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Morphology and SSR fingerprinting of newly developed *Cynara cardunculus* genotypes exploitable as ornamentals

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Summary

The species *Cynara cardunculus* includes the globe artichoke (var. *scolymus*), the cultivated cardoon (var. *altilis*) and the wild cardoon (var. *sylvestris*). The three *taxa* are sexually compatible and originate fertile F1 progenies, which, given the high heterozygosity of the species, are highly segregating. We report the characterization of two F_1 populations, one bred from a cross between globe artichoke and cultivated cardoon, and the other between globe artichoke and wild cardoon. Both populations featured a wide array of phenotypes in relation to several traits, and some of the newly developed genotypes are of interest for the ornamental market. The two populations were genotyped at 50 microsatellite (SSR) loci: in the globe artichoke x wild cardoon and globe artichoke x cultivated cardoon progenies 116 and 97 alleles were respectively detected. SSR pattern scores were used to produce an UPGMA dendrogram and a PCoA plot. A set of nine SSR loci, evenly dispersed across the genome, was shown to be sufficient to unambiguously identify each segregant. The molecular fingerprinting is useful for establishing the true to type correspondence of propagative materials in nurseries and ensures the effective correspondence between the real and the declared identity of a clone.

Key words: *Cynara cardunculus*, ornamentals, phenotypic diversity, molecular fingerprinting

Introduction

The *Asteraceae* family includes a number of popular ornamentals, such as ageratum, aster, chrysanthemum, dahlia, marigold, zinnia, calendula and gerbera. Other members of the family have utility as leaf vegetables (lettuce, endive), and a further group is exploited as a source of comestible oil (sunflower, safflower). *Cynara cardunculus* var. *scolymus* (the globe artichoke), along with its close relatives the cultivated (var. *altilis*) and the wild (var. *sylvestris*) cardoon, also belongs to this botanical group.

The immature capitula of the globe artichoke are for the most part consumed as a vegetable, and as a result, most commercial varieties are classified on the basis of capitulum appearance. Some varieties are particularly suitable for fresh consumption, but others are more appropriate for industrial transformation (Lanteri et al. 2004a; Lanteri and Portis, 2008; Mauro et al. 2011). The cultivated cardoon is mainly grown in Southern Europe, where its young leaves form an ingredient of certain traditional dishes. DNA fingerprinting has confirmed that the two cultivated forms must have evolved independently through anthropogenic selection from the wild cardoon (Lanteri et al. 2004b; Portis et al. 2005a); and the assumption is that the most likely location of the earliest selection activity was in Sicily (Mauro et al. 2008)

Other than as a vegetable, the species has a wide range of uses, its lignocellulosic biomass has been proposed as a potential source of bioenergy, its seed oil is similar in composition to that of both safflower and sunflower (Foti et al. 1999; Ierna and Mauromicale 2010; Maccarone et al. 1999), and the plant produces a number of pharmaceutically active compounds, like phenolic esters, sequiterpenes and inulin (Comino et al. 2009; Comino et al. 2007; Lattanzio et al. 2009; Lombardo et al. 2010; Menin et al. 2010; Pandino et al. 2010).

The various *C. cardunculus* taxa feature a range of plant architecture. The foliage varies in colour from green to ash-grey, plant height can reach 3 m, there is a variable amount of branching (Porceddu et al. 1976), and the number of inflorescences (capitula) per plant ranges from five to 15 in the globe artichoke and from 30 to 40 in cultivated cardoon. The main capitulum is invariably the largest. The immature capitula are polymorphic with respect to size and shape; some develop spiny outer bracts of various shades of green, and which later during development may turn purple (Cravero et al. 2005; Pochard et al. 1969). The mature capitulum can be white or violet.

Beyond all the possible cited uses, *C. cardunculus* is also exploited as ornamental, both as garden plants and as cut flowers (Cocker 1967): fresh specimens have a long vase life, while dried forms are popular in floral arrangements. The use of the species as ornamental is increasing since, notwithstanding the assortment of ornamental crops is already very large, novelty are constantly in demand by consumers.

The highly heterozygotic nature of the species produces a wide range of phenotypes, especially among progeny of crosses between the different taxa (Cravero et al. 2005; Foury 1969; Lopez-Anido et al. 1998; Mauromicale and Ierna 2000).

