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Congruence between morphological and molecular markers inferred from the analysis of the intra-morphotype genetic diversity and the spatial structure of *Oxalis tuberosa* Mol.

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Abstract *Oxalis tuberosa* is an important crop cultivated in the highest Andean zones. A germplasm collection is maintained ex situ by CIP, which has developed a morphological markers system to classify the accessions into morphotypes, i.e. groups of morphologically identical accessions. However, their genetic uniformity is currently unknown. The ISSR technique was used in two experiments to determine the relationships between both morphological and molecular markers systems. The intra-morphotype genetic diversity, the spatial structures of the diversity and the congruence between both markers systems were determined. In the first experience, 44 accessions representing five morphotypes, clearly distinct from each other, were analyzed. At the molecular level, the accessions exactly clustered according to their morphotypes. However, a genetic variability was observed inside each morphotype. In the second experiment, 34 accessions gradually differing from each other on morphological base were analyzed. The morphological clustering showed no geographical structure. On the opposite, the molecular analysis showed that the genetic structure was slightly related to the collection site. The correlation between both markers systems was weak but significant. The lack of perfect congruence between morphological and molecular data suggests that the morphological system may be useful for the morphotypes management but is not appropriate to study the genetic structure of the oca. The spatial structure of the genetic diversity can be related to the evolution of the species and the discordance between the morphological and molecular structures may result from similar selection pressures at different places leading to similar forms with a different genetic background.

Keywords Genetic diversity · ISSR markers · Morphotype · oca · *Oxalis tuberosa* · Spatial structure

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Introduction

Oca (*Oxalis tuberosa* Molina) is a crop cultivated in the highest zones of the Andes. This starchy tuber crop provides, in association with other tuber species, a varied diet for the local population. Although the phenotypic diversity of oca is known to be high, the genetic diversity of the species has been poorly investigated until recently and appears to be quite low (Pissard et al. 2006). This low level may be related to the dominating vegetative reproduction. Indeed, oca is propagated essentially by tubers and botanical seed propagation by farmers has never been observed (Trognitz et al. 1998). However, the species is able to reproduce sexually, at least in controlled conditions, and possesses an intact system of heteromorphic stylar incompatibility which mainly controls the extent of both fruit and seed productions (Gibbs 1976; Trognitz et al. 1998, 2000). Although hybridization in fields has never been demonstrated, cropping practices such as the coexistence of different varieties in the same field or in the neighbouring fields (Terrazas and Valdivia 1998) and the sympatry of both cultivated and related wild forms (Arbizu and Tapia 1992) are favorable to genetic exchanges by

potential cross-pollination. Consequently, occasional new genotypes of oca may be created by putative outcrossings, which could contribute sporadically to the heterozygosity of the cultivated *Oxalis tuberosa* germplasm (Gibbs 1976).

The morphological diversity of the tubers is high, particularly its pigmentation that varies from white to purple with a variable color distribution according to the different varieties. The local names given by the peasants reflect the external appearance of the tubers and subsequent uses (Bianco and Sachs 1998; Ramirez 2002). Peasants recognize advantages and disadvantages of their local varieties for production, food, culinary uses and medicinal properties. Moreover, the farming of many varieties contributes to preserve the harvest from major crop losses due to new pest strains or extreme abiotic stresses (Terrazas and Valdivia 1998; Cadima et al. 2003). The knowledge of the local agricultural system transmitted through the generations including technical information about the products and the agricultural practices has contributed to the conservation of this morphological diversity. Nevertheless, the increase of the rural depopulation in the Andean region endangers the traditional farming system which maintains and uses the crop diversity. A reduction of the number of oca varieties has been observed between 1980 and 1990 and has been attributed to an increase in the production of a small number of commercial varieties. This tendency to reduce the diversity is associated with the loss of traditional knowledge about the uses of oca biodiversity (Terrazas and Valdivia 1998). In this context, a better understanding of the morphological and genetic diversity of the oca crop is essential to develop conservation strategies appropriate to this species.

More than 400 Peruvian accessions of oca are maintained in the ex situ collection of CIP (International Potato Center, Peru). The passport descriptors that have been devised for ex situ conservation (Ford-Lloyd and Maxted 1997) provide the basic information used for the general management of the accessions. These parameters, that are usually recorded when the accession is originally collected, are available for a great part of the oca accessions. After their collection, the accessions have been maintained in fields for many years and a great number of morphological characters have been recorded. The stable morphological characters have been retained to describe the accessions (IPGRI/CIP 2001). This morphological markers system allowed to classify the accessions into morphotypes, i.e. groups sharing all the same morphological characters (Arbizu et al. 1997). The considerable morphological diversity present in the oca has lead to the definition of 240 morphotypes in 2003 (C. Arbizu, unpublished results). Although the use of morphological and other field measurements is extremely valuable, it may suffer from several disadvantages. Indeed, it is an indirect method for the measurement of the genetic diversity, the environmental conditions may influence plant performances for many morphological traits, and finally, the number of morphological characters that can be reasonably measured in field trials is relatively small. Nevertheless, morphological markers still play a central role in the analysis of variation in crop species because their use does not require sophisticated technology and some of them are of clear agronomic importance (Newbury and Ford-Lloyd 1997). In addition to this information, molecular techniques can provide very accurate assessments of the quantity of variation within populations (Ford-Lloyd and Maxted 1997). Moreover, the determination of genetic relationships in genebank is an important aspect of genetic resources management (van Treuren et al. 2004). Molecular marker data can be used in association with passport data and morphological analysis to reduce the redundancy in the collection and consequently, the maintenance cost.

As vegetatively propagated species cultivated in traditional farming systems have been poorly studied until now, the study of the oca diversity aims to contribute to better understand the evolution of this kind of crop. The specific aim of this study is to determine the relationships between two different markers systems used to characterize the oca germplasm: the morphological and genetic markers. The intra-morphotype genetic diversity, the spatial structures of the morphological and molecular diversity and finally, the congruence between both markers systems will be determined.

