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RESEARCH ARTICLE

Wild sunflower diversity in Argentina revealed by ISSR and SSR markers: an approach for conservation and breeding programmes

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Keywords
Conservation; genetic variability; *Helianthus annuus*; microsatellites; naturalised population.

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Abstract

Wild sunflower *Helianthus annuus* originates from North America and has naturalised in Argentina where it is considered invasive. The present study attempts to assess the genetic diversity using two different molecular marker systems to study the wild genetic patterns and to provide data applicable to conservation and breeding uses. Ten natural populations sampled throughout the wild range and six inbred lines were studied using inter-simple sequence repeat (ISSR) and simple sequence repeats (SSR) markers. A total of 64 ISSR bands and 29 SSR alleles were produced from 106 wild and cultivated plants. We found 9 ISSR private bands and 21 SSR private alleles in wild accessions, but no private bands/alleles were found in cultivated sunflowers. Molecular variability in wild populations was approximately 60% higher than in inbred lines. Local wild sunflowers kept considerable diversity levels in comparison with populations in the centre of origin (approximately 70%) and therefore they might possess a potential for adaptive evolutionary change. Analysis of molecular variance (AMOVA) indicated population structure with nearly 20% of genetic variability attributable to between-population differentiation. Principal coordinate analyses (PCO) grouped wild populations from different geographic locations, and a Mantel test showed low congruence between genetic distance (GD) and geographic distances (GGD); hence, molecular data could not rule out multiple wild introduction events. Low correlations were found between ISSR and SSR GD at individual and population levels; thus, divergent evolutionary groups were not evident in local wild sunflowers. Several genetic diversity criteria were utilised to assign conservation value and certain wild populations emerged as interesting sites for more extensive sampling.

Introduction

The wild or common sunflower *Helianthus annuus* (Asteraceae), an annual, diploid ($x = 17$) native to North America, is a very variable species, naturalised and invasive in several countries (Dry & Burdon, 1986; Muller *et al*., 2009). In the central area of Argentina devoted to agriculture, early reports mentioned *H. annuus* as an unusual species (Cabrera, 1974), but Zuloaga & Morrone (1999) and Poverene *et al*. (2002, 2008) showed that this species has increased its geographic distribution reaching, at present, seven provinces. Wild sunflower was probably introduced in Argentina as a forage crop (Bauer, 1991) but unintentional introductions as seed impurities or the occurrence of several entries for ornamental or breeding purposes cannot be discarded.

The *H. annuus* species comprises weedy, wild and domesticated forms (Heiser *et al*., 1969). Wild sunflower populations of *H. annuus* together with its annual relative *Helianthus petiolaris*, are undergoing an invasive process

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Abstract

Wild sunflower *Helianthus annuus* originates from North America and has naturalized in Argentina where it is considered invasive. The present study attempts to assess the genetic diversity using two different molecular marker systems, to study the wild genetic patterns, and to provide data applicable to conservation and breeding uses. Ten natural populations sampled throughout the wild range and six inbred lines were studied using ISSR (inter-simple sequence repeat) and SSR (simple sequence repeats) markers. A total of 64 ISSR bands and 29 SSR alleles were produced from 106 wild and cultivated plants. We found nine ISSR private bands and 21 SSR private alleles in wild accessions, but none private band/alleles were found in cultivated sunflower. Molecular variability in wild populations was approximately 60% higher than in inbred lines. Local wild sunflower kept considerable diversity levels in comparison to populations in the centre of origin (ca. 70%) and therefore they would possess a potential for adaptive evolutionary change. AMOVA indicated population structure with nearly 20% of genetic variability attributable to between-population differentiation. PCO grouped wild populations from different geographic locations, and a Mantel test showed low congruence between genetic and geographic distances; hence molecular data could not rule out multiple wild introduction events. Low correlations were found between ISSR and SSR genetic distances at individual and population levels, thus divergent evolutionary groups were not evident in local wild sunflower. Several genetic diversity criteria were utilized to assign conservation value and certain wild populations emerged as interesting sites for more extensive sampling.

Keywords: *Helianthus annuus;* Genetic variability; Conservation; Naturalized population; Microsatellites.

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The *H. annuus* species comprises weedy, wild, and domesticated forms (Heiser et al., 1969). Wild sunflower populations of *H. annuus* together with its annual relative *H. petiolaris*, are undergoing an invasive process throughout the central region of Argentina (Cantamutto et al., 2008). Wild populations highly hybridize with the crop in the invaded area (Ureta et al., 2008a,b), as it was documented in the centre of origin (Burke et al., 2002). Gene flow between domestic plants and wild relatives can cause ecologically significant changes in recipient plant populations (Ellstrand, 2003). Genes coding novel traits such as herbicide tolerance, insect resistance, or environmental stress adaptation, could be transferred to wild populations. Some of these characters may confer adaptive advantages and ability to spread into the wild, promoting changes towards a more invasive plant growth habit (Keeler, 1989; Snow et al., 2003). In Argentina, sunflower varieties bearing imidazolinone tolerance have been adopted by farmers in the last decade (Ministry of Agriculture, Livestock, Fisheries and Food of Argentina, 2008) and the transference of the herbicide tolerance to local wild populations has been observed (Alejandro Presotto, personal communication). Gene flow from wild to crop in regions devoted to sunflower hybrid seed production, may also yield considerable seed contamination, which constitutes a present constraint in some areas of the US and Europe (Anfinrud, 1997; Muller et al., 2006).

The common sunflower, *H. annuus*, occurs through a wide geographical range in its centre of origin (Rogers et al., 1982) suggesting that a large amount of genetic diversity is available for germplasm conservation and crop breeding. Several new inbred lines has developed by hybridization with wild relatives (Jan & Seiler, 2007) as sources of cytoplasmic male sterility (Leclercq, 1969), pathogen resistance (Miller & Gulya, 1999), herbicide and salt tolerance (Miller & Seiler, 2003; Miller & Al-Khatib, 2004). In recent years, sunflower production in Argentina has been advanced toward more marginal western environments (De la Vega et al., 2007). Naturalized populations of wild sunflower constitute potentially useful germplasm adapted to local environments.

The knowledge about the genetic structure of a wild species is essential to define conservation strategies. Molecular markers could provide information about the introduction process and the involved amount of genetic variation which finally determines the potential for adaptive evolutionary change. This topic deserves special attention when the species follows an invasive pattern of spreading (Bossdorf et al., 2005).