Molecular markers represent a reliable alternative to morphological descriptors for varietal identification, since they are not influenced by the growing environment, by the developmental stage of the plant, or by the identity of the tissue (Collard et al. 2005). Microsatellites (SSRs) are particularly suited to DNA fingerprinting as well as for identifying potential parental genotypes for highly segregating populations development, given their robustness and informativeness. Over the last few years, a substantial set of globe artichoke SSR assays has been developed (Acquadro et al. 2003; 2005a; 2005b; 2009), and these have been used to assess genetic diversity (Mauro et al.

2008; Portis et al. 2005a; 2005b; 2005c) and to construct genetic linkage maps (Lanteri et al. 2006; Portis et al. 2009).

Here we report on phenotypic diversity released in progenies obtained by crossing a genotype of globe artichoke with both one of cultivated and one of wild cardoon, as well as on the SSR-based molecular fingerprinting of the 184 newly generated genotypes, with a particular focus on segregants showing potential for ornamental use.

Materials and Methods

Plant material and DNA isolation

Two F_1 populations were created, both involving the globe artichoke clone 'Romanesco C3' as female; the male parents were the cultivated cardoon genotype 'Altilis 41' (Progeny 1), and the wild cardoon accession 'Creta 4' (Progeny 2). 'Romanesco C3' is a late-maturing variety, which forms large purple-green capitula (each weighing up to 400g); its mature capitulum develops violet coloured florets. 'Altilis 41' is a selection made by the University of Catania on the basis of its high biomass yield potential; its foliage is grey and its florets white. 'Creta 4' was collected from a population in Crete; it produces a large number of capitula, forms green-violet bracts and violet florets. A few days before anthesis, the outer bracts of the parental capitula were removed and the remaining structure bagged. Pollen was collected from the male parent and stored at 3±1° C for up to three days. Prior to the hybridization itself, the female capitulum was rinsed in tap water, and after 2 h the pollen was applied with a soft brush. The bag was then replaced over the capitulum and kept in place until the achenes had matured (~40 days). Ripe achenes were extracted from dry heads with a mini-thresher.

The mature achenes were germinated in moist peat, and after 50 days, surviving seedlings (154 from each cross), which by then had developed four true leaves, were transplanted into the field, each spaced 1m from its nearest neighbour. DNA was extracted from the young plants following (Lanteri et al. 2001), and used to confirm hybridity by SSR genotyping. A selection of 188 true hybrids (94 from Progeny 1 and 94 from Progeny 2) was made from the confirmed ones.

Assessment of morphology and segregations of traits

The morphology of the hybrid individuals and three vegetatively propagated plants of each parental genotype was assessed over two seasons. The assessment included traits of interest for ornamental use like the measurement of plant height, the distance between the first basal leaf and the upper tip of the main capitulum, the number of days to flowering, the duration of flowering (the number of days between the blooming of the main and the last-produced capitulum), capitulum colour, floret colour, the number of capitula per plant, the maximum longitudinal and transverse diameter of the fully developed main capitulum, and a main capitulum shape index (the ratio between the two previously mentioned diameters).

The goodness-of fit between observed and expected segregation data according to previously proposed inheritance models for the heads colour, florets colour and presence of spines was assessed using the chi-square (χ^2) test.

SSR fingerprinting

An initial set of 93 genomic SSR assays developed in our laboratory (Acquadro et al. 2003; 2005a; 2005b; 2009) was used to establish informativeness by amplifying the

DNA of the three parents and 6 plants of each progeny. Each 20 µl PCR contained 10ng genomic DNA, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 U Taq polymerase, 0.2 mM dNTP, 200 nM unlabelled reverse primer and 200 nM IRD700-labelled forward primer. The touchdown PCR protocol reported by Acquadro et al. (2005a) was applied. The resulting amplicons were separated by denaturing 6% polyacrylamide gel electrophoresis using a LI-COR Gene ReadIR 4200 device, as described by Jackson and Matthews (2000).

Fifty primer pairs identifying polymorphism in both segregating populations were applied to the full set of genotypes in study, following the protocol reported above.