Materials and methods

Plant materials

The plant materials were obtained from the ex situ germplasm collection of CIP (International Potato Centre, Lima, Peru). Accessions classified into morphotypes on the basis of the morphological markers system as previously described have been used. The first experiment was designed to study the intra-morphotype genetic diversity. A total of 44 accessions, representing five morphotypes, each of which including from six to ten accessions, were analyzed (Tables 1 and 2). The morphotypes were selected according to their morphological characters, determined in fields in 2003, in order to maximize the inter-morphotypes diversity. All accessions belonging to a same morphotype always arose from the same or close collection sites. The second experiment was designed to investigate the spatial structures of the morphological and genetic diversity and to evaluate the congruence between morphological and molecular markers. A sample of 34 accessions, constituting 34 distinct morphotypes and coming from the different

Table 1 The five morphotypes (44 accessions) of oca (*Oxalis tuberosa*) analyzed by ISSR markers in order to determine the intra-morphotype genetic diversity: cultivar name, department, region, locality and geographical position (latitude and longitude) of the collection site

Morphotype no.	COL no.	CIP no.	Cultivar name	Department	Region	Locality	Latitude (°S)	Longitude (°W)
1	COC 048	202462	–	–	–	–	–	–
1	COC 047	202461	–	–	–	–	–	–
1	UNC O 226	202581	Oca blanca	–	–	–	–	–
1	COC 180	202478	–	–	–	–	–	–
1	COC 423	202490	–	–	–	–	–	–
1	MU 003	202366	Janq'u qhiñi	Puno	Chucuito	Tuntachawi Yaqari	16.20	69.47
1	MU 004	202367	Janq'u quiñi	Puno	Chucuito	Qaxi (Caje)	16.22	69.45
1	MU 007	202368	Janq'u quiñi	Puno	Yunguyo	Cuturapi - Samsuni	16.27	69.17
1	MU 010	202369	Janq'u quiñi	Puno	Yunguyo	Chambi Kimsacruz-Ch'ujña Ch'uj	16.27	69.07
1	MU 012	202371	Janq'u quiñi	Puno	Yunguyo	Chikani Uma - Santuku uma	16.32	69.05
24	COC X 34	202519	–	–	–	–	–	–
24	COC 540	202498	–	–	–	–	–	–
24	MU 025	202237	Q'illu apilla	Puno	P. Yunguyo	Chambi Kimsacruz (Yunguyo)	16.27	69.07
24	ARV 5343	202521	K'ello	Puno	Chucuito	Desaguadero	–	–
24	UNC O 338	202580	Manzanilla apilla	–	–	–	–	–
24	MU 026	202238	Q'illu qhiñi	Puno	P. Yunguyo	Chikani Uma (Yunguyo)	16.32	69.05
24	COC 013a	202447	–	–	–	–	–	–
24	MH 0335	202175	Oca	Oruro	P. Cercado	Oruro	17.40	66.17
24	COC 03 08 009a	202449	–	–	–	–	–	–
169	AMM 5152	202076	Oca	Ancash	Carhuaz	Huellapu	–	–
169	AMM 5146	202075	Oca	Ancash	Yungay	Huashcau	9.18	77.63
169	AMM 5132	202013	Oca	Ancash	Yungay	Cochayó	9.23	77.72
169	AMM 5154	202017	Oca	Ancash	Carhuaz	Huellapu	–	–
169	AMM 5145	202074	Oca	Ancash	Yungay	Huashcau	9.18	77.63
169	AMM 5165	202078	Oca	Ancash	Carhuaz	Llipta	9.22	77.60
183	AJA 5247	202033	Salteada	Cajamarca	Cajamarca	Chaquil-El Capulí	7.11	78.35
183	O 145 84	202444	Oca amarilla	La Libertad	Sánchez Carrión	La Ramada	–	–
183	O 053 83	202421	Oca señorita	Cajamarca	Hualgayoc	San Antonio Alto	–	–
183	O 036 83	202416	Oca susana o mulla	Cajamarca	Celendín	Cruz Conga	–	–
183	O 044 83	202419	Oca blanca	Cajamarca	Cajamarca	La Encañada	–	–
183	AMM 5222	202081	Oca	Ancash	Yungay	Poncos	9.25	77.74
183	AJA 5256	202091	Canastita	Cajamarca	Cajamarca	Chaquil-El Capulí	7.11	78.35
183	O 089 84	202434	Chumpak	Ancash	Bolognesi	Mercado Chiquián	–	–
183	AMM 5144	202073	Oca	Ancash	Yungay	Huashcau	9.18	77.63
183	CLON 012	202592	S/N	Cajamarca	Cajamarca	Pariamarca	–	–
207	ARB 5050	202058	Oke	Cajamarca	Cajamarca	Chamis	7.13	78.55
207	CLON 041	202601	S/N	Cajamarca	Cajamarca	Choropunta		
207	AGM 5080	202007	Oca mulla grande	Cajamarca	Chota	Rojaspampa-Choctapata	6.55	78.63
207	CLON 035	202598	Susana	Cajamarca	Cajamarca	Choropunta	–	–
207	AJA 5243	202029	Mulla	Cajamarca	Cajamarca	Tartar Chico-Pampa de la culebra	7.12	78.46
207	AJA 5246	202032	Oque	Cajamarca	Cajamarca	Chaquil-El Capulí	7.11	78.35
207	AGM 5084	202009	Oca mulla	Cajamarca	Chota	Rojaspampa-Choctapata	6.55	78.63

Table 1 continued

Morphotype no.	COL no.	CIP no.	Cultivar name	Department	Region	Locality	Latitude (°S)	Longitude (°W)
207	CLON 034	202597	Sorocona	Cajamarca	Cajamarca	Huacataz	–	–
207	CLON 045	202604	S/N	Cajamarca	Cajamarca	Huacataz	–	–

geographical regions of Peru, was chosen (Table 3). Morphotypes analyzed in both experiments were defined on the same morphological characters.

DNA extraction

DNA was isolated from fresh leaflets tissue at CIP and shipped to UCL with appropriate authorization delivered by the Ministry of Agriculture of Peru (INIEA). The extraction was performed as described in Pissard et al. (2006).

PCR and electrophoresis conditions

Nine ISSR primers were used to perform the analysis (Pissard et al. 2006). The amplification reactions were performed in a 10 µl volume containing 10 ng genomic DNA, 0.4 µmol/l primer (Eurogentec SA, Seraing, Belgium), 80 µmol/l each dNTP, 1U Taq polymerase and 1× Taq DNA Poly buffer (Amersham Bioscience Corp., Piscataway, N.J.). The PCR amplifications were performed using a PTC 100 thermal cycler (MJ Research, Waltham, Mass.) according to the following reaction conditions. For the primers Nos. 1–6, a denaturation step of 10 min at 95°C, 35 cycles [30 s at 95°C, 45 s at T_a (annealing temperature empirically determined—Table 4) 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 [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. The PCR products were analyzed by electrophoresis using 1.5% w/v agarose gel carried out at a constant voltage of 90 V. A DNA ladder (Smartladder, Eurogentec SA, Seraing, Belgium) was used in each electrophoresis gel as molecular weight marker. Gels stained with ethidium bromide were visualized under UV light and recorded with the video image analyser Biocapt (Vilbert-Lourmat, Marne-La-Vallée, France).