Isozyme variability in 20 *H. anuuus* populations from Argentina showed a considerable diversity and divergence between populations (Poverene et al., 2004). However, the limited number of available

loci and low polymorphism did not allow a complete characterization of genetic resources. ISSRs (inter-simple sequence repeat) (Zietkiewicz et al., 1994) exhibit levels of polymorphism suitable for subspecies analysis and a great potential for natural population studies (Wolfe et al., 1998; Wang et al., 2007). ISSR markers have been used for genetic polymorphism study in other Asteraceae (Chapman et al., 2000; Slotta et al., 2005; Archibald et al., 2006; Haouari & Ferchichi, 2008). Microsatellite or SSR (simple sequence repeats) (Litt & Luty, 1989) have been utilized in *Helianthus* species for molecular characterization of inbred lines (Paniego et al., 2002), QTL mapping (Mokrani et al., 2002; Darvishzadeh et al., 2007; Baack et al., 2008), and population structure analysis (Ellis et al., 2006). Tang et al. (2003) developed multiplex PCR for SSR loci in cultivated sunflower.

It is unknown if a high genetic diversity of wild *H. annuus* is responsible of its successful invasive process across a wide geographical area in Argentina. If the wild populations were derived from a single introduction event, a significant narrowing of the native genetic variability might be detected. Alternatively, if a high genetic diversity were observed, multiple introduction events and/or novel recombination could not be discarded.

The objectives were: (1) to study the genetic structure of naturalized *H. annuus* populations, (2) to assess the levels of polymorphism detected by two different molecular methods, (3) to compare genetic patterns of cultivated and wild materials, and (4) to provide data applicable to wild sunflower conservation and breeding uses.

Materials and methods

Plant sampling and DNA isolation

Wild *Helianthus annuus* populations spread from 31º58’ to 37º31’S, and 60º33’ to 68º14’W in Argentina, following a patchy distribution on different soil types, in a range of agro-ecological conditions. Most populations comprise ca. 1000 to 10,000 plants, on areas of less than 1000 m^2 to more than 50,000 m^2 with mean density of 1-3 pl.m^{-2} (Poverene et al., 2008).

Ten representative populations were sampled throughout its range. The seeds were sown in a greenhouse and seedlings were transplanted in a common garden at the experimental field of the Agronomy Department, Universidad Nacional del Sur, Bahia Blanca, Argentina (S 38º41’38’’, W 62º14’53’’).

Leaf material from 10 randomly selected plants from each location was collected. The locations and their provinces were as follows: (1) A Alsina, Buenos Aires (ALS), (2) Carhue, Buenos Aires (CAR), (3) J. Celman, Córdoba (JCE), (4) Río Cuarto, Córdoba (RCU), (5) La Carlota, Córdoba (LCA), (6) Rancul, La Pampa (RAN), (7) Colonia Barón, La Pampa (CBA), (8) Media Agua, San Juan (MAG), (9) Malvinas, Mendoza (MAL) and (10) Diamante, Entre Ríos (DIA). ALS, RAN and CBA populations grew adjacent to sunflower fields when collection was performed. Six inbred lines (HA367, HAR274, HAR2, HAR3, HAR5, and HA89) that had previously shown to be polymorphic at SSR loci (Paniego et al., 2002) were included in the analysis and considered as a sample of cultivated genotypes.

Leaf material was stored at –70°C and then lyophilized. Total genomic DNA was isolated from leaf tissue using a modified CTAB method (Hoisington et al., 1994).

ISSR markers

Twenty ISSR primers were analyzed. Ten of them were anchored with one or more bases. Amplification reactions were performed in 25 µl volume containing: 1.5 U of Taq DNA polymerase (PB-L), 2.5 mM $MgCl_2$, 350 ng of primer, 0.2 mM of each dNTP, 0.25 µl of BSA 100X and 20 ng of genomic DNA template in buffer 1X. Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for each primer. The reactions were placed in a MJ Research Thermal Cycler programmed for an initial denaturation step of 94°C for 1 min 30 s, followed by 40 cycles of 94°C for 40 s, specific annealing temperature for 45 s, and 72°C for 1 min 30 s with a final extension at 72°C for 5 min. (modified from Zietkiewicz et al., 1994). PCR products were separated on 2.5% agarose gels with 1X TAE electrophoresis buffer at 80 V for 160 min and stained with ethidium bromide. The gels were exposed to UV-light and photographed. Ten wild individuals and three individuals from sunflower inbred lines were loaded onto each gel. The presence or absence of ISSR band was visually determined on the photographs. The inbred lines patterns and a 100 pb ladder were used to compare the profiles among gels.

SSR markers

Tang et al. (2003) established 13 SSR marker groups suitable for PCR-multiplexes based on primer compatibility, genotyping performance, allele length range, map position and heterozygosity. From these PCR-multiplex groups, the set 1 which comprises six SSR loci was chosen. Because the primers were not fluorescently labeled, the original group was split in two PCR-multiplex sub-groups. The SSR markers were sorted by allele-length range and a 100p difference was kept between SSR loci to minimize the risk of non-allelic band co-migration. The SSR marker loci amplified by each multiplex were ORS-297, ORS-456 and ORS-1222 (Set A) and ORS-342, ORS-533 and ORS-543 (Set B). An additional SSR locus, ORS-399 was separately analyzed (Table 1). Amplification reactions were performed in 20µl volumes containing: 1.5 U of Taq DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$, x pmol of specific primer (Table 1), 0.4 mM of each dNTP, and 30 ng of genomic DNA template in buffer 1.6X. Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for set A (62°C), set B (58°C) and ORS-399 (58°C). By Touchdown PCR, the annealing temperature was decreased 1°C starting with 5°C over the set annealing temperature. A MJ Research Thermal Cycler was programmed for initial denaturation step of 95°C for 3 min, followed by 10 touchdown cycles of 94°C for 30 s, touchdown annealing temperature for 30 s and 72°C for 45 s. PCR products were subsequently amplified for 34 cycles at 94°C for 30 s, annealing temperature for 30 s, and 72°C for 45 s with a final extension at 72°C for 20 min (modified from Tang et al., 2003). Amplifications were initially checked on 1.5% agarose gels. PCR products were analyzed on 6% denaturing polyacrylamide gel, 1X TBE electrophoresis buffer at 60 W for 75 min. Forty wild individuals and six individuals from inbred lines were loaded onto each gel. The bands were then visualized by silver staining and scanned.

Data scoring and genetic variability

For the ISSR data it was assumed that each band corresponded to one locus with two alleles and therefore the loci were scored for band presence (1) or absence (0) to create binary matrices. The presence of band represented the homozygote (AA) or heterozygote (Aa) genotypes, and the absence represented a recessive homozygote (aa). Allele frequency p(A) and q(a) were estimated by assuming independent nuclear loci and Hardy-Weinberg equilibrium (Lynch & Milligan 1994) as follows: $q = \sqrt{q^2}$, where q^2 = frequency of absent band and p = 1 – q.