Amplicon sizes were estimated from the migration of an IRD700-labelled 50-350 bp ladder. The SSR data were collected by e-Seq software (DNA Sequencing and Analysis Software) v3.0 and analysed using the GenAlex Excel package (Peakall and Smouse 2006). A co-phenetic distance matrix was generated as described by Smouse and Peakall (1999) and used to construct a UPGMA-based dendrogram (Sneath and Sokal 1973) within the NTSYS software package v2.10 (Rohlf 1998). A principal coordinate analysis (PCoA) was then performed, based on the triangular matrix of genetic similarity estimates, and the two axes were plotted graphically, according to the extracted Eigenvectors.

The minimum number of SSR loci needed to fully discriminate all individuals was determined first by selecting the set of most informative SSRs; subsequently, a second set was identified on the basis of the location of the SSRs in different linkage groups on the reference genetic linkage map (Portis et al. 2009). Mantel tests (Mantel 1967) were performed to establish correlations between the similarity matrices generated by the two sets of SSRs with the one generated from the complete data set.

Results and Discussion

Phenotypic variation within the F_1 populations

The two progeny sets showed an astonishing phenotypic variation (Figure 1), with some individuals, the result of specific events of chromosomal segregation and recombination, displaying aspects of morphology not previously observed in either cultivated or wild types; some of these may have high potential in the context of developing ornamental varieties of the species.

Progeny 1 showed the most variability in terms of flowering time (203-242 days), vegetative growth, plant height (56-127cm) and the number of capitula per plant (1-32) (Table 1). Plant height may be a trait of interest for garden plants but not for cut flowers; on the other hand both branching of the floral stem and the duration of flowering period, which varied from 48 to 55 days (Table 1) in both progenies, are key traits for both end-uses, since they both influence the profitability of a genotype grown for cuttings as well as its aesthetic value in a garden.

In both sets of progenies, capitulum shape was either round (shape index <1.1) or partially elongated and conical (shape index 1.2-2.0) (Table 1, Figure 1). Both the longitudinal and transverse diameter of the capitulum were variable (in Progeny 1, the former by 6.0 cm and the latter by 4.6 cm; in Progeny 2 by, respectively 4.2 cm and 4.8 cm; see Table 1). Considerable variation was also released for the intensity of capitulum pigmentation, a character known to be particularly sensitive to temperature within cultivated germplasm.

The current genetic model for capitulum colour assumes the presence of two dominant genes, *P* and *U* (Cravero *et al.* 2005): *P*_ allows anthocyanin production,

resulting in purple bracts, while *pp* inhibits anthocyanin production resulting in green bracts; *U_* results in an uneven distribution of anthocyanin pigments encoded by *P*, while *uu* results in an even distribution of pigment in the presence of *P*. Among the two F_1 populations it was possible to identify the predicted three classes of capitulum colouration (uneven purple, even purple and green) segregating overall in the expected digenic ratio of 9:4:3 (Table 2). Superimposed on this pattern was a gradation in capitulum colour, along with streaks of different colour intensity, suggesting the existence of pigmentation modifier genes.

At flowering the capitula produce 600-1500 hermaphroditic florets. Anthesis begins at the periphery and moves, over the subsequent 3-5 days, into the centre. As the floret opens, the stigma elongates through the tube of dehiscing anthers.

The blue-violet colour of the style and stigma was monomorphic in both populations, despite the fact that the cultivated cardoon parent's florets are white (Figure 1). Floret colour is known to be a monogenic trait in which the blue-violet type is the product of the dominant allele at *B* (Basnitzki and Zohary 1994; Foury and Aubert 1977). Hence, also on the basis of our previous observation, the likely allelic state of the three parental lines was *BB* in 'Romanesco C3', *bb* in 'Altilis 41' and *BB* or *Bb* in 'Creta 4'.

Spiny and non spiny types were represented in both progeny sets; this trait is also under monogenic control, with the dominant allele (*Sp*) specifying the non-spiny trait (Lanteri et al. 2006). Both the globe artichoke and cultivated cardoon parent were non-spiny, while the wild cardoon was spiny. The 1:1 (50 spiny, 44 non-spiny) segregation among Progeny 2, and the lack of any segregation in Progeny 1 showed that the allelic constitution of the globe artichoke must have been *Spsp*, that of the cultivated cardoon

SpSp and that of the wild cardoon *spsp* (Table 2). In both progeny sets, some of the non-spiny types developed small thorns on both the leaves and capitula, while in others, small thorns developed only on the leaves, leaving the apex of the bracts flat or even indented with a small thorn in its centre (data not shown). The absence of thorns (or, if they are present, then they are small and soft) is clearly a positive trait in terms of handling the plant for cut flower purposes.