Data analysis

The 18 morphological markers used for the characterization of morphotypes were analyzed with SAS 8.02 (SAS Institute Inc.). The Gower's distance (Gower 1971) was

Table 2 Morphological characters of the five morphotypes of oca (*Oxalis tuberosa*) analyzed by ISSR markers in order to determine the intra-morphotype genetic diversity (see IPGRI/CIP 2001 for numeric codes signification)

Morphotype (CIP no.)	1	24	169	183	207
C1 = Stem colour	1	1	2	3	4
C2 = Pigmentation in leaf axils	0	1	0	0	0
C3 = Foliage colour	1	2	1	2	1
C4 = Leaves reverse colour	1	3	1	3	1
C5 = Petiole colour	1	3	2	2	3
C6 = Flowering intensity	3	3	7	5	5
C7 = Petal colour	1	1	2	2	2
C8 = Heterostyly	2	1	1	1	1
C9 = Corolla shape	2	2	3	2	2
C10 = Sepal colour	1	1	3	1	1
C11 = Pedicel and peduncle colour	1	2	2	2	2
C12 = Predominant tuber skin colour	1	4	3	5	8
C13 = Secondary tuber skin colour	0	9	0	4	2
C14 = Distribution of the secondary tuber skin colour	0	1	0	3	3
C15 = Predominant tuber flesh colour	1	4	3	4	1
C16 = Secondary tuber flesh colour	0	0	0	0	10
C17 = Distribution of the secondary tuber flesh colour	0	0	0	0	2
C18 = Tuber shape	4	4	2	2	2

Table 3 The 34 accessions of oca (*Oxalis tuberosa)* analyzed in order to determine the spatial structures of the morphological and genetic diversity: department, region, locality and geographical position (latitude/longitude) of the collection site

COL no.	CIP no.	Department	Region	Locality	Latitude (°S)	Longitude (°W)
ARB 5052	CIP 202003	Cajamarca	Cajamarca	Chamis	7.13	78.55
AGM 5077	CIP 202005	Cajamarca	Chota	Calmarca	6.55	78.73
AMM 5140	CIP 202015	Ancash	Yungay	Cochayó	9.2	77.68
AMM 5151	CIP 202016	Ancash	Carhuaz	Huellapu	9.25	77.68
AMM 5167	CIP 202020	Ancash	Carhuaz	Llipta	9.22	77.6
AMM 5173	CIP 202024	Ancash	Carhuaz	Hualcán	9.21	77.61
ARB 5236	CIP 202028	Cusco	Calca	Amaru	13.37	71.81
AJA 5249	CIP 202034	Cajamarca	Cajamarca	Tartar Chico-Chin Chin	7.1	78.31
AJA 5252	CIP 202037	Cajamarca	Cajamarca	Puylucana Alta	7.13	78.44
AJA 5255	CIP 202039	Cajamarca	Cajamarca	Tartar Chico-Pampa de la Culebra	7.12	78.46
AJA 5264	CIP 202040	Cajamarca	Cajamarca	Chaquil-El Capulí	7.1	78.31
AJB 5295	CIP 202045	Cajamarca	Chota	Rambrampata	6.33	78.39
ARB 5329	CIP 202049	Lima	Yauyos	Tintin	12.3	75.8
ARM 5036	CIP 202052	Piura	Piura	Piura	5.54	80.87
AMM 5222	CIP 202081	Ancash	Yungay	Poncos	9.25	77.74
AMM 5223	CIP 202082	Ancash	Yungay	Huaypán	9.9	77.44
AJB 5298	CIP 202114	Cajamarca	Chota	Choctapata	6.54	78.6
ARV 5351	CIP 202126	Puno	Chucuito	Imicate	16.27	69.13
ARV 5353	CIP 202128	Puno	Chucuito	Tinicachi	16.19	68.95
ARV 5355	CIP 202129	Puno	Chucuito	Acari	16.38	69.2
ARV 5356	CIP 202130	Puno	Chucuito	Apillani	16.22	69.06
ARV 5358	CIP 202132	Puno	Chucuito	Unicachi	15.53	69.53
MU 21	CIP 202235	Puno	Chucuito	Ch'imu Alto Ayriwa (Zepita)	16.46	69.1
MU 25	CIP 202237	Puno	Yunguyo	Chambi Kimsacruz (Yunguyo)	16.26	69.06
MU 31	CIP 202240	Puno	Yunguyo	Cuturapi (Yunguyo)	16.26	69.16
MU 36	CIP 202241	Puno	Chucuito	Qaxi, Caje (Juli)	16.21	69.45
MU 46	CIP 202245	Puno	Yunguyo	Queñuani (Yunguyo)	16.26	69.16
MU 60	CIP 202251	Puno	Puno	Añu Callejón (Platería)	15.88	69.85
MU 63	CIP 202252	Puno	Puno	Cucho Esqueña (Acora)	16.01	69.8
PRODEKON 3	CIP 202291	Ancash	Pallasca	Toldobamba	8.23	77.82
PRODEKON 7	CIP 202295	Ancash	Pallasca	Potrero	8.15	78.01
PRODEKON 12	CIP 202300	Ancash	Pallasca	Cayarenga	8.31	77.83
O 85-84	CIP 202433	Ancash	Bolognesi	Mercado Chiquian	10.15	77.15
O 136-84	CIP 202442	La Libertad	Otuzco	Motil	7.98	78.55

Table 4 ISSR primers and their optimal annealing temperature (T_a)

Primer no.	Primer sequence $5' \rightarrow 3'$	T_a (°C)
1	DHB-$(CGA)_5$	53
2	VHV-$(GT)_7$-G	51
3	BDB-$(CAC)_5$	55
4	$(AG)_8$-YT	47
5	$(GA)_8$-YC	51
6	$(AC)_8$-G	47
7	$(AG)_8$-T	50
8	$(GA)_8$-A	50
9	DDC-$(CAC)_5$	55

used to generate dissimilarity matrices. Molecular patterns were analyzed using Gene Profiler (Scanalytics, Inc.). The intensity of the bands was not taken into account for the general scoring and only clearly scorable and reproducible bands on multiple independent runs were considered. These bands were scored qualitatively as present (1) or absent (0) and both polymorphic and monomorphic bands were included in the final data sets. The genetic distances were estimated using Jaccard similarity coefficient (Jaccard 1908). Note that Gower's and Jaccard's distances are equivalent in the case of presence–absence markers like ISSR ones. The UPGMA (unweighted pair-group method with arithmetic averaging) clustering method was used to

generate dendrograms with Treecon (Van de Peer and De Wachter 1994).

Reproducibility of the ISSR technique

All plant materials designed for both experiments i.e. 44 and 34 accessions for the first and second experiments respectively, were used to evaluate the reproducibility of the technique. At least two independent runs were realized by two research workers (A-M. Faux and S. Paulet) for the first experiment and by one researcher (A. Pissard) for the second one. For both experiments, the fingerprintings were compared for all bands and the reproducibility of the ISSR technique was estimated by the absolute error rate. This parameter was calculated by the ratio between the number of non matching bands and the total number of bands.