For the SSR loci, homozygous and heterozygous genotypes were inferred from the banding patterns and allele frequencies (p_i) calculated directly from them. The absence of band (null allele) was registered as missing data.

Mean expected heterozygosity values (*He*) and percentage of polymorphic loci (%P) were calculated for ISSRs and SSR loci:

$He = 1 - \sum p_i^2$; $P\% = \frac{L_p}{LT} \times 100$, where p_i is the frequency of the i-th allele, L_p is the number of polymorphic loci and LT is the total number of loci.

Hardy Weinberg equilibrium was tested using Chi-Squared Test:

$\chi^2 = \sum_{i=1}^{K} \frac{(O_i - E_i)^2}{E_i}$, where O_i is the observed number of individuals of the i-th genotype, E_i is the expected number under equilibrium hypothesis and K is the total number of genotypes. Degrees of freedom for the Chi-Squared test were calculated as DF = [Na(Na-1)]/2, where Na is the number of alleles at the locus.

The inbreeding coefficient (F) was calculated for SSR on a per locus basis from observed (Ho) and expected (He) heterozygosity: F = (He - Ho) / He .Values close to zero are expected under random mating, while substantial positive values indicate inbreeding or undetected null alleles. Negative values indicate an excess of heterozygotes, due to negative assortative mating, or selection (Hartl & Clark, 1997).

Distance metrics

We calculated individual genetic distances (GD) for the ISSR data according to Huff et al. (1993): $GD = n\left[1 - \frac{2n_{xy}}{2n}\right]$, where n is the total number of polymorphic bands and $2n_{xy}$ is the number of markers shared by two individuals. In this measure, both band presence and absence are considered informative. This is a true Euclidean metric as required for the subsequent analysis of molecular variance.

The calculation of individual by individual genetic distances for SSR followed the method introduced by Peakall et al. (1995) and further explained in Smouse and Peakall (1999). For the analysis of a SSR single-locus, first step involves the vectors calculation by additive genotype scoring convention

per individuals (see Peakall et al., 1995). Subsequently, the squared distance (d^2) between any two genotypes is one-half the Euclidean distance between their respective pair of vectors as follow:

$d_{ij}^2 = \frac{1}{2}\sum_{k=1}^{K}\left(y_{ik} - y_{jk}\right)^2$, where i and j are the genotypes and k is the scoring character. Squared distances range from 0, when individuals share the same alleles, to 4 when individuals are homozygous for different alleles. Genetics distance matrices for each locus were summed across loci under the assumption of independence.

At population level, a φ_{PT} (analogue of F_{ST}) obtained from AMOVA was used as an estimate of population genetic differentiation with ISSR and SSR markers. This method avoids to the assumption of equilibrium and gives the same treatment both to dominant and co-dominant markers maintaining the assumptions of independence among loci. Principal coordinate analyses (PCO) were performed on genetic distance matrices.

To measure physical distance between populations we estimate a Geographic Distance (GGD) for latitude (*X*) / Longitude (*Y*) values:

$$GGD = \sqrt{(X_i - X_j)^2 + (Y_i - Y_j)^2}$$

The correlation between ISSR and SSR genetic and geographic distance matrices was analyzed by the Mantel test of matrix correspondence (Mantel, 1967; Smouse et al., 1986; Peakall et al., 1995) at individual and population levels. Statistical significance was determined by random permutation, with the number of permutations set to 1000.

Analysis of molecular variance

The individual pairwise genetic distance matrices obtained with ISSR and SSR data set were subjected to analysis of molecular variance (AMOVA) (Excoffier et al., 1992; Peakall et al., 1995). Total genetic variation was partitioned into three levels: between individuals, between populations within regions, and between regions. Regions were established according to the following criteria: distance to crop at collecting time, phytogeographic region (Pampa, Espinal, Monte) according to Cabrera (1971) and sunflower production zones (crop area reported by the Ministry of Agriculture, Livestock, Fisheries and Food of Argentina). Variation was summarized both as the proportion of the total variance and as φ-Statistics or *F*-Statistic analogues; φ_{PR}, φ_{RT} and φ_{PT}. φ_{PR} represents the correlation between individuals within a population relative to that of individuals from the same region, φ_{RT} is the correlation of individuals from the same region, relative to that of individuals from the species, and φ_{PT} is the correlation between individuals within a population, relative to that of individuals from the species.

Pairwise φ_{PT} values between all population pairs and between the regions pairs were calculated. Statistical significance was tested by 1000 random permutations. Genetic variability measures, distance metrics, principal co-ordinates (PCO) analysis, correlation analysis and AMOVA were analyzed using GenAlEx 6 (Genetic Analysis in Excel; Peakall & Smouse, 2006), available at: http://www.anu.edu.au/BoZo/GenAlEx. Cluster analysis was performed using INFOSTAT software (Di Rienzo et al, 2008), through the unweighted pair-group method with arithmetical average (UPGMA) algorithm.

Results

Ten out of twenty ISSR primers rendered amplification products (Table 1). The ISSR primers $(AC)_8A$, $(GT)_8G$, $(CT)_8T$, $(AG)_7TC$, $(ACC)_5$, $(AGA)_5$, $CAA(GA)_5$, $(GTAT)_4$, $(ACTG)_4$ and $(AAAG)_4$ failed in amplify. Molecular profiles were markedly affected by annealing temperature. Five primers (9, 14, 16, 19 and 20) were selected based on banding pattern quality. These selected primers were amplified at 52°C as optimal annealing temperature. Multiplex PCR allowed the amplification of six SSR loci and a separated PCR was performed for the seventh locus (Table 1). The ORS-543 and ORS-399 primers did not generate clear patterns in wild samples and therefore were not included in the analysis.

ISSR polymorphism

A total of 64 reproducible ISSR bands were produced from 106 wild and cultivated plants and 55 amplified fragments (85.9 %) were found to be polymorphic (Table 2, Fig. 1A). The mean polymorphism level calculated as the number of polymorphic bands/total bands per primer was 86.3% ranging from 80.0% to 90.0% among the five primers. The percentage of polymorphic bands was higher between wild sunflower (84.4%) than within elite inbred lines (31.3%). Mean expected heterozygosities estimated from polymorphic ISSR loci only, were 0.370 between wild sunflower and 0.138 between inbred lines. Heterozygosity for individual loci ranged from 0.110 to 0.500 between wild sunflower and 0.159 to 0.488 between inbred lines.

