 0.24 to 0.27, which is equivalent or slightly greater than the intracountry genetic distances of the same countries. The greatest intercountry genetic distances were observed between Peru and the other three countries.

Table 3. Pairwise genetic distances (mean ± SD) between accessions within a country and between countries.

	ARG	BOL	CHL	PER
ARG	*0.23±0.12*			
BOL	0.24±0.08	*0.24±0.07*		
CHL	0.27±0.05	0.27±0.05	*0.23±0.11*	
PER	0.34±0.05	0.34±0.06	0.36±0.05	*0.30±0.08*

Note: ARG, Argentina; BOL, Bolivia; CHL, Chile; PER, Peru. Italics indicate pairwise genetic distances between accessions within a country.

Discussion

Amplification of ISSR markers

ISSR markers are easy to apply once the specific conditions have been determined. As already observed by Huang and Sun (2000), the annealing temperature seems to be the key parameter determining pattern quality and reliability. Optimization of the primers does not seem to be affected by the length of the repeat unit (2 or 3), since more than 70% of primers were optimized in both cases. The two tetranucleotide motifs tested did not give scorable patterns. In agreement with other studies, both di- and trinucleotide repeats are very useful for amplifying polymorphic bands (Barth et al. 2002). Primers with AG and GA repeats are more efficient than those containing a combination of nucleotides A and C. The AC and CAC repeats, in particular, produce patterns with many undiscernable bands presenting no (or very few and very doubtful) polymorphism. These motifs seem to be abundant in the oca genome. Such a result is in agreement with different ISSR studies on plant genomes (Bornet et al. 2002; Provan et al. 1996; Ruas et al. 2003). The anchor position did not show any influence on the number of amplified bands or their size in opposition to some previous surveys (Fang et al. 1997; Sankar and Moore 2001). However, the number of anchored nucleotides may influence pattern quality. This has been already observed

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and may be related to the more restrictive amplification conditions (Martín and Sánchez-Yélamo 2000).

Polymorphism of ISSR markers

As already demonstrated, the ISSR technique can reveal a high percentage of polymorphism per primer. The amplification of microsatellite areas with 5′-anchored primers should permit the acquisition of co-dominant markers and reveal polymorphism in repeated regions. However, it is likely that most of the small size variations of repeated regions are not scorable on agarose gels or lead to unmanageable fingerprints, as observed for some primers. Consequently, it must be considered that the ISSR technique generally provides dominant markers that reveal the same kind of polymorphism as RAPD markers. Because SSR sequences are the binding sites for ISSR primers, their distribution across the genome is important. They may be concentrated or distributed randomly on the chromosomes, so that the potential use of this marker depends on the variety and frequency of microsatellites, each of which changes with the species and with the targeted SSR motifs (Morgante and Olivieri 1993). An uneven distribution could explain an unsuitability of this marker to highlight the genetic diversity, as was shown for *Arabidopsis thaliana* (Barth et al. 2002). Since this is the first study on oca diversity based on molecular markers, no comparison is possible. However, the coherence of the dendrogram, the FCA, and the geographical data strongly support the reliability of the marker system used in this study.

In ISSR studies such as this, some of the amplified fragments are not scored owing to their weak intensity and probably also because of the low resolving power provided by agarose gels (Liu and Wendel 2001). Moreover, ISSR tends to over-emphasize differences between closely related populations and to attribute less variation to differences over larger geographical distances (Nybom 2004). Therefore, one should refer mainly to the grouping pattern and not the genetic values presented, which could be slightly biased.

Genetic diversity of the oca

Compared with other ISSR studies, the level of genetic diversity in the oca gene pool (0.28) seems relatively low. Indeed, the Argentinian cultivated potatoes possessed a minimum genetic distance between cultivars of 0.38 (Bornet et al. 2002) and the average genetic dissimilarity between rice cultivars was 0.45 (Blair et al. 1999). However, a level of genetic diversity revealed by ISSR similar to that of oca has been demonstrated for *Calamagrostis porteri* subsp. *insperata*, which presents the same breeding characteristics (vegetative propagation, auto-incompatibility, rare sexual reproduction given the infrequent flowering) (Esselman et al. 1999). The low level of genetic diversity may be related to the dominating vegetative reproduction preventing any genetic recombination from meiosis. However, occasional new genotypes may be created by somatic mutation, putative outcrossing between cultivated forms or gene flow with wild forms. Furthermore, they may be maintained and propagated by tubers given that the peasant farmers possess a detailed knowledge of their crop and preserve its diversity from year to year, identifying and conserving new forms which may occasionally occur (Ortega 1997). Under a traditional farming system where mixing several genotypes is common (Terrazas and Valdivia 1998), plants originating from unmanaged sexual reproduction could be incorporated into recognized cultivars and so, into the material of propagation like it was already assumed by Gibbs (1976) and confirmed for potato in the Andes (Ortega 1997) and for cassava (Elias et al. 2001). If sexual reproduction is quite rare today, it may have been more common or possibly the dominating reproduction mode once (Gibbs 1976). This author suggested that the first cultivated *Oxalis tuberosa* would have been an outbreeding species and hence one with a high level of heterozygosity from which the rich variety of tuber types could be culled and fixed by preferential selection. With continued asexual propagation by means of tubers, the rate of cultivar diversification could be expected to slow down owing to genetic erosion. If this is the case, the genetic diversity revealed here could represent diversity from the eventual new genotypes in addition to the relictual diversity.

Results obtained with clustering and FCA suggest that ISSR markers are able to distinguish cultivated genotypes according to their collection site. Indeed, all of the Peruvian accessions were clearly separated from the others. However, accessions from Bolivia and Argentina clustered together with the Chilean group. This may be explained by the relative proximity of oca cultivation areas and the great germplasm movements occurring in these regions. Indeed, Chilean and Argentinian oca are cultivated exclusively in the northern regions of these countries, near the Bolivian border. Likewise, a Bolivian accession originating from Quilima (accession No. 10) clusters together with the Peruvian group. This is not surprising, since this locality is situated in the north of Bolivia near the Peruvian border. The correlation between genetic distances and geographical coordinates (latitude and longitude) supports the fact that the genetic structure of oca is influenced by the geographical distance between cultivation areas.

The genetic relationships between oca accessions (Figs. 2 and 3) show that the Peruvian accessions formed the most distant clusters. Moreover, the genetic diversity observed inside Peru is slightly greater than that found in other Andean countries. Consequently, the Peruvian germplasm appears to constitute a distinct group of greater genetic diversity. This could support the hypothesis advanced by different sources that oca's domestication happened in the regions of Peru and north of Bolivia (Arbizu and Tapia 1992; Hodge 1951; Emshwiller and Doyle 2002).

This study has developed ISSR markers on oca that have been used to demonstrate a relatively low level of genetic diversity and to establish the genetic structure of the species across the Andes. Further investigations are necessary to confirm these first results and to elucidate the evolution of the species, which could be considered a genetic model of a vegetatively propagated crop influenced by human selection and cultural practices. This could help to define accurate strategies for the conservation of genetic resources on a large scale in the Andes, i.e., in situ conservation strategies in diverse eco-geographic environments.

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