First experiment: analysis of the intra-morphotype genetic diversity

Morphological and molecular distances were calculated within and between morphotypes. Factorial correspondence analyses (FCA) based on both morphological and ISSR markers were performed using XLSTAT-Pro V7.5.2 (Addinsoft, 1995–2004). ISSR data were subjected to an analysis of the molecular variance (AMOVA) to determine the components of the molecular variance between and within morphotypes. In order to evaluate the differentiation between morphotypes, Chi^2 tests based on band frequencies were realized between each pair of morphotypes. Assignments tests were realized to determine the assignment likelihood of the accessions to the morphotypes. They were performed by comparing each accession to five groups corresponding to the five morphotypes using the Pop Assign option of GenAlex6 (Peakall and Smouse 2005), which is based on the assignment test of Paetkau (2004). For each sample a log likelihood value was calculated for each group using the allele frequencies of the respective population. A sample was assigned to a group with the highest log likelihood. The 'leave-one-out' procedure, in which the sample is removed from the dataset before calculating the adjusted frequencies to be used in the assignment likelihood, was used. In order to test the correlation between both types of markers, a Mantel test (Mantel 1967) was calculated between the matrices of morphological and genetic distances.

Second experiment: analysis of the spatial structures of the morphological and genetic diversity

A geographical distances matrix was designed for the 34 morphotypes based on the latitude and longitude positions of their collection site. The geographical data were compared to the genetic and morphological data. Mantel tests were performed between the matrices of geographical, genetic and morphological distances. Partial correlations were calculated to describe the relationships between each pair of variables whilst taking away the effects of the third one. The spatial structures of the morphological and genetic diversity were investigated by the spatial autocorrelation method of Smouse and Peakall (1999) available in GenAlex6. The *r*-autocorrelation coefficients among geographical distance classes were summarized by correlograms.

Results

Reproducibility of the ISSR technique

For both experiments, the comparison of fingerprintings showed few differences between the binary data obtained independently. For the first experiment, the absolute error rate between both researchers' runs (i.e. the percentage of divergent scoring) was 4%. For the second one, the absolute error rate between independent runs realized by one researcher was very low (1.3%). These low levels of absolute error rate showed that the reproducibility of the technique was high.

Intra-morphotype genetic diversity

Morphological analysis

The average Gower's pairwise distance among all accessions was 0.411 ± 0.221 (mean ± sd). The average distance between morphotypes was 0.505 ± 0.011 (mean ± sd) and ranged from 0.354 (between morphotype 183 and 24) to 0.658 (between morphotype 207 and 24) (Table 5). The distances between accessions of the same morphotype were, of course, zero since all accessions of a morphotype share the same morphological characters. In the 2-D factorial correspondence, the morphotypes were clearly separated by the first two axes that explained respectively 30.53% and 28.54% of the total variance between accessions (Fig. 1A).

Molecular analysis

Amplifications using the 9 primers selected generated a total of 69 reliable and reproducible bands, from which 32 (46.38%) were polymorphic. The total number of bands scored ranged from 5 to 11 and the number of polymorphic bands from 0 to 7 per primer.

The average pairwise distance between all accessions was 0.185 ± 0.095 (mean ± sd) with distances ranging

Table 5 Genetic and morphological distances between and within the morphotypes

	Morphotype 1	Morphotype 24	Morphotype 169	Morphotype 183	Morphotype 207
Morphotype 1	0,004/0	0,524	0,488	0,593	0,596
Morphotype 24	0,117	0,004/0	0,628	0,354	0,658
Morphotype 169	0,271	0,296	0,019/0	0,383	0,459
Morphotype 183	0,256	0,224	0,177	0,048/0	0,360
Morphotype 207	0,238	0,254	0,154	0,234	0,021/0

Diagonal: genetic distances within morphotypes (first number) and morphological distances within morphotypes (second number). Above diagonal: morphological distances between morphotypes. Below diagonal: genetic distances between morphotypes

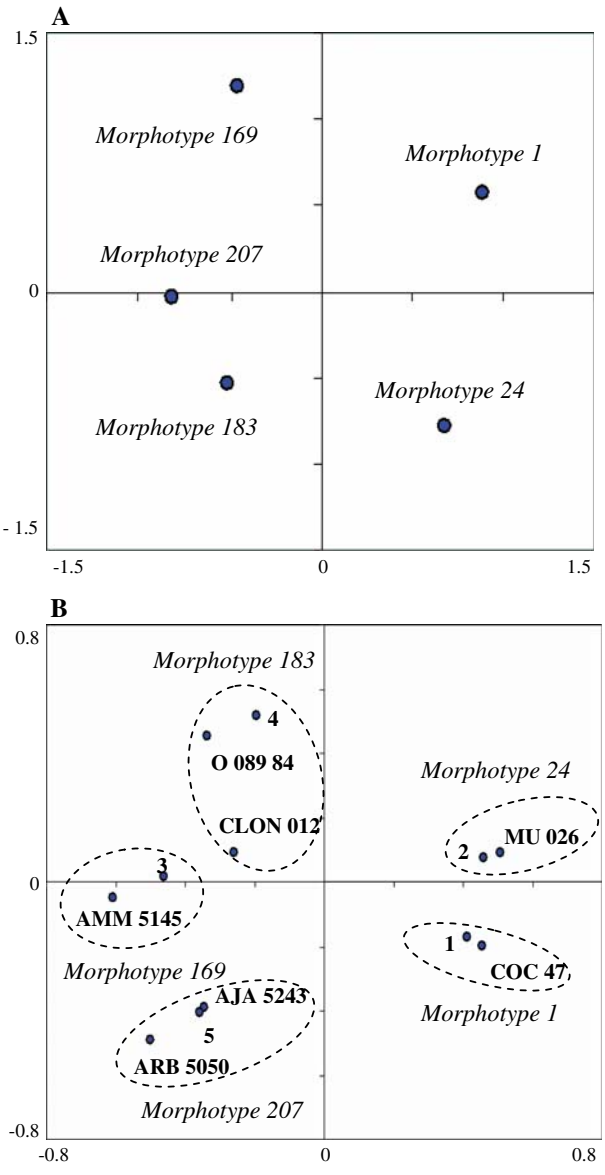

Fig. 1 Factorial correspondence analysis plot of the five oca morphotypes based on morphological (**A**) and genetic (**B**) markers. Circles surround the accessions belonging to the same morphotypes

from 0 to 0.3. The average distance between morphotypes was 0.22 ± 0.056 (mean ± sd) with distances between morphotypes ranging from 0.117 to 0.296. The analysis of the accessions within morphotypes (intra-morphotype genetic diversity) revealed a low, but different from zero, average distance of 0.019 ± 0.018 (mean ± sd) with distances ranging from 0.004 (morphotypes 1 and 24) to 0.048 (morphotype 183) (Table 5). The percentage of polymorphic bands per morphotype was 1, 4, 17 and 7% for morphotypes 1 and 24, 169, 183 and 207 respectively.