SSR polymorphism

The five selected SSR loci were polymorphic and produced 29 alleles from 106 wild and cultivated individuals (Table 2, Fig. 1B). The mean number of alleles per locus was 5.8 ranging from 2 to 9 among the five primers. This value was greater between wild sunflower (5.8) than inbred lines (1.6). The percentage of polymorphic loci was higher between wild sunflower (100%) than inbred lines (40%). Mean expected heterozygosities were 0.591 between wild sunflower and 0.178 between inbred lines. Heterozygosity for individual loci ranged from 0.247 to 0.855 between wild sunflower, and 0 to 0.611 between inbred lines.

Population variability

Measures of genetic diversity in populations are shown in Table 3. Differences in variability were observed between populations: CAR and JCE showed the highest values whereas the lowest values were registered for MAL and RAN. Equilibrium tests were non significant in 38 out of 47 cases, indicating random mating within populations (Table 4). All except one of the significant deviations could be addressed to heterozygote deficit based on high and positive F inbreeding coefficient.

In this analysis, no private band/alleles of cultivated individuals were found for ISSR and SSR markers, instead they displayed a subset of the wild population markers. Wild sunflowers showed nine

ISSRs private band and 21 SSRs private alleles. Some unique markers were determined in particular crop-wild population comparisons (Table 5).

Genetic structure

Principal coordinate (PCO) analysis of the pairwise individual ISSR distance matrices is shown in Fig. 2A. The first two axes explained 45.3 % of the variation. Wild individuals were scattered and grouping according to populations was loose. The PCO based on the population φ_{PT} distance matrices is shown in Fig 2B. The first two axes explained 66.7% of the variation. Inbred lines were placed on an edge close to MAL, ALS and RAN wild samples and therefore a clear-cut separation between cultivated and wild materials was not detected. Similar associations were observed from a dendrogram based on the population φ_{PT} ISSR distance matrix (Figure 2C), through average linkage (r = 0.72). Figure 3A shows PCO analysis of the pairwise individual SSR distance matrix. The first two axes explained 49.1 % of variation, whereas the PCO based on the population φ_{PT} distance matrices explained 58% of the variation (Fig. 3B). Cluster analysis based on the population φ_{PT} SSR distance matrix is shown in Figure 2C (r = 0.88). The general pattern was similar to that obtained with ISSR markers although cultivated materials constituted a more distinct group. In the ISSR and SSR analyses, closely related populations often came from distant locations and all the between-population comparisons displayed significant φ_{PT} except the ALS-CBA comparison based on SSR.

Analysis of molecular variance (AMOVA) for the total marker data set in wild populations is shown in Table 6. Genetic diversity was high within populations. Between-populations variance estimated with ISSR and SSR was significant and around 20% of the total variation, being 19% with ISSR and 22% with SSR. When the AMOVA was calculated considering cultivated and wilds as groups, genetic differentiation between them was significant but differences were observed in the variance distribution depending on the selected marker. A higher between-groups component was obtained using SSR (36%) than ISSR (12%) markers. To evaluate the existence of genetic differentiation patterns, regions established using different criteria were compared through φ_{RT} from AMOVA (Table 6). Non significant differences were observed between regions using ISSR markers. For SSR, we found a significant portion of variance between phytogeographic regions and production zones.

Correlations between matrices

At the individual level, a significant positive low correlation (r=0.108, P=0.021) was found between ISSR and SSR genetic distance matrices. At the population level, population φ_{PT} distance matrices for ISSR and SSR showed a low and positive but non significant correlation (r=0.156, P=0.284). The correlation between ISSR and geographic distance matrices at the individual and population levels by Mantel test was low (r=0.244, P=0.001; r=0.243, P=0.184, respectively). Similar results were found with the correlation between SSR and geographic distance matrices at individual and population levels (r=0.115, P=0.018; r=0.311, P=0.124, respectively).

Discussion

There is scarce ISSR information available for sunflower, therefore the knowledge on optimal amplification conditions and level of polymorphism become useful data for other research. ISSRs markers displayed highly reproducible patterns. Amplification products were obtained using primers with different repeat motif length and with or without anchors bases. Compound primers carrying two or more types of adjacent repeats (i.e primer 19) gave high quality patterns. Gupta et al. (1994) also described the banding pattern dependence on primer sequence and annealing temperature, although he observed that primers based on quadruple repeats were most effective in amplifying DNA markers. Mohsen & Ali (2008) found a similar percentage of polymorphic loci in other Asteraceae using primer here coded as 14. Our experiments confirmed that ISSR patterns are highly modified by the annealing temperature; however 52° C can be suggested as a start point for studies.

Our results demonstrated that different sets of SSR primers developed for multiplex dye labeled analysis can be assembled according to their allele sizes and therefore the routine use of acrylamide silver stain becomes possible.

The finding of crop private alleles for gene flow studies still remains a difficult task. Only 15 of 1341 microsatellite alleles were unique to elite inbred lines (Tang & Knapp 2003) A high degree of enzymatic and cpDNA sequence similarity was observed between wild and domesticated *H. annuus*, and domesticated *H. annuus* contained a subset of the alleles and cpDNAs found in wild *H. annuus* (Rieseberg & Seiler, 1990). No unique ISSR loci or SSR alleles for domestic sunflower were found. Functional polymorphisms such as SNP (Fusari et al., 2008) or EST-SSR markers could be more informative since they can target some specific genes (Heesacker et al., 2008), some of which have experienced strong selection during sunflower breeding process such as seed oil content and composition (Burke et al., 2005) and for which a larger wild-crop divergence is expected.

Despite the absence of general crop-specific alleles, crop-wild gene flow could be studied in some sites, such us MAL, MAG, DIA and RAN populations where several crop markers were absent. Whereas MAG and DIA populations grow far from sunflower crop (Poverene et al., 2008), RAN population constitutes an interesting site to study the long-term effects of crop-wild introgression given its lack of some cultivated alleles and also crop and wild plants co-occur very often.

Several ISSR and SSR unique bands/alleles of wild sunflower were found. These markers become useful tools in the assessment of wild-crop gene flow, and their use would allow the detection of undesirable foreign pollination at hybrid seed production zones, where the presence of wild individuals increase the risk of seed contamination (Reagon & Snow, 2006). The occurrence of several private alleles of wild sunflower demonstrates that these populations do not arise from volunteers.