Leaf shape and upper and lower leaf surface tomentosity were also highly variable in both progeny sets. The former varied from deeply divided to lobed, while the intensity of tomentosity strongly influenced leaf colour, which ranged from an ash-grey (typical of the wild cardoon) to deep green (like that of the leaves of the globe artichoke) (data not shown).

SSR-based genotyping

Cross-pollination in the globe artichoke is largely assured by protandry, but a degree of self-pollination does occur, because the stigma remains receptive to pollen for 4–5 days after pollen shed, and so peripheral florets can be fertilized by pollen from central florets. Self-pollination can also occur via pollen transfer between asynchronous capitula produced by the same plant. Thus it was important to verify the hybridity of the progeny obtained by cross-pollination, and this was achieved using SSR profiling at two informative loci.

All 93 SSR assays initially applied generated a profile consisting of either one or two alleles per template, as expected from a diploid species, and the allelic constitution. Among them, 50 loci segregated in both F_1 populations for at least one parent and were then applied to the full set of hybrid individuals (Table 3). Within Progeny 1, the

segregation of 17 loci was consistent with a 1:1:1:1 ratio; of these, 12 displayed a four allele segregation (segregation type <ab x cd>), and the other five a three allele segregation (<ab x ac>). One locus was consistent with a 1:2:1 ratio (segregation type <ab x ab>) while the remaining 32 loci segregated within only one of the parents, producing a segregation pattern consistent with a 1:1 ratio (Table 3). Within Progeny 2, 25 loci proved to be heterozygous in both the parents, all of them segregating in a 1:1:1:1 ratio (16 with four alleles and nine with three alleles); the other 25 loci segregated within only one of the parents (1:1 ratio). As also noted by Portis et al. (2009), the level of heterozygosity in the cultivated cardoon was less than in the wild cardoon, and so more of the loci segregated only for the globe artichoke alleles (Table 3).

The segregation analysis also revealed a number of null alleles, which occur at SSR loci when one (or both) primer annealing sites are lost by mutation or deletion (Jones et al. 1998; Holm et al. 2001; Pemberton et al. 1995). The effect of null alleles is to over-estimate the frequency of homozygosity, since it becomes no longer possible to discriminate homozygotes from heterozygotes (Pekkinen et al. 2005). In this situation, the options are either to disregard the affected loci, to score segregation in the same way as for a dominant marker (Rodzen and May 2002), to attempt to redesign the primers (Shaw et al. 1999; Van Oosterhout et al. 2004), or to adjust allele frequencies on the basis of a global estimate of the frequency of null alleles. However, for the present populations, it was generally possible to identify the presence of null alleles. Within Progeny 2, null alleles were inferred at 12 of the 50 SSR loci. As described in Figure 2, seven of these loci displayed the pattern named a) and five pattern b). Patterns c) and d) detected monomorphic null alleles, and thus were not taken into consideration. The null

alleles at two of the SSR loci displayed a segregation pattern which was ambiguous (Figure 3) and so were excluded from any further analysis. Within Progeny 1, null alleles were identified at just five of the 50 loci, presumably as a consequence of the lower phylogenetic distance between the two parental genotypes. Indeed the highest similarity value detected between pairs of genotypes in the Progeny 1 was 0.73 while in the Progeny 2 it was 0.82.

On the basis of the whole 50 SSR primers set it was possible to obtain a specific molecular fingerprinting for each individual in both segregating progenies. The initial attempt to identify the minimum number of SSR loci needed to fully discriminate between all individuals was based on a selection of 16 SSRs at which four alleles were detected in Progeny 2 (Table 3). These were used to create a similarity matrix between each individual; the most similar pair was 78% similar to one another; the correlation between this matrix and the total similarity matrix indicated a good fit between these two representations of the genetic relationships ($r = 0.87$).

Nine of the 16 SSRs (CELMS-01, -15, -23, -24, -37, -41, -58 and -60, and CMAL-21) were then chosen on the basis that these loci are dispersed across different linkage groups (Portis et al. 2009). The similarity matrix obtained from this set was well correlated with the one obtained using the whole data set ($r = 0.78$). The most similar pair of individuals from Progeny 1 were 90% similar to one another, while those from Progeny 2 were 85% similar to one another. The nine SSR loci were able to fully discriminate all members of both progeny sets. The UPGMA-based clustering (Figure 4) separated the genotypes into two major clades, corresponding to the two F1 populations, with the common parent (‘Romanesco C3’) at an intermediate position.