The 2-D factorial correspondence analysis realized on the ISSR markers showed five groups of points separated along the two first axes and corresponding to the five morphotypes defined on the morphological basis (Fig. 1B). Each of these groups was composed of a set of accessions genetically identical, represented by a unique point on the FCA plot (points 1–5), and by some accessions positioned near these points, i.e. genetically slightly different from the rest of the morphotype (COC 47, MU 026, AMM 5145 for respectively the morphotype 1, 24 and 169, CLON 012 and O 089 84 for the morphotype 183, and AJA 5243 and ARB 5050 for the morphotype 207). The first axis, that explained 33.59% of the total variation, separated the morphotypes 1 and 24 from the morphotypes 169, 183 and 207. The second axis of the FCA explained 20.88% of the total variation and separated the morphotype 1 and 207 from respectively the morphotypes 24 and 183.

The cluster analysis showed clearly five clusters corresponding exactly to the five morphotypes (Fig. 2). This clustering confirmed and clarified the results of the 2-D FCA. The morphotypes 169 and 207 clustered first, and further joined to the morphotype 183. They finally clustered to the group composed by the morphotypes 1 and 24. The clear separation of the morphotypes was confirmed by the results of the molecular variance analysis, which showed a highly significant genetic differentiation between morphotypes (92%, P-value < 0.001). Moreover the results were in agreement with the low level of intra-morphotype genetic diversity compared to the level of genetic diversity between morphotypes (Table 6). Finally, Chi^2 tests based on band frequencies showed significant differences at the

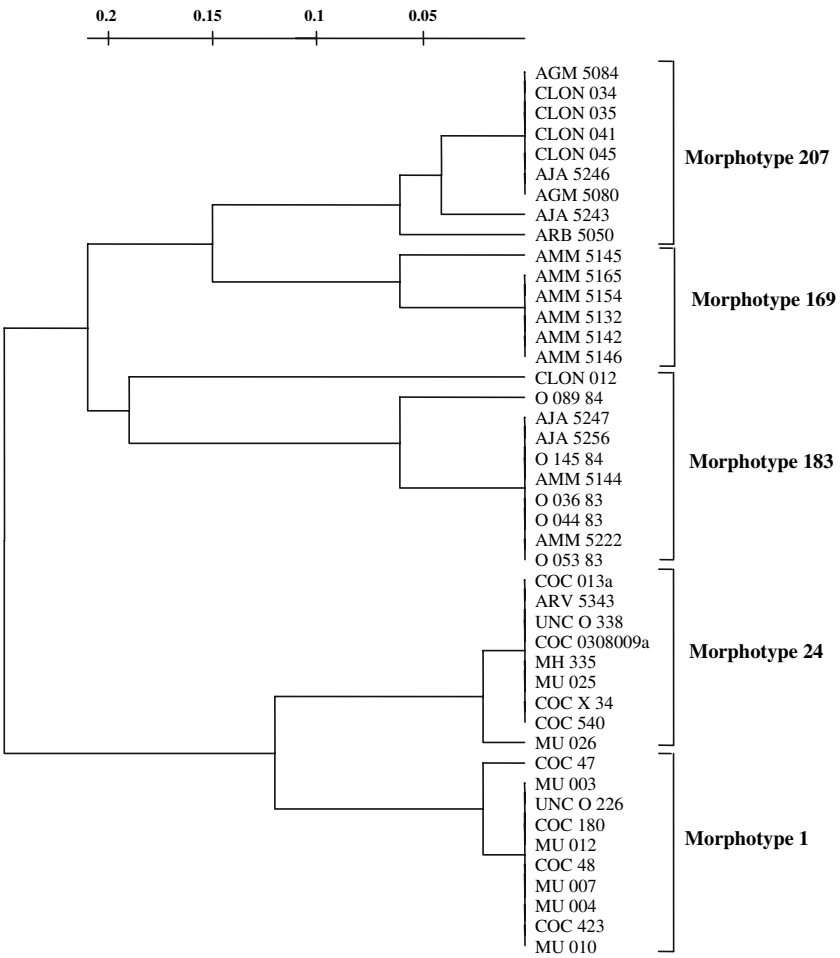

Fig. 2 Dendrogram (UPGMA method) of 44 oca accessions based on 69 ISSR markers in relation with the morphotypes determined on the basis of 18 morphological characters. The scale bar on the top of the figure represents the pairwise genetic distances between accessions (Jaccard's distance)

0.001 level between all pairs of morphotypes, except between morphotypes 1 and 24, and 169 and 207 where differences were significant at the 0.05 and 0.01 levels, respectively (Table 7).

Assignment tests revealed that the accessions were assigned to the morphotypes they belong to, except for one accession only: CLON 012 did not match with the morphotype 183, to which it corresponded morphologically, but with the morphotype 169 (Table 8).

The Mantel test performed between the morphological and genetic distances matrices resulted in a highly significant correlation between both types of markers ($r = 0.823$; P-value < 0.001).

Spatial structures of the morphological and genetic diversity

Morphological analysis

The average morphological distance was 0.397 ± 0.123 (mean ± sd) with distances between accessions ranging from 0.019 to 0.695. The clustering based on the morphological distances showed two main groups, each of which including accessions from different regions or departments (Fig. 3). No clustering of the accessions according to their collection site was observed.

The Mantel test revealed that correlation between the morphological (x) and geographical (z) distances was very

Table 6 Analysis of molecular variance (AMOVA) for ISSR variation between morphotypes

Source of variation	df	Mean squared deviations	Expected mean squared deviations	Percent of total molecular variance	P value[a]
Between morphotypes	4	204.539	5.792	91.78	<0.001
Within morphotypes	39	20.233	0.518	8.22	
Total	43	224.773	6.310		

[a] Probability of obtaining a larger component estimate. Number of permutations: 999

Table 7 Chi^2 values based on band frequencies between morphotypes

	Morphotype 1	Morphotype 24	Morphotype 169	Morphotype 183	Morphotype 207
Morphotype 1	0				
Morphotype 24	51.84*	0			
Morphotype 169	122.81***	131.27***	0		
Morphotype 183	139.51***	113.74***	64.90***	0	
Morphotype 207	124.61***	136.44***	56.39**	113.43***	0

* Significant at $P < 0.05$;
** Significant at $P < 0.01$;
*** Significant at $P < 0.001$

weak although highly significant ($r_{xz} = 0.130$; $r^2 = 0.016$; P-value < 0.01). Moreover, this correlation was reduced when the genetic effect was removed ($r_{xz.y}$ partial = 0.107). The correlogram showed weak r-values among all distance classes, which ranged from 1 (corresponding to within-department distances) to 14 (corresponding to distances between the most distant departments) (Fig. 4A). The spatial structure of the morphological characters was never highly significant, showing that there was no general relationship between the morphology and the collection site.

Genetic analysis

The nine ISSR primers generated a total of 98 reliable and reproducible bands, from which 67 (68.4%) were polymorphic. The average pairwise genetic distance between accessions was 0.29 ± 0.056 (mean ± sd) with distances ranging from 0.01 to 0.43.