Hardy-Weinberg equilibrium within population was not found in some cases. Heterozygote deficiency was observed in DIA, CBA, JCE and RAN populations, with positive inbreeding coefficient. This could be addressed to undetected null alleles. In fact, 5% of the total amount of PCR reactions showed absence of the corresponding SSR product. Also, wild plants are highly self-incompatible, but some populations comprise a low number of plants, which favors mating among relatives. On the whole,

inbreeding does not seem to be significant given that most loci showed genotype frequencies close to those expected by equilibrium.

SSR data in wild populations of the centre of origin gave He ca. 0.8 (Tang & Knapp, 2003). Local sunflower populations showed He ca. 0.6, indicating that populations maintain approximately 70% of the original diversity. Moreover, taking into account the observed progressive expansion of the wild sunflower distribution range in Argentina (Cantamutto et al., 2008), wild *H. annuus* may be considered an invasive species with significant adaptive potential. This species is not yet perceived as an agricultural weed in this country, but some *H. annuus* populations in North America have evolved towards weedy forms from nearby wild populations and persist in agricultural systems (Kane & Rieseberg, 2008). The rapid adoption of herbicide tolerant hybrids in Argentina points to the need of understanding wild-crop interaction, in order to prevent an environmental and economic impact due to the increased use of herbicides.

Since two independent and different marker classes gave similar molecular variance distribution, ca. 20% seems to be a confident estimate of genetic differentiation between wild populations. SSR markers were more efficient than ISSR markers in finding differences between wild and inbred lines (36 vs. 12%, $P<0,001$).

Multivariate analysis of molecular variability showed that individuals from the same population do not form definite groups. The similarity between some wild and cultivated materials might be a consequence of gene flow from adjacent sunflower fields, either in local conditions or in the centre of origin, although similarity by ancestry cannot be rejected. A significant genetic differentiation between populations via AMOVA shows some genetic structure for *H. annuus* in Argentina. Most variation was found within populations, as it is expected for an allogamous, self-incompatible and insect pollinated species (Hamrick & Godt, 1989). Yatabe et al. (2007) reported similar results in the centre of origin, finding most genetic diversity (ca. 93%) within populations. Gene flow between *H. annuus* and a second annual wild species, *H. petiolaris*, also naturalized in Argentina could be another source of the observed diversity. Using SSR and EST sequences, Kane et al. (2009) observed that in spite of prominent morphological, ecological, reproductive and karyotypic differences between *H. annuus* and *H. petiolaris* populations in the US, the extent of gene flow between these species was surprising. Gutierrez et al. (2010) showed that under local agro-ecological conditions inter-specific hybridization is a frequent event.

A low genetic-geographic correlation is generally attributed to multiple introductions effects (Wang et al., 2007). A lack of geographical signature in the pattern of population genetic variation can also be explained by human activities on seed dispersal and genetic drift (Knapp & Rice, 1998), basically associated to population size or founder effect. In the present study, the lack of relationship between genetic and geographical data agrees with that observed using morphological traits (Cantamutto et al., 2010). However, some populations in close proximity were found to be genetically similar which could reflect a common origin or the occurrence of gene flow. The significant variation between phytogeographic zones could indicate an undergoing adaptive process of wild populations to different climate, soil and agro-ecological conditions. To elucidate this process, studies using morpho-physiological traits directly modulated by natural selection would be needed.

It has been proposed that independent different marker systems increase their correlation values from individual to group, i.e populations, subspecies, since the different markers delineate the same evolutionary important groups (Peakall et al., 1995; Powell et al., 1996; Maguire et al., 2002). Our data showed low congruence between ISSR and SSR genetic distances at both individual and population comparisons. Therefore, separate or divergent evolutionary groups are not evident in local wild sunflower.

Implications for conservation

Domestication and breeding have dramatically narrowed genetic diversity in sunflower from wild populations to elite oilseed inbred lines (Tang & Knapp, 2003). The molecular variability in Argentinean wild sunflower was approximate 60% higher than in inbred lines. Several genetic criteria have been used to identify both germplasm accessions and natural populations of high conservation value: i) allele richness or number of alleles per locus, ii) locally common alleles, defined as alleles that are common in one to several populations but not at the species as a whole and iii) number of private or unique alleles (Marshall & Brown, 1975; Brown & Briggs, 1991). The allelic structure of wild sunflower populations obtained with these criteria is shown in Table 7. Based on these values the JCE population combines high values for all those criteria, followed by CBA, LCA, CAR and DIA. Based on typical diversity measures shown in Table 3, the highest score corresponds to JCE, CAR, CBA, ALS and RCU. In fact, RCU and JCE are localized in the area supposed to be the first entry point of the species in Argentina (Bauer, 1991). The presence of private bands in wild populations in comparison to inbred lines, points to JCE, LCA, CAR and CBA populations. Combining all the listed criteria, the JCE, CBA, LCA and CAR populations constitute interesting targets for more extensive sampling.

Conclusions

This study demonstrated that wild *H. annuus* of Argentina constitutes an important reservoir of genetic variability. The high level of genetic diversity and the lack of correlation between geographical and genetic distance address to a multiple event hypothesis for the introduction of wild *H. annuus* in Argentina or to novel recombination. Considering their genetic variability and habitat diversity adaptation, wild populations could be a source of novel genes for breeding and therefore valuable for conservation and breeding programs. At present, local populations are being evaluated for variability in cold and drought tolerance, virus resistance, male sterility and functional markers polymorphisms, in order to assess a complete germplasm characterization.

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Table 1: Sequences and amplification results for ten ISSR primers, for six sunflower SSR markers assembled into two three-plexes PCR-multiplex (Sets A y B) and for an additional sunflower SSR marker. MB: multi band pattern, FB: few band pattern, S: smear, C: clear pattern, U: unclear pattern.

ISSR[1]	Sequences	Results	SSR[1]	Sequences	pmol[2]	Results
4	$(GA)_9T$	MB-S	**Set A**			
6	$(CA)_6AC$	FB-S	ORS-297-F	GTGTCTGCACGAACTGTGGT	6	C
7	$(CT)_8TG$	FB-C	ORS-297-R	TGCAAAGCTCACACTAACCTG	6	
8	$(GTG)_3GC$	MB-C	ORS-456-F	CCAAGGAATTCTAACAAGAGTTTAAG	8	C
9	$(CAA)_5$	MB-C	ORS-456-R	GATTTCTCACTTCACTCCTCTATGC	8	
10	$(AGC)_5$	MB-C	ORS-1222-F	GGCATTGTTGTCATTTCATCTCT	8	C
14	$(CAC)_4GC$	MB-C	ORS-1222-R	ATCATGTCGGAATAGCTTGTTGA	8	
16	$(GACA)_4$	MB-C	**Set B**			
19	$(GATA)_2(GACA)_2$	MB-C	ORS-342-F	TGTTCATCAGGTTTGTCTCCA	8	C
20	$(ACTG)_2ACCGACTG$	MB-C	ORS-342-R	CACCAGCATAGCCATTCAAA	8	
			ORS-533-F	TGGTGGAGGTCACTATTGGA	14	C
			ORS-533-R	AGGAAAGAAGGAAGCCGAGA	14	
			ORS-543-F	CCAAGTTTCAGTTACAATCCATGA	8	U
			ORS-543-R	GGTCATTAGGAGTTTGGGATCA	8	
			Additional loci			
			ORS-399-F	CGTACGGTGTAGTTCTCATGGT	5.5	U
			ORS-399-R	GGATCACGTGGCGTTCTATT	5.5	

[1]ISSR, inter-simple sequence repeat; SSR, simple sequence repeats.