The first two axes of the PCoA scatter plot (Figure 5) explained, respectively, 39% and 23% of the overall genetic variation; the former largely discriminated between the two populations and highlighted the higher variability present within Progeny 2. Indeed in Progeny 2 a molecular fingerprinting of each genotype might be obtain by applying only 7 (i.e. CELMS-01, 15, 24, 37, 41, 58 and CMAL-21) of the 9 selected SSRs. A further reduction in the number of SSR loci needed for molecular fingerprinting could be possible if the focus is restricted to types suitable for the development of ornamental varieties.

Conclusions

Here we have reported the release of a large amount of phenotypic diversity by crossing the globe artichoke with either the wild or the cultivated cardoon. On the basis of our previous studies, due to the heterozygotic nature of the species, highly segregating progenies may be also obtained following selfing or by crossing spiny with non spiny globe artichoke varietal types; however, the amount of released phenotypic diversity is by far less pronounced than the one we observed in our inter-taxa progenies. A number of the segregants displayed aspects of morphology which have high potential as ornamentals. A particular advantage of this species is that any individual can be readily immortalised by vegetative propagation, either by isolating basal growing shoots or semi-dormant shoots which develop on the underground stem. Indeed, a set of thirty of the most promising genotypes has been vegetatively propagated by isolating basal growing shoots, and are currently under evaluation in different environments with the goal of identifying the most valuable for future marketing. Micropropagation is a further option, as protocols for meristem culture have been well established for the species, also

starting from meristem tips, with the goal to obtain virus free plants for the production of sanitary controlled propagative material (Acquadro et al. 2010; Barba et al. 2004; Papanice et al. 2004).

A set of nine SSR loci, evenly dispersed across the genome, was shown to be sufficient to unambiguously identify each segregant. The DNA profiles generated by these SSR assays will have utility in establishing the genetic identity of vegetatively propagated materials.

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Table 1. Phenotypic variation observed for seven traits in the two inter-subspecies hybrid populations [globe artichoke x cultivated cardoon (Progeny 1) and globe artichoke x wild cardoon (Progeny 2)].

Trait	**Progeny 1**			**Progeny 2**		
	Mean	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**
Plant height (cm)	95	56	127	54	28	89
Days to flowering	216	203	242	223	214	234
Flowering period (days)	25	7	55	22	2	48
Number of heads plant^{-1}	7.4	1	32	5.7	1	20
Head longitudinal diameter (cm)	6.9	4.4	10.4	6.4	4.0	8.2
Head transversal diameter (cm)	5.1	3.2	7.8	4.4	2.8	7.6
Shape index	1.37	1.04	1.94	1.45	1.07	1.97

Table 2. Observed and expected segregation for the heads colour, florets colour and presence of spines in the two inter-subspecies hybrid populations [globe artichoke x cultivated cardoon (Progeny 1) and globe artichoke x wild cardoon (Progeny 2)].

Number of individuals	**Globe artichoke 'Romanesco C3'**	**Cultivated cardoon 'Altilis 41'**	**Wild cardoon 'Creta 4'**	**Progeny 1**	**Progeny 2**
Purple-green heads	X	X	X	53	52
Green heads				24	27
Purple heads				17	15
Expected ratio				9 : 4 : 3	9 : 4 : 3
χ^2				0.03 ns	0.39 ns
Putative genotype	PpUu	PpUu	PpUu	[P_U_ (9)] : [ppU_ + ppuu (4)] : [P_uu (3)]	
Violet florets	X		X	94	94
White florets		X		0	0
Expected ratio				1 : 0	1 : 0
χ^2				ns	ns
Putative genotype	BB	bb	BB (or Bb)	Bb	BB (or BB + Bb)
Spiny heads			X	0	44
Not spiny heads	X	X		94	50
Expected ratio				1:0	1:1
χ^2				ns	0.38 ns
Putative genotype	Spsp	SpSp	spsp	SpSp + Spsp	[Spsp (1)] : [spsp (1)]

Table 3. Segregation patterns and number of polymorphic/informative alleles detected in the in two inter-subspecies hybrid populations [globe artichoke x cultivated cardoon (Progeny 1) and globe artichoke x wild cardoon (Progeny 2)].