The clustering analysis showed one isolated accession and three major clusters of accessions (Fig. 5). The isolated accession was the only one from which collection site was located in Cusco. The remaining accessions were divided into three groups: a cluster that gathered all the accessions collected in the department of Puno (Group I), except two, and two other clusters containing accessions collected in the departments of Ancash, Cajamarca, Lima and La Libertad (Groups II and III).

The Mantel test between molecular (y) and geographical (z) distances resulted in weak although highly significant correlation ($r_{yz} = 0.190$; P-value < 0.01). The correlation was reduced removing the morphological variable ($r_{yz.x} = 0.175$). Spatial autocorrelation analysis showed a clear decrease of the autocorrelation among the distance classes (Fig. 4B). The r-values decreased from 0.066 (distance class 1) to –0.062 (distance class 14). Positive values were observed in the four first distance classes. Highly significant genetic spatial structure was observed for the first distance class ($r = 0.066$; P-value < 0.001).

Congruence between morphological and molecular markers

The Mantel test between morphological (x) and molecular (y) distances matrices resulted in weak although significant correlation ($r_{xy} = 0.137$; P-value < 0.05). This correlation was reduced when the geographical (z) variable was removed ($r_{xy.z} = 0.115$).

Discussion

Reproducibility of the ISSR technique

The low absolute error rates for both experiments performed in the present study demonstrated the reliability of the ISSR technique. Indeed, this technique is known to be highly reproducible because of the long length of the primers (Bornet and Branchard 2001; Kim et al. 2002). It has clearly been demonstrated for potato (Prevost and Wilkinson 1999). However, competition during the simultaneous amplification of different fragments has been proved to be a source of genotyping errors in RAPD analysis (Halldén et al. 1996). Although to a lower extent, the same phenomenon is supposed to occur in ISSR analysis (Mc Gregor et al. 2000). Moreover, the 4% of inconsistency between the runs observed in the first experiment could be due, at least partially, to the involvement of two different researchers. The difficulty in scoring bands which has been already reported in ISSR analysis (Sankar and Moore 2001), could lead to different interpretations according to the researcher. This is supported by the very low absolute error rate highlighted in the second experiment, where only one researcher performed the comparison between runs.

Molecular and morphological approach for oca genebank management

The nine ISSR primers were able to differentiate all the morphotypes. Moreover, all the accessions from a same morphotype always clustered together (Fig. 2). The dendrogram also revealed the existence of a genetic variation between accessions inside each morphotype. In fact, ISSR markers were able to distinguish 12 genetic profiles among the five morphotypes analyzed, each morphotype presenting two or three distinct molecular profiles. The concern of many genebanks is to save management and operating costs by reducing redundancy in their collection. The

Table 8 Assignment values of the accessions to the five morphotypes on the basis of molecular data expressed by the Log likelihoods and assigned morphotype

Morphotype	Accession	Morphotye 1	Morphotype 24	Morphotype 169	Morphotype 183	Morphotype 207	Assigned Morphotype
1	COC 47	–4,000	–28,102	–64,475	–50,743	–54,318	1
1	COC 423	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	MU 010	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	COC 180	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	MU 012	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	MU 003	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	UNC O 226	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	COC 48	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	MU 007	–0,102	–24,102	–60,475	–46,743	–50,318	1
1	MU 004	–0,102	–24,102	–60,475	–46,743	–50,318	1
24	MH 335	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	MU 025	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	COC X 34	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	COC 540	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	UNC O 338	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	COC 03 08 009a	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	MU 026	–28,092	–4,000	–72,475	–44,834	–62,318	24
24	COC 013a	–24,092	–0,116	–68,475	–40,834	–58,318	24
24	ARV 5343	–24,092	–0,116	–68,475	–40,834	–58,318	24
169	AMM 5152	–60,092	–68,102	–0,581	–26,743	–30,318	169
169	AMM 5145	–72,092	–80,102	–12,000	–31,764	–29,930	169
169	AMM 5146	–60,092	–68,102	–0,581	–26,743	–30,318	169
169	AMM 5165	–60,092	–68,102	–0,581	–26,743	–30,318	169
169	AMM 5154	–60,092	–68,102	–0,581	–26,743	–30,318	169
169	AMM 5132	–60,092	–68,102	–0,581	–26,743	–30,318	169
183	CLON 012	–56,092	–56,102	–33,873	–38,113	–42,318	169
183	O 145 84	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	AMM 5144	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	AJA 5247	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	O 089 84	–68,092	–60,102	–40,669	–10,829	–49,930	183
183	AJA 5256	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	O 036 83	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	O 044 83	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	AMM 5222	–56,092	–48,102	–36,475	–1,344	–50,318	183
183	O 053 83	–56,092	–48,102	–36,475	–1,344	–50,318	183
207	AJA 5246	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	AGM 5080	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	CLON 035	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	CLON 041	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	AJA 5243	–52,092	–60,102	–29,873	–39,947	–8,348	207
207	AGM 5084	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	CLON 034	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	CLON 045	–52,092	–60,102	–29,873	–39,947	–0,580	207
207	ARB 5050	–64,092	–72,102	–36,669	–47,764	–12,232	207

Note that Clon 012 was the only accession that was not assigned to its own morphotype

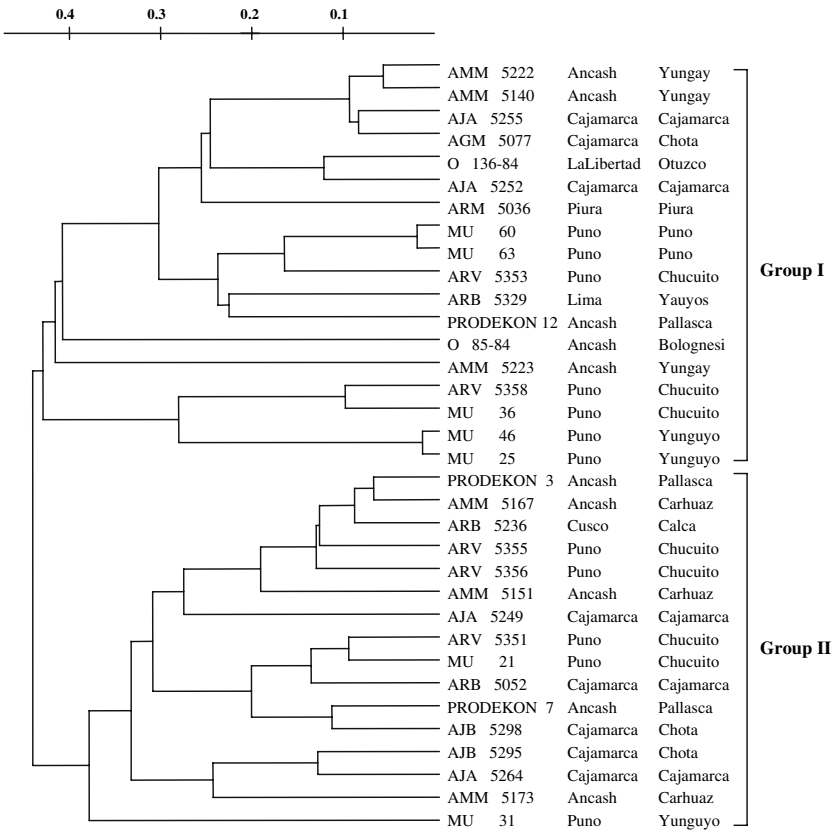

Fig. 3 Dendrogram (UPGMA method) of 34 oca accessions based on morphological markers according to their department and region of collection site. The scale bar on the top of the figure represents the pairwise morphological distances between accessions (Gower's distance)