[2] Concentrations for SSR primers.

Table 2: Genetic diversity parameters for ISSR and SSR markers loci in *Helianthus annuus*. Total number of loci (T), polymorphic loci (P), percentage of polymorphic loci (%P), SSR allele number (A), expected (He) and observed (Ho) heterozygosity between inbred lines ($_I$), wild populations ($_W$) and the whole sample ($_T$).

ISSR	**T**	**P_I**	**P_W**	**P_T**	**$\%P_I$**	**$\%P_W$**	**$\%P_T$**	**He_I**	**He_W**	**He_T**
9	15	7	12	12	46.7	80	80	0.244	0.382	0.385
14	8	1	7	7	12.5	87.5	87.5	0.070	0.426	0.422
16	10	2	9	9	20	90	90	0.100	0.402	0.398
19	16	5	14	14	31.25	87.5	87.5	0.121	0.353	0.350
20	15	5	12	13	33.33	80	86.7	0.122	0.324	0.335
All loci	64	20	54	55	31.25	84.38	85.9	0.138	0.370	0.371

SSR	**A_I**	**A_W**	**A_T**	**He_I**	**He_W**	**He_T**	**Ho_I**	**Ho_W**	**Ho_T**
ORS-297	3	7	7	0.611	0.775	0.782	0.000	0.638	0.600
ORS-456	1	2	2	0.000	0.339	0.387	0.000	0.227	0.214
ORS-1222	1	4	4	0.000	0.247	0.234	0.000	0.194	0.182
ORS-342	1	7	7	0.000	0.737	0.728	0.000	0.489	0.460
ORS-533	2	9	9	0.278	0.855	0.851	0.000	0.588	0.553
All loci	8	29	29	0.178	0.591	0.596	0.000	0.427	0.402

Table 3: Mean genetic diversity parameters for ISSR and SSR polymorphic markers in 10 wild populations and a set of inbred lines (CUL) of *Helianthus annuus*. Allele number (A), percentage of polymorphic loci (P), expected heterozygosity (He) and observed heterozygosity (Ho). Standard errors are in parentheses. ALS: Alsina, MAL: Malvinas, DIA: Diamante, RAN: Rancul, MAG: Media Agua, CBA: Colonia Barón, JCE: Juarez Celman, RCU: Río Cuarto, CAR: Carhue, LCA: La Carlota, CUL: Sunflower inbred lines.

		ALS	**MAL**	**DIA**	**RAN**	**MAG**	**CBA**	**JCE**	**RCU**	**CAR**	**LCA**	**CUL**
A	ISSR	1.49 (0.09)	1,42 (0.09)	1.56 (0.09)	1.36 (0.09)	1.40 (0.10)	1.60 (0.08)	1.66 (0.07)	1.56 (0.09)	1.64 (0.08)	1.55 (0.10)	1.20 (0.10)
	SSR	3.60 (0.68)	2.40 (0.25)	2.40 (0.40)	2.40 (0.40)	3.00 (0.84)	3.80 (0.80)	4.00 (0.84)	3.40 (0.87)	3.40 (0.75)	3.60 (0.93)	1.6 (0.40)
P	ISSR	60	53	64	47	53	64	69	67	69	60	36
	SSR	100	100	100	80	80	100	100	80	100	100	40
He	ISSR	0.27 (0.03)	0.21 (0.03)	0.28 (0.03)	0.18 (0.03)	0.22 (0.03)	0.24 (0.03)	0.26 (0.03)	0.28 (0.03)	0.27 (0.03)	0.23 (0.03)	0.14 (0.03)
	SSR	0.52 (0.10)	0.44 (0.06)	0.33 (0.09)	0.38 (0.14)	0.46 (0.15)	0.52 (0.12)	0.63 (0.07)	0.44 (0.14)	0.48 (0.13)	0.53 (0.10)	0.18 (0.12)
Ho	SSR	0.52 (0.15)	0.51 (0.12)	0.21 (0.05)	0.22 (0.12)	0.45 (0.17)	0.32 (0.12)	0.46 (0.12)	0.41 (0.15)	0.54 (0.14)	0.57 (0.07)	0.00 (0.00)

Table 4: Summary of chi-square tests for Hardy-Weinberg equilibrium and inbreeding coefficient (F) by locus and by population, estimated from SSR markers in natural populations of *Helianthus annuus*. DF: degrees of freedom, Mon: monomorphic. ALS: Alsina, MAL: Malvinas, DIA: Diamante, RAN: Rancul, MAG: Media Agua, CBA: Colonia Barón, JCE: Juarez Celman, RCU: Río Cuarto, CAR: Carhue, LCA: La Carlota.