Segregation type	Segregation ratio	N° informative alleles/locus	Progeny 1		Progeny 2	
			N° loci	Total N° of alleles	N° loci	Total N° of alleles
<ab x cd>	1:1:1:1	4	12	48	16	64
<ab x ac>	1:1:1:1	3	5	15	9	27
<ab x ab>	1:2:1	2	1	2	0	0
<ab x aa> <ab x cc> <aa x ab> <aa x bc>	1:1	1	32	32	25	25
total			**50**	**97**	**50**	**116**

Figure 1:

Examples of the phenotypic variation released in two inter-subspecies hybrid populations. White flower of the cultivated cardoon parent are also included.

Figure 2:

Segregation patterns which allow for the identification of null alleles (indicated by a white band). A null allele was inferred to be segregating when a single product or no product was observed.

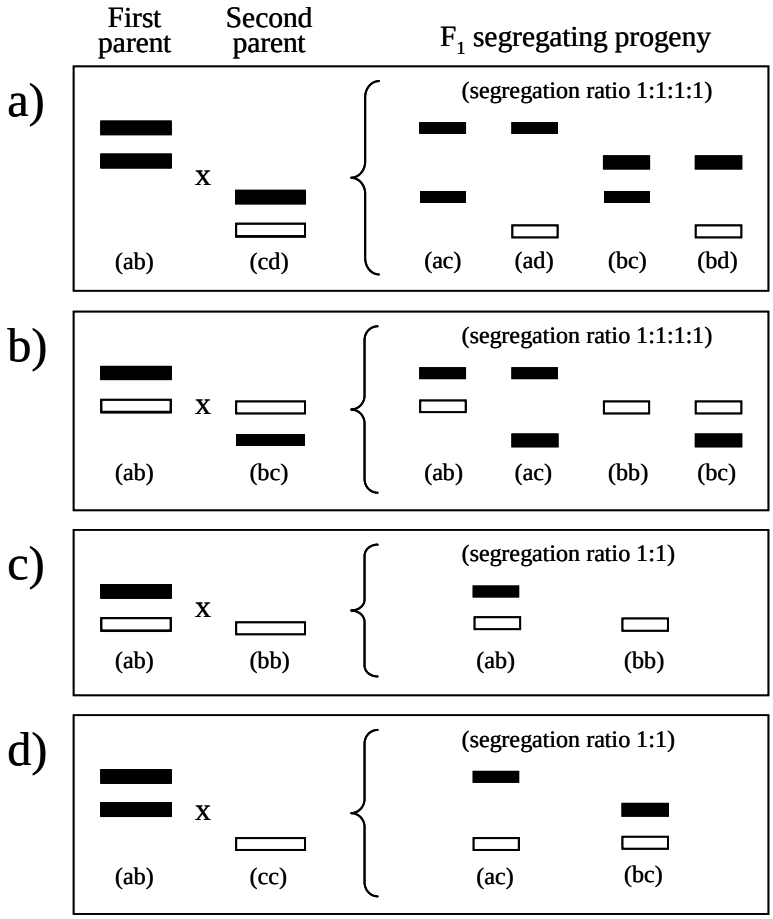


Figure 3:

Segregation pattern which does not allow for the identification of null alleles (indicated by a white band). Both homozygous and heterozygous individuals amplify only a single product.

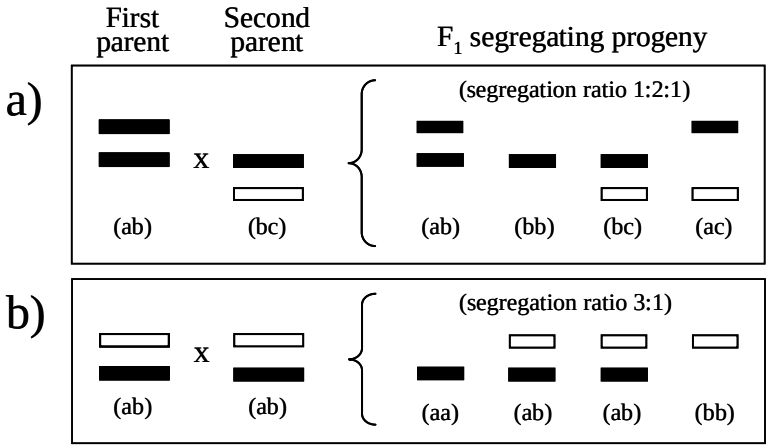


Figure 4:

UPGMA-based cluster analysis of the segregants arising in two inter-subspecies hybrid populations, based on their allelic constitution at nine SSR loci. In the dendrogram parental genotypes are indicated.