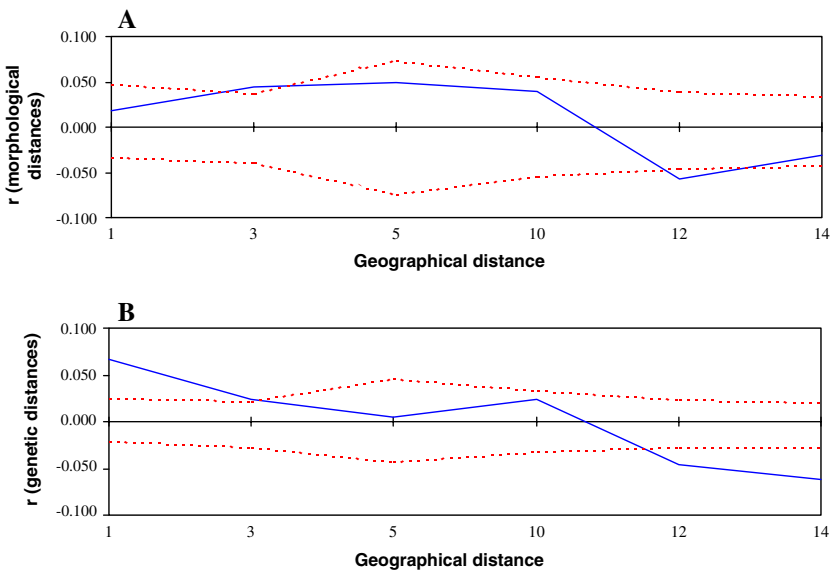

Fig. 4 Correlogram of the spatial autocorrelation coefficient (r) per distance class for morphological distances (**A**) and for genetic distances (**B**), with 95% null hypothesis confidence regions indicated by dotted lines. *Note:* A geographical distance class of 1 corresponds approximatively to a geographical distance of 111 km

elimination of all duplicates in the present sample of 44 accessions would result in the conservation of only five profiles on a morphological basis versus 12 on a molecular basis. Therefore, the morphological approach alone to eliminate duplicates of oca could result in a loss of genotypes. This could be partially prevented by the use of molecular markers, although the remaining duplicates identified by the molecular approach were not necessarily completely identical.

The greater discriminatory capacity of ISSR markers compared to the morphological markers may be explained by their genetic determinism. The number of genes involved in the 18 morphological characters is not known. Most of these characters are qualitative and could be

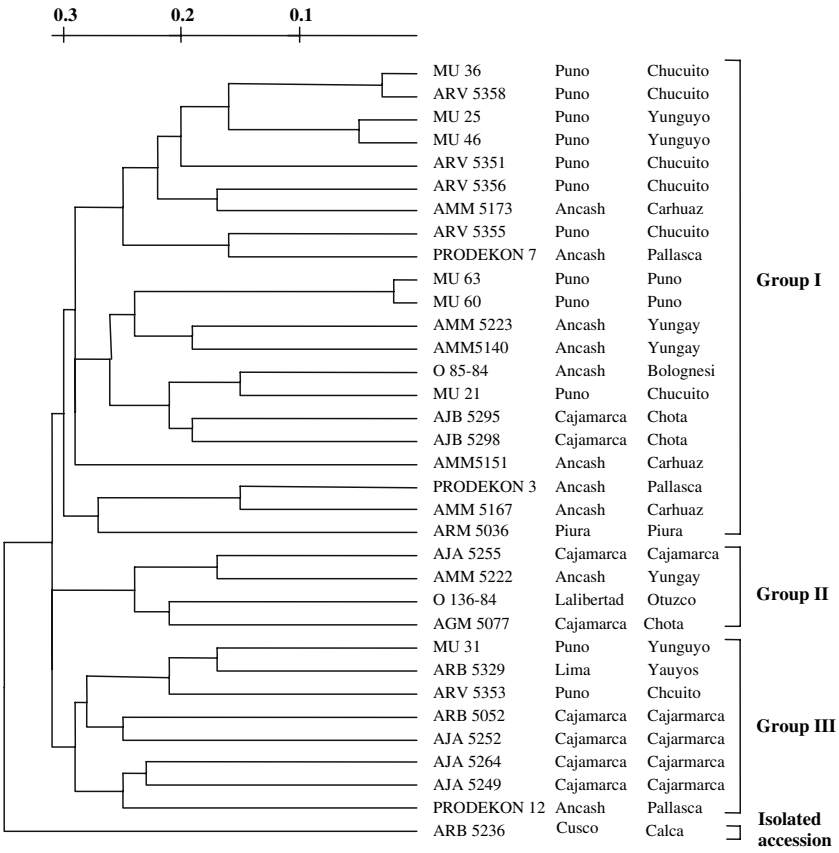

Fig. 5 Dendrogram (UPGMA method) of the 34 oca morphotypes based on ISSR analysis according to their department and region of collection site. The scale bar on the top of the figure represents the pairwise genetic distances between accessions (Jaccard's distance)

therefore under monogenic or oligogenic control. In addition, ten characters refer to the colour of vegetative organs and thus, could be controlled, at least partially, by the same genes. Indeed, the colouration of organs is due to the accumulation of flavonoids, including anthocyanins. Expression of anthocyanins has been studied in *Solanaceae* and is controlled by orthologous genes located in the same genomic region. Moreover, a linkage between anthocyanin and tuber-shape loci has been highlighted (Chaim et al. 2003). It has been confirmed in potato for which tuber shape is controlled by a single locus Ro located on the chromosome 10 and presents high heritability (van Eck et al. 1994). Flower colour is controlled by three loci, including one on the chromosome 10 (van Eck et al. 1993). On the opposite, it is likely that most of the 32 polymorphic ISSR markers considered here correspond to independent loci. However, an exact comparison of the number of loci involved in the different markers systems is not feasible.