Pop	**Locus**	**DF**	**ChiSq**	**F**	**Pop**	**Locus**	**DF**	**ChiSq**	**F**
	ORS-297	10	20.78*	-0.189		**ORS-297**	10	24.00**	0.495
	ORS-456	1	5.38*	0.733		**ORS-456**	1	9.00**	1.000
ALS	**ORS-1222**	1	0.14	-0.125	**CBA**	**ORS-1222**	1	0.03	-0.053
	ORS-342	10	12.39	0.007		**ORS-342**	6	1.39	-0.101
	ORS-533	6	4.95	-0.194		**ORS-533**	15	20.46	0.359
	ORS-297	3	6.69	-0.513		**ORS-297**	15	16.94	-0.046
	ORS-456	1	1.33	-0.385		**ORS-456**	1	0.28	0.167
MAL	**ORS-1222**	1	0.26	-0.169	**JCE**	**ORS-1222**	1	6.09*	0.780
	ORS-342	1	0.31	-0.176		**ORS-342**	10	25.56**	0.441
	ORS-533	3	4.81	0.532		**ORS-533**	10	15.96	0.195
	ORS-297	1	5.28*	0.766		**ORS-297**	6	4.44	-0.416
	ORS-456	1	0.03	-0.053		**ORS-456**	1	0.14	-0.125
DIA	**ORS-1222**	1	0.63	-0.250	**RCU**	**ORS-1222**	Mon		-
	ORS-342	1	0.12	-0.111		**ORS-342**	6	9.98	0.422
	ORS-533	6	15.26*	0.613		**ORS-533**	15	16.47	0.129
	ORS-297	3	5.88	0.389		**ORS-297**	6	3.14	-0.022
	ORS-456	1	0.03	-0.053		**ORS-456**	1	0.31	-0.176
RAN	**ORS-1222**	Mon		-	**CAR**	**ORS-1222**	1	0.03	-0.053
	ORS-342	3	14.00**	1.000		**ORS-342**	3	4.44	-0.441
	ORS-533	3	0.35	-0.026		**ORS-533**	15	18.40	0.000
	ORS-297	3	6.11	-0.248		**ORS-297**	21	21.56	0.188
	ORS-456	1	3.70	0.608		**ORS-456**	1	0.63	-0.250
MAG	**ORS-1222**	Mon		-	**LCA**	**ORS-1222**	1	0.63	-0.250
	ORS-342	3	4.99	-0.009		**ORS-342**	6	2.74	-0.176
	ORS-533	15	15.86	0.103		**ORS-533**	3	1.12	-0.138

*P<0.05, **P<0.01.

Table 5: Number of privates bands/alleles (NP) of wild and cultivated sunflower at the population level. ALS: Alsina, MAL: Malvinas, DIA: Diamante, RAN: Rancul, MAG: Media Agua, CBA: Colonia Barón, JCE: Juarez Celman, RCU: Río Cuarto, CAR: Carhue, LCA: La Carlota.

NP		**ALS**	**MAL**	**DIA**	**RAN**	**MAG**	**CBA**	**JCE**	**RCU**	**CAR**	**LCA**
Wild populations	ISSR	5	5	7	4	6	6	8	4	6	6
	SSR	10	8	6	6	9	11	13	11	11	13
Inbred lines	ISSR	2	2	2	2	3	0	2	2	0	1
	SSR	0	3	2	2	2	0	1	2	2	2

Table 6: Analysis of molecular variance (AMOVA) and sources of variation for wild *Helianthus annuus* populations. φ_{PR}, φ_{RT} and φ_{PT} defined in Materials and Methods.

Region criteria	**None**		**phytogeographic region ***		**production zones****		**Sympatric with the crop**	
	SSR	ISSR	SSR	ISSR	SSR	ISSR	SSR	ISSR
% Variance	%	%	%	%	%	%	%	%
Between Regions	-	-	5	0	5	0	0.3	0
Between pops./regions	22	19	18	20	19	20	21.7	20
Indiv./within Pops	78	81	77	80	76	80	78	80
φ_{RT}	- -	- -	0.047 P=0.001	0 P=0.984	0.052 P=0.002	0 P=0.987	0.003 P=0.315	0 P=0.596
φ_{PR}	- -	- -	0.191 P=0.001	0.192 P=0.001	0.203 P=0.001	0.187 P=0.001	0.218 P=0.001	0.194 P=0.001
φ_{PT}	0.217 P=0.001	0.195 P=0.001	0.229 P=0.001	0.192 P=0.001	0.245 P=0.001	0.187 P=0.001	0.220 P=0.001	0.194 P=0.001

* Pampa, Espinal and Monte, **According to Ministry of Agriculture, Livestock, Fisheries and Food of Argentina.

Table 7: Summary of genetic criteria used to identify natural populations of high conservation value estimated from polymorphic marker loci. N: number of bands/alleles, NL: number of locally common bands/alleles (<=50%), NP: number of privates bands/alleles. ALS: Alsina, MAL: Malvinas, DIA: Diamante, RAN: Rancul, MAG: Media Agua, CBA: Colonia Barón, JCE: Juarez Celman, RCU: Río Cuarto, CAR: Carhue, LCA: La Carlota.

		ALS	**MAL**	**DIA**	**RAN**	**MAG**	**CBA**	**JCE**	**RCU**	**CAR**	**LCA**
N	ISSR	49	49	51	49	48	53	53	49	52	52
	SSR	18	12	12	12	15	19	20	17	17	18
NL	ISSR	1	0	2	2	1	2	3	1	1	2
	SSR	5	3	2	2	4	7	6	6	6	6
NP	ISSR	0	0	0	0	0	0	0	0	0	0
	SSR	1	0	1	0	0	0	1	0	1	0

Figure legends:

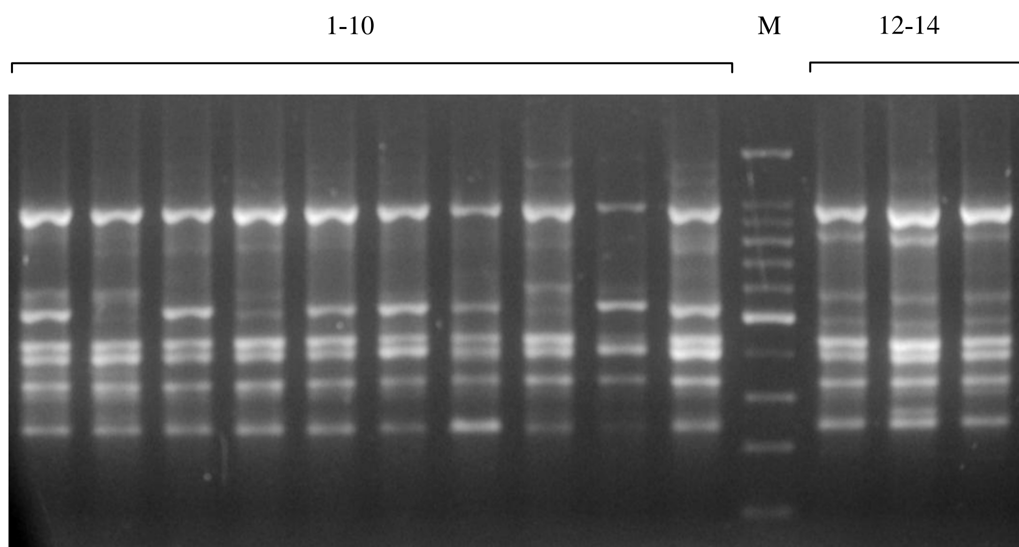
Figure 1. A: Patterns of ISSR markers amplified with primer 20: 1-10 wild RCU plants; 12-14 inbred lines HA367, HAR2, and HA89. **B:** SSR alleles for ORS-533 and ORS-342 loci: 1 to 41 wild plants from RAN (1-10); MAG (11-20); CBA (21-30); RCU (31-40); 42-47 inbred lines HA367, HAR274, HAR2, HAR3, HAR5 and HA89. M: 100 bp ladder.