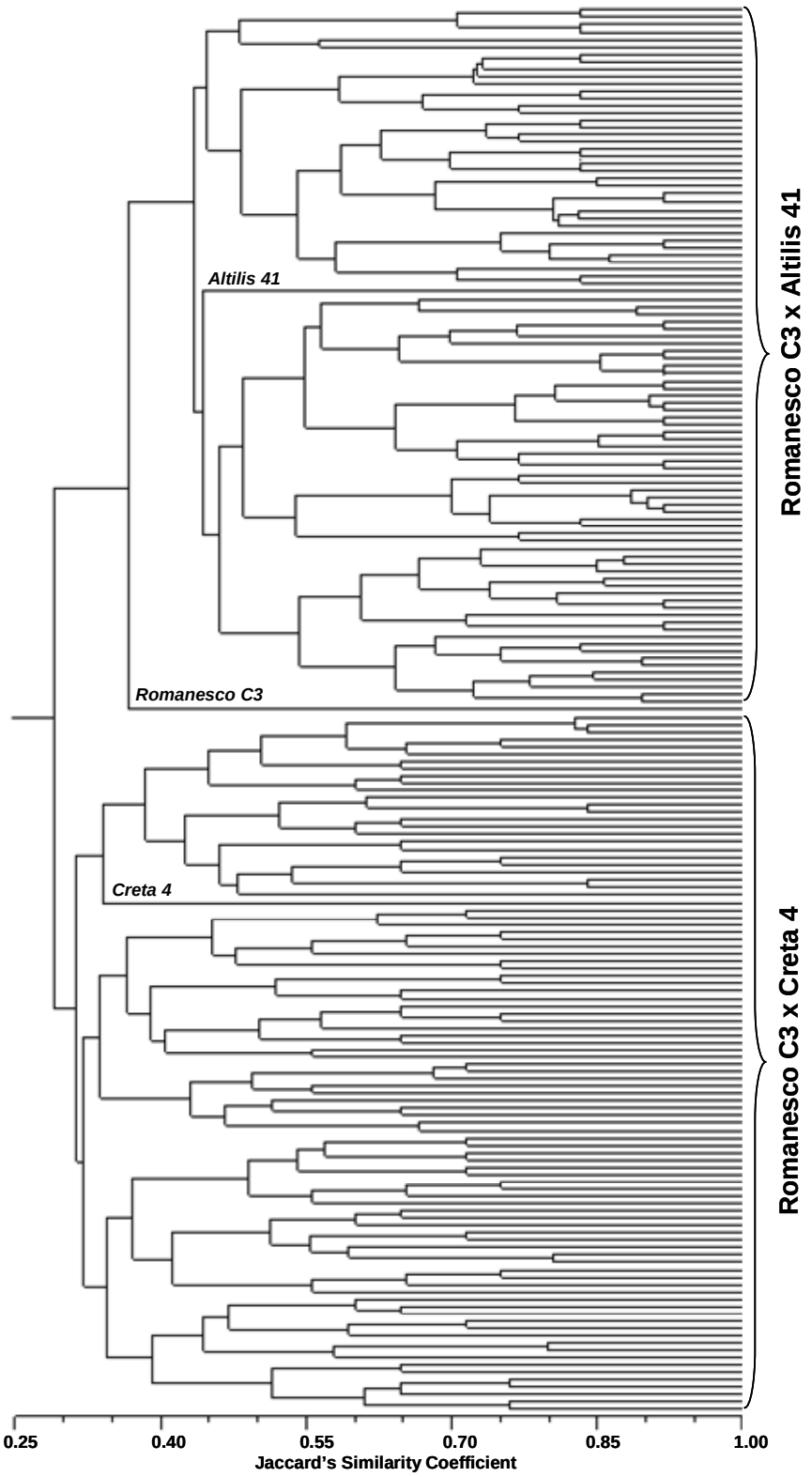
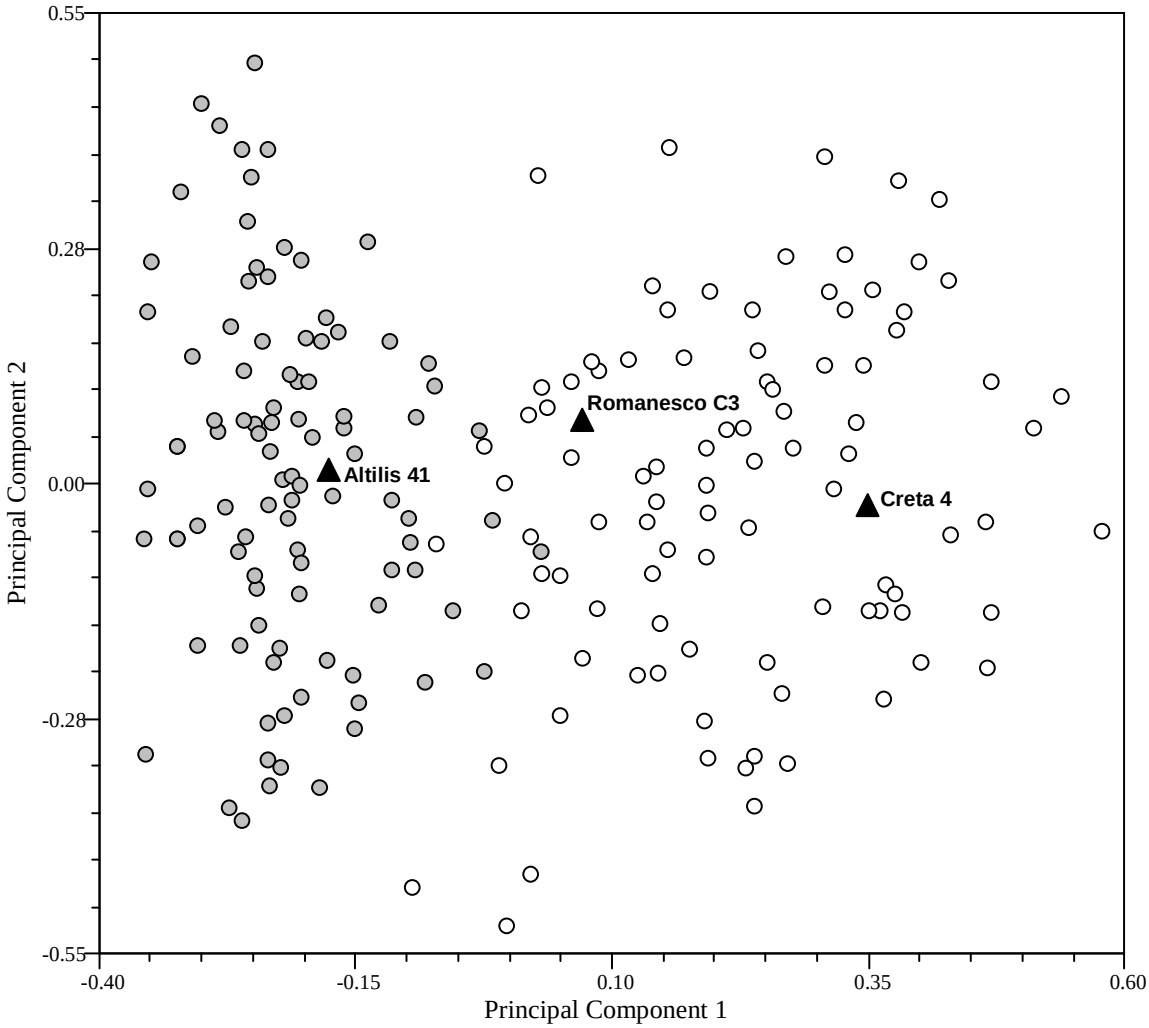


Figure 5:

Principal coordinate analysis (PCoA) based on SSR-based genotypic data, depicting the genetic relationship between the segregants arising in two inter-subspecies hybrid populations. Progeny 1 shown as grey circles, Progeny 2 as white circles. The parents are indicated by triangles.