Intra-morphotype diversity

An intra-morphotype genetic diversity was highlighted in this study. However, little genetic variability was observed within the morphotypes. Indeed, all the accessions of a same morphotype clustered at low distances (maximum 0.06), except one accession, CLON 012, which greatly diverged from its morphotype. This resulted in a relatively low average genetic distance between all accessions compared to the higher genetic diversity revealed at the species level (Pissard et al. 2006). The genetic divergence of CLON 012 from the morphotype 183 showed on the FCA plot and on the dendrogram was confirmed by the assignment tests. The results let suppose that CLON 012 could belong to morphotype 169. The inconsistency concerning this accession was confirmed by the morphological characterizations subsequently carried out (2004–2005), which showed its belonging to another morphotype (C. Arbizu, unpublished data). Indeed, the characterization is made every year and changes may occur in the intensity of expression of some characters, leading to slightly different classifications over years. This highlights a limitation of the morphological descriptors which are known to be more or less subjective and to suffer from the influence of the environment, leading to a certain variation in the description according to the year or location of assessment (Ortiz et al. 2004).

The intra-morphotype genetic diversity revealed here allowed us to make some assumptions about the genetic variability of oca varieties. As the varieties are differentiated by the peasants mainly on the basis of a few tuber characters, collecting plants presenting the same tuber morphology is likely to allow the existence of some

genotypic diversity inside these varieties. This confusion of genetically distinct individuals that share similar morphological features could lead to intravarietal polyclonality as observed in cassava (Elias et al. 2000, 2001). The genetic variability of the oca varieties has never been investigated up to now and the 'morphotype' unit, as defined in a very precise way in the oca genebank of CIP, could be considered as a taxonomic unit distinct from the peasant cultivar or variety.

The spatial structures of the morphological and genetic diversity

The morphological clustering suggested that there was no geographical structure of the morphological diversity. In fact, accessions clustered independently of their collection site. Each cluster contained accessions coming from different regions or departments. This was confirmed by the autocorrelation analysis that showed low *r*-values among all distance classes and by the very low partial correlation between both morphological and geographical variables. On the contrary, the ISSR analysis suggested that the genetic structure was slightly influenced by the collection site. The molecular dendrogram showed that the accessions coming from the same collection site tended to cluster together. Indeed, accessions coming from the southern part of the country (department of Puno), except two, belonged to one group. The remaining accessions coming from the central part of Peru (departments of Ancash, Cajamarca, Lima and La Libertad) formed two other groups. The influence of the geographical distance on the oca genetic structure has already been observed (Pissard et al. 2006). The imperfect clustering of the accessions by collection site could be explained by the dissemination of tubers over long distances by peasants or collectors. This leads to misidentification of the accession's geographical origin, the term collection site being therefore more appropriate than geographical origin. These occasional great germplasm movements could play a role in the dynamics of the oca genetic diversity and could complement the in situ conservation of the varieties.

The influence of the geographical distance on the genetic structure can be observed more precisely on the correlogram where accessions with short distance classes were genetically more closely related to each other than those more distant geographically. This is particularly the case for the smallest distance class i.e. for accessions collected in the same department. This scale may be related to the geographical area over which genotypes could be easily dispersed via tuber exchanges. Indeed, oca varieties flow in and out of the individual stock and the community in a dynamic pattern. The numerous exchanges between peasants generates inter- and intracommunal seed flows (Terrazas and Valdivia 1998). Peasants may also acquire fresh or new tubers from the markets which can provide oca tubers from relatively close communities (about 5–10 km) as well as from more distant ones (about 80 km) (Ramirez 2002). At greater distances classes, movements of tubers are likely to be less frequent. Consequently, isolation by distance may occur as suggested by the spatial autocorrelation analysis.

This spatial structure of the genetic diversity can be related to the evolution of the species. If *Oxalis tuberosa* may have developed in the southern Peru or northwestern Bolivia and have radiated southward and especially northward along the Andean axis (Arbizu and Tapia 1992; Emswhiller 2002), it is likely that distinct genetic groups may have developed in the same time. Indeed, mutations associated with this geographical radiation could explain the genetic distinction between accessions collected in the different parts of the country. Moreover, sexual reproduction may have occurred during the domestication of the species and its long history of cultivation contributing sporadically to the continued heterozygosity of the germplasm oca (Gibbs 1976). The discordance between the spatial structures of the morphological and molecular diversity may result from similar selection pressures at different places leading to similar forms with a different genetic background. Indeed, it is likely that selection for similar agronomic and morphological characters have been applied by farmers independently in different parts of the country leading to a weak correspondence between the morphological and geographical data as revealed by the partial correlation.

Even if geographical separation does not always predict genetic differences, it is usually considered as an important parameter when collecting germplasm. As the goal of germplasm conservation in genebanks is to maximize genetic diversity, adequate sampling procedures during explorations are of great importance for the efficiency of the collecting trip. In this context, the identification of factors associated with genetic differences is essential. As the genetic structure of the oca species seems to be influenced by the collection site, this factor should be taken into account when collecting samples. Sampling across a large range of geographical coordinates may result in maximizing the sampled genetic variability.

Congruence between morphological and molecular markers

Exclusive vegetative propagation is expected to transmit the genome without any genetic recombination. Therefore, association between neutral molecular markers and loci involved in morphological characters could be observed. This could lead to some correspondence between the

structure of molecular and agro-morphological variability (Elias et al. 2001). A high correlation between morphological and molecular markers was revealed in the first experiment. However accessions within morphotypes came from the same or close collection sites (department, region or even locality). Consequently, it is likely that these accessions are closely related. In that case, the great concordance between both types of markers is not surprising and may not be considered as an evidence of congruence between the markers. In fact, the second experiment revealed little concordance between both markers systems as it could be observed by the comparison between the morphological and genetic clusterings. This was confirmed by the weak correlation revealed by the Mantel test. In conclusion, while the congruence between both types of markers seems to be high in the case of accessions genetically very similar, the same pattern must not be concluded for a larger range of accessions. It could mean that these morphological markers may be very useful for morphotypes management in genebanks but may not be appropriate to estimate the genetic diversity and relationships between accessions.

In summary, these results suggested that the morphological characters could be used as an accurate tool for the identification of morphotypes by grouping the accessions according to their similarities. The relatively low cost of the morphological characterization makes it an interesting tool to manage the germplasm collection. However, since genetic variability within morphotypes exists, molecular markers could be subsequently used to the morphological grouping to characterize more precisely the accessions when identifying duplicates of a collection. Molecular markers are also more convenient to reflect the genetic relationships of the species. Finally, this study confirmed previous results showing that the genetic structure of the oca is influenced by the geographical distance. The research on the oca species and other Andean tuber crops, such as mashua (*Tropaeolum tuberosum* Ruíz & Pavón) and ulluco (*Ullucus tuberosus* Caldas), contributes to a better understanding of the genetic diversity and structure of these species, which may be considered as potential genetic models for vegetatively propagated plants cropped in traditional farming systems. This could help define appropriate strategies of genetic resources conservation for this kind of crop.

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