Figure 2. A: PCO plots based on the individual ISSR distance matrix. **B:** PCO plots based on the population (φ_{PT}) ISSR distance matrix. **C:** UPGMA dendrogram based on the population (φ_{PT}) ISSR distance matrix. ALS: Alsina, MAL: Malvinas, DIA: Diamante, RAN: Rancul, MAG: Media Agua, CBA: Colonia Barón, JCE: Juarez Celman, RCU: Río Cuarto, CAR: Carhue, LCA: La Carlota, CUL: Sunflower inbred lines.

Figure 3: A: PCO plots based on the individual SSR distance matrix. **B:** PCO plots based on the population (φ_{PT}) SSR distance matrix. **C:** UPGMA dendrogram based on the population (φ_{PT}) SSR distance matrix. ALS: Alsina, MAL: Malvinas, DIA: Diamante, RAN: Rancul, MAG: Media Agua, CBA: Colonia Barón, JCE: Juarez Celman, RCU: Río Cuarto, CAR: Carhue, LCA: La Carlota, CUL: Sunflower inbred lines.

Figure 1.

A)



B)

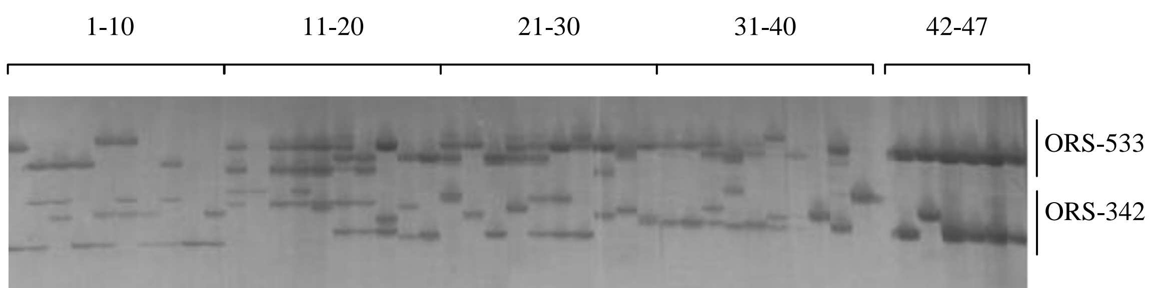


Figure 2.

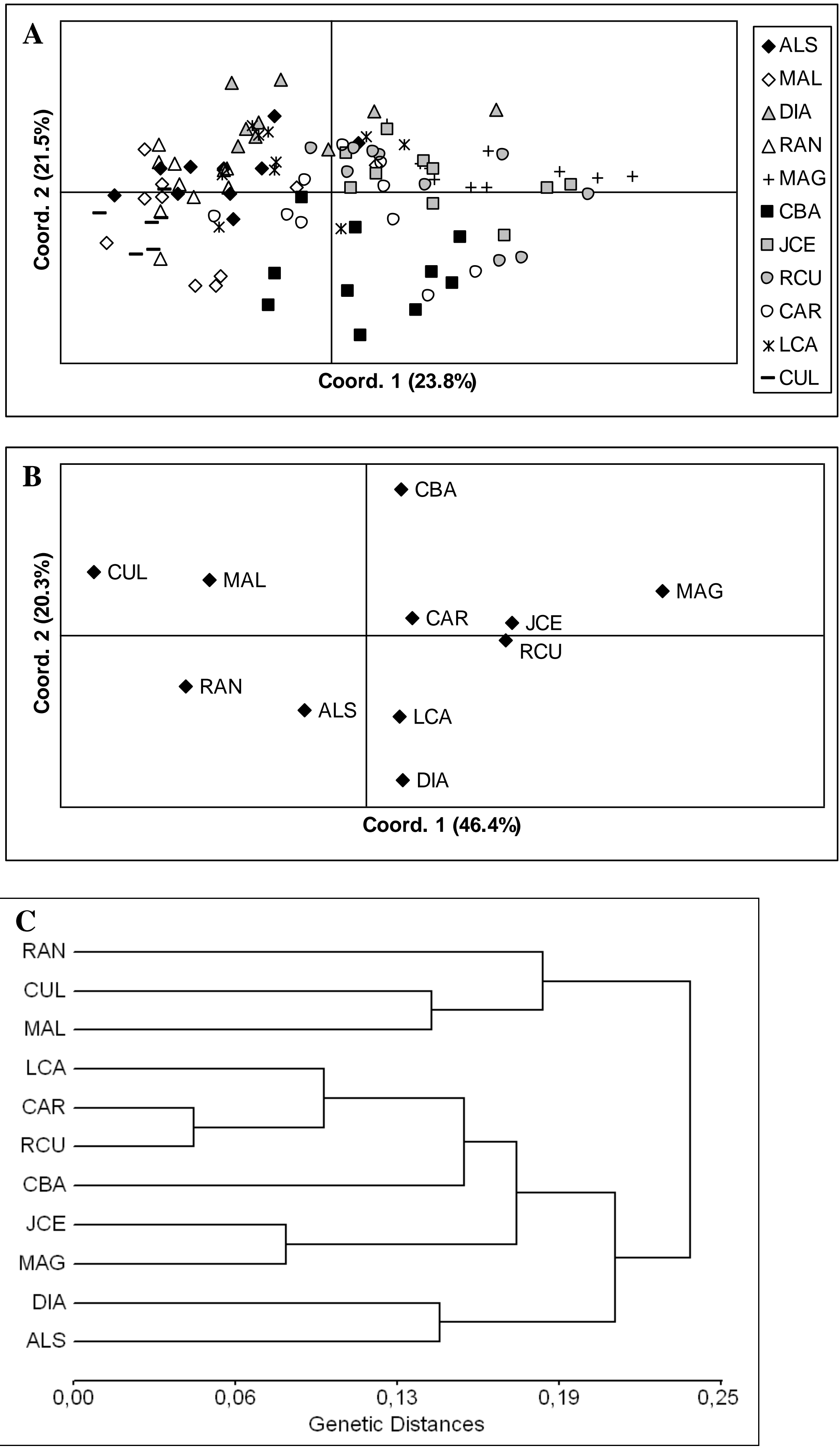


Figure 3: