Ferriol Molina, M.; Garmendia, A.; Ruiz, J.; Merle Farinós, HB.; Boira Tortajada, H. (2012). Morphological and molecular analysis of natural hybrids between the diploid Centaurea aspera L. and the tetraploid C. seridis L. (Compositae). Plant Biosystems. 146(1):86-100. doi:10.1080/11263504.2012.727878.

Running title: Hybrids in polyploid complex *Centaurea*

Corresponding author:

María Ferriol Molina

Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia

Camino de Vera s/n

46021 Valencia, Spain

Telephone number: 34 96 3879339

Fax number: 34 96 3879269

e-mail: mafermo@upvnet.upv.es

Morphological and molecular analysis of natural hybrids between the diploid *Centaurea aspera* L. and the tetraploid *C. seridis* L. (Compositae)

María FERRIOL MOLINA[1], Alfonso GARMENDIA SALVADOR[1], Juan José RUIZ MARTINEZ[2], Hugo MERLE FARINOS[3] & Herminio BOIRA TORTAJADA[1]

Con formato: Español (España, internacional)

Abstract

Polyploidy and hybridisation are the basis of the evolution of *Centaurea* (Compositae). At the El Saler dune field (eastern Spain), the diploid *Centaurea aspera* ssp. *stenophylla* and the tetraploid *C. seridis* ssp. *maritima* form a polyploid complex in which *C. x subdecurrens* individuals occur. This polyploid complex was analysed morphologically and genetically, using RAPD and TBP markers. Flow cytometry showed that the hybrids are triploid, which is a rare finding in *Centaurea*. Morphologically, in contrast to leaf characters, flowering characters clearly discriminated the three taxa. The genetic analyses confirm that *C. x subdecurrens* is a result of the hybridisation between *Centaurea aspera* ssp. *stenophylla* and *C. seridis* ssp. *maritima,* and suggest that backcrossing events and gene flow are very rare or absent. Although the hybrids likely represent true F1 offspring, they displayed some genetic diversity that is probably due to the combination of alleles. Genetic diversity was higher in diploid than in tetraploid individuals. This fact, and the high degree of sterility of the triploid hybrids, may reflect a cytotype minority exclusion effect. This may cause spatial segregation, which effectively takes place in the study area. Dune disturbance may lead to an overlapping of the parents' distribution areas, facilitating hybridisation.

Keywords: Centaurea x subdecurrens, Polyploid complex, RAPD, TBP, Triploid hybrid

Polyploidy can provide evolutionary advantages and is consequently widespread in nature (Comai 2005). It is believed that the proportion of polyploid angiosperm plants varies from 30% to 70% (Bennet 2004; Pellicer et al. 2010), and genomic data even indicates ubiquity among them (Soltis 2009). Polyploid formation is the major mode of sympatric speciation in flowering plants as it can occur by immediate reproductive isolation (Coyne & Orr 2004; Seo et al. 2010). Diploid and related polyploid taxa often coexist in contact zones. This coexistence may result in the production of hybrid offspring, generally triploid individuals, and may influence the dynamics and evolution of polyploid complexes (Petit et al. 1999). Even if in some hybrid zones triploids may act as a bridge between different ploidy levels, in most, triploid offspring is totally or highly sterile, acting as a major reproductive barrier (Husband 2004). In these cases, the survival of the mixed-ploidy populations is threatened by a frequency-dependent selection, called the minority cytotype principle (Levin 1975). This model assumes that, in a hybrid zone with both a rare and a common cytotype, the latter would pollinate the rarer cytotype to a greater extent. Consequently, the rarer cytotype produces a higher proportion of inviable triploid offspring, leading to a reduction of its proportion, and, ultimately, to its extinction. However, different factors may sustain and promote the survival of the two ploidy levels in the contact zone. The two cytotypes may differentiate themselves by means of spatial segregation, meaning they may specialise ecologically in separate habitats (Stuessy et al. 2004; Thorsson et al. 2007), or by temporal segregation, in which case they diverge in terms of flowering time (Osborn 2004; Husband & Schemske 2000). The rarer cytotype may also increase its selfing rate if deleterious alleles are progressively purged to avoid inbreeding depression. Furthermore, chromosome doubling can affect organ size.

25 Consequently, if the rarer cytotype is an insect-pollinated polyploid, increasing the
26 flower size may lead to an increase in pollinator attractiveness (Petit et al. 1999).

27 *Centaurea* (Compositae) is a modern genus that originated between the Pliocene
28 and the Pleistocene. In this genus, the cycle of polyploidy and descending dysploidy, as
29 well as hybridisation events, are at the origin of its diversification and speciation
30 (Hellwig 2004; Romaschenko et al. 2004). Some polyploid complexes have been
31 reported, such as those including diploid and tetraploid cytotypes of *C. jacea* L. (Hardy
32 et al. 2000), *C. phrygia* L. (Koutecky 2007), and *C. stoebe* L. (Spaniel et al. 2008). In
33 addition, the diploid species *C. aspera* L. (2n = 22, Cueto & Blanca, 1986) and the
34 tetraploid *C. seridis* L. (2n=44, Parra et al. 1998) coexist in a contact zone forming
35 another polyploid complex. These taxa belong to the *Seridia* section, which displays the
36 Jacea pollen type and includes 7 species distributed throughout the western
37 Mediterranean according to *Flora Europaea* (Tutin et al. 1972). These species are
38 mainly found in coastal habitats, such as dunes and places with periodical inundations
39 and rocks (Hellwig 2004). On the east coast of Spain, three of the seven species coexist:
40 *C. aspera*, *C. seridis* and *C. sonchifolia*, the latter two of which are considered
41 synonyms by Bolòs and Vigo (1995). Only certain subspecies grow in this area: *C.*
42 *aspera* ssp. *aspera*, *C. aspera* ssp. *stenophylla* (Dufour) Nyman, and *C. seridis* ssp.
43 *maritima* (Dufour) Dostal, which is also considered to be a synonym of *C. sonchifolia*
44 ssp. *maritima* by *Flora Europaea* (Tutin et al. 1972, updated at http://rbg-
45 web2.rbge.org.uk/FE/fe.html).

46 Morphologically, *C. seridis* is differentiated from *C. aspera* by its winged stems,
47 decurrent and hairy cauline leaves, greater flower size, and higher involucral scale spine
48 number. The hybridisation between the two species gives rise to morphologically
49 transitional forms, whose taxonomical adscription is still very confusing (Mateo &

Crespo 2009; Merle et al. 2010). *Centaurea* x *subdecurrens* was first described by Pau (1898), and is a synonym of *C. x valentina* Rouy. Subsequently, different names were applied to hybrids between species and subspecies: *C. x aemiliae* Font Quer, *C. x segobricensis* Pau, *C. x auricularis* Pau, and *C. x albuferae* Costa. However, none of these hybrids are recognised by *Flora Europaea*. At present, some are considered to be nothosubspecies and even varieties (Susanna 1988; Crespo & Mateo 1990). These intermediate forms have been described in all the areas where the parental species and subspecies coexist: the coastal sand dunes at El Saler and Pinedo (Valencia), those at Dehesa de Campoamor (Alicante), and those near Cabo Palos (Murcia) (Susanna 1988).

In this study, we examined the *C. aspera* ssp. *stenophylla* / *C. seridis* ssp. *maritima* hybrid zone on the dunes of El Saler in order to clearly identify the genetic origin of *C. x subdecurrens* individuals and to elucidate the nature of this contact zone in terms of ploidy levels and genetic and morphological diversity. This preliminary work will allow us, in the future, to study the ecological and genetic mechanisms underlying the coexistence, competition, and demography of both species. We made use of random amplified polymorphic DNA (RAPD) and tubulin-based polymorphism (TBP) markers. RAPD markers have been used to study hybrid progeny among several plant groups (Caraway et al. 2001; Droogenbroeck et al. 2006; Conte et al. 2007; Repplinger et al. 2007; D'Andrea et al. 2008; Ducarme et al. 2010). In regards to *Centaurea* specifically, RAPDs have been used to assess its genetic diversity and clarify its taxonomy (Yildirim et al. 2009; Sozen & Ozaydin 2010). TBP is based on the presence of intron-specific DNA polymorphisms of the plant beta–tubulin gene family (Bardini et al. 2004). The designed primers amplify the first intron of different beta-tubulin isotypes, revealing specific fingerprints. This molecular marker is particularly suited to ecological studies because it amplifies regions of genes of known function and

because the beta-tubulin polypeptide has a multifunctional role in various cell mechanisms. These markers have shown to be very useful for comparisons of diploid and tetraploid accessions of *Lotus* (Bardini et al. 2004) as well as other plant species with different ploidy levels (Breviario et al. 2008).

The three main objectives of the present study are: (1) to determine the ploidy level of *C. aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima*, and *C. x subdecurrens* individuals, (2) to assess the morphological differentiation between the different cytotypes and morphotypes encountered, as present taxonomic treatments largely rely on a morphology-based concept, and (3) to identify individuals in terms of parental and hybrid genealogies on the basis of genetic markers unique to each species and to thereby infer their genetic diversity.

Materials and Methods

Study area

The study was carried out in the El Saler dunefield on the east coast of Spain (39º20´N, 0º19´W), 12 km south of the city of Valencia. It occupies approximately 17 ha between the Albufera lagoon and the Mediterranean Sea (Figure 1).

In this area, stabilised dunes, separated by temporarily flooded interdune flats, lie parallel to the beach. Pioneer communities colonise the dunes closer to the sea, while communities with larger vegetation cover and floristic diversity colonise the semi-fixed dunes located farther inland. Finally, a shrubland grows on the old fixed dunes (Costa & Mansanet 1981).

Study organisms

The diploid *C. aspera* and the tetraploid *C. seridis* are perennial herbaceous plants. Only one subspecies of each grows in El Saler: *C. aspera* ssp. *stenophylla* and *C. seridis* ssp. *maritima.* The former is found in dry lands at low altitudes in eastern Spain, in nitrophilous plant communities of *Ammophiletea* and *Oleo-Ceratonion*, although it has also been cited in more continental areas (Bolòs & Vigo 1995; program ANTHOS 2011). *Centaurea seridis* ssp. *maritima* has a narrower distribution area. It is sabulicolous and grows on coastal sand dunes that frequently exhibit a high level of anthropic disturbance and nitrophilia, near buildings, roads, parking areas, and walking paths (Costa & Mansanet 1981; program ANTHOS 2011). Rarely does it advance inland (Mateo et al. 1987).

The habitat differentiation for these taxa can be observed on a small scale in relation to the distance to the sea at the study area (Costa & Mansanet 1981). *Centaurea aspera* ssp. *stenophylla* appears in fixed and semi-fixed dune shrublands, relatively far

from the sea. However, its distribution area overlaps with that of *C. seridis* ssp. *maritima* on disturbed, coastal, semi-fixed dunes, where both subspecies coexist with *C. x subdecurrens* plants.

All the studied taxa are insect-pollinated. The flowering periods of diploids and tetraploids overlap during at least three months: *C. aspera* ssp. *stenophylla* sets flowers from March to June, and *C. seridis* ssp. *maritima* from April to July (Mateo & Crespo 2003). We observed that *C. x subdecurrens* individuals set flowers from May to July, and that these flowers were sterile, producing aborted achenes.

Plant material

Sampling was carried out during the spring and early summer of 2004. Thirty-four individuals of *Centaurea,* growing in the dunes of El Saler (Valencia), were used in this study. Eleven individuals represented *C. aspera* ssp. *stenophylla*, 12 individuals represented *C. seridis* ssp. *maritima*, and 11 individuals represented *C. x subdecurrens*. These individuals were selected to maximise the range of morphological diversity and habitat origins and at the same time avoid samples originating from one clone (which is common in plants growing very close together). Geographical coordinates were recorded using G.P.S. (Garmin eTrex Vista HCx). Leaves were transported in a cooler and frozen plant tissues were stored at -80ºC. Voucher specimens were also collected, then dried by being pressed in absorbent paper, stored at room temperature, and kept in the Herbarium of the Polytechnic University of Valencia (VALA). For the molecular analyses, one individual of *C. paui* Loscos ex Willk., collected in the Espadán Mountains (Castellón, Spain), was used as an outgroup.

Assessment of ploidy level

The ploidy level of *C. aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima*, and *C. x subdecurrens* was determined by flow cytometry, which allows the rapid determination of the DNA content of the nucleus. The analyses were performed at the Flow Cytometry Area (Scientific – Technical Services) of the University of Oviedo. Leaf samples were sent from the study area by rapid refrigerated transport (approx. 12 hours). Nuclear suspensions were prepared according to the protocol of Galbraith et al. (1983). Nuclei from chicken red blood cells (CRBC; 2C = 3.14 ± 0.16 pg of DNA, Cires et al. 2009), kept at the University of Oviedo, were used as a DNA reference standard. This animal reference standard has proven to work well in plant ploidy determinations, and a single blood sample can be used for all runs (Cires et al. 2009). Fixed CRBCs were self-prepared and stored at low temperatures (J. Dolezel, unpublished results). A razor blade was employed to chop up 150 mg of the sampled leaf tissue in a glass Petri dish containing 1 mL of ice-cold Otto's nuclear isolation buffer [Otto 1: 100 mM citric acid monohydrate, 0.5% (v/v) Tween 20 (pH approx. 2-3); Otto II: 400 mM $Na_2HPO_4.12H_2O$ (pH approx. 8-9)] (Otto 1990; Dolezel & Gödhe 1995). Nuclei from a CRBC reference standard were added after preparing the plant nuclei suspension. The samples were maintained at an ice-cold temperature following isolation of the nuclei in order to decrease nuclease activity. The nuclear suspension was subsequently filtered through a 42-µm nylon filter to remove large debris, and 50 µg mL^{-1} of propidium iodide (PI, Sigma) were added to strain the DNA. As propidium iodide is an intercalating fluorescent dye that binds to DNA as well as to double-stranded RNA, the samples were treated with RNase (50 µg mL^{-1}, Sigma) to avoid the staining of double-stranded RNA. After mixing well, the samples were put on ice and kept in darkness for a 30 min period before being analysed. Experiments were carried out using a Cytomics FC 500 (Beckman Coulter) with 488-nm excitation from an argon ion laser. Data

analysis was performed using the program Cytomics RXP Analysis (Beckman Coulter). Analyses were performed in triplicate for each dye, and at least 5,000 nuclei were analysed per sample.

Morphometric measurements

Morphometric measurements were recorded at the flowering peak (May) and as quickly as possible in order to diminish the seasonal effect. A total of 38 vegetative and 21 flowering characters were measured for each individual (Table I). The characters included those traditionally used for differentiation of the taxa, as can be found in determination keys and floras, as well as several others that seemed potentially useful based on field observations. Ten leaf characters were evaluated separately in 8 leaves of each plant: 2 basal rosette leaves, 2 lower leaves (but not rosette), 2 middle leaves, and 2 upper leaves. Length and width of the petiole were not measured in the upper leaves, as they were sessile in all individuals. Flowering characters included involucral characters from 2 fresh and 2 dry involucres for each individual, and flower characters from 3 inner and 3 outer flowers that developed in each of the two measured fresh involucres for each plant.

DNA Extraction

Genomic DNA was isolated from young leaves by the modified CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1990). For each individual, 50 mg of ground leaf tissue were suspended in 250 µl of extraction buffer [20 mM EDTA, 0.1 M Tris-HCl (ph 8.0), 1.4 M NaCl, 2% (w/v) CTAB, and 5µl of beta-mercaptoethanol]. The suspension was mixed well and incubated at 60ºC for 30 min, which was followed by chloroform-isoamyl alcohol (24:1) extraction and

precipitation with 0.67 vol isopropanol at -20ºC. The pellet formed after low-speed centrifugation for 5 min was washed with 76% (v/v) ethanol and 10 mM NH_4OAc. The DNA was then suspended in TE buffer. The resulting DNA concentration was measured in a 1% (w/v) agarose gel stained with ethidium bromide using known size lambda DNA digested with Hind III.

Molecular characterisation using Random Amplified Polymorphic DNA (RAPD)

Single arbitrary 10-base primers were tested for their ability to amplify scorable and reproducible DNA fragments. Primers resulting in faint or irreproducible bands were excluded from the analysis. Nineteen primers out of 27 were accepted for subsequent analysis (A02: TGCCGAGCTG, A03: AGTCAGCCAC, A07: GAAACGGGTG, A09: GTGACGTAGG, B17: AGGGAACGAG, AB21: CCCAAGGTCC, AB22: GGTGCGGGAA, AB31: CCCGGCATAA, AB214: TGCGGCTGAG, M16: GTAACCAGCC, M18: CACCATCCGT, N2: ACCAGGGGCA, B2: TGATCCCTGG, B5: TGCGCCCTTC, 262: CGCCCCCAGT, 300: GGCTAGGCCG, OPA05: AGGGGTCTTG, OPC12: TGTCATCCCC, OPB14: TCCGCTCTGG). Each 25 µl PCR reaction mixture consisted of 20 ng genomic DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.3 µM primer, 10x Taq buffer, and 1 unit of Taq polymerase (Boehringer Mannheim). Samples were subjected to the following thermal profile for amplification in an oven thermocycler (Eppendorf Mastercycler Gradient): 5 min of denaturing at 94ºC, 40 cycles with three steps each: 1 min of denaturing at 94ºC, 1 min of annealing at 35ºC, and 2 min of elongation at 72ºC, with a final elongation step of 5 min at 72ºC. The visualisation of amplification fragments was accomplished on a 2% agarose gel in 1XTBE buffer stained with ethidium bromide.

213 *Molecular analysis using Tubulin-based polymorphism (TBP)*

214 The forward and reverse primers used, the PCR reaction mixture, and the
215 thermal profile for amplification are described in Bardini et al. (2004). Separation of the
216 amplified fragments was carried out on 12% polyacrylamide gels [acrylamide-
217 bisacrylamide (29:1), TBE 1X] at 650 V for 5 h. The gels were stained with $AgNO_3$ to
218 visualise the TBP fragments and were then dried overnight.

219

220 *Data analysis*

221 The number of morphotypes was defined by K-means clustering method (KM)
222 classification analysis (MacQueen, 1967), with 2, 3, and 4 groups, in order to observe
223 consistency. Afterwards, each sampled plant was assigned to its morphotype.
224 Differences in each variable for each morphotype were compared using ANOVA and
225 Fisher Less Significant Differences (LSD) Post Hoc test.

226 Morphological differentiation was also studied by discriminant analysis, using
227 morphotypes as predefined groups. This method reduces the dimensions of the original
228 data and allows a visual interpretation of the relationships among individuals. Before
229 carrying out this analysis, character selection was performed. As rosette leaves started to
230 dry off when the plants began to flower, and could therefore not be measured for several
231 individuals, characters from this leaf type were excluded to avoid the bias caused by a
232 series of blank values. The variables in which variances were non-homogeneous were
233 also discarded using Cochran's test and Levene's test. Furthermore, if one or more
234 variables had a high correlation coefficient (higher than 0,85), only one of them was
235 retained, being that which corresponded to the most easily measurable character. As a
236 result of this selection, eight independent characters were used for subsequent analyses
237 (LNL, UAL, UBT, FBN, ITL, IUL, ILL, ISL, see Table 1). Due to the fact that, in some

238 floras, only vegetative characters are used to discriminate C. *aspera* from *C. seridis*
239 (Bolòs & Vigo 1995), or the subspecies within them (Mateo & Crespo 2003), two data
240 sets were used for the discriminant analysis: vegetative characters only and a
241 combination of both vegetative and flowering characters.

242 All the analyses were performed using Statgraphics Plus 5.1 and Systat 11.

243 In the molecular analysis, data scored as presence (1) or absence (0) of
244 amplification fragments were used to calculate genetic distances ($1 - S_{ij}$) among
245 genotypes according to the Nei and Li (1979) similarity coefficient $S_{ij} = 2a/(2a+b+c)$,
246 where S_{ij} is the similarity between the two individuals i and j; a is the number of shared
247 bands; b is the number of bands exclusively amplified by i; and c is the number of bands
248 exclusively amplified by j. The distance matrix was subjected to cluster analysis by the
249 Unweighted Pair-Group Method (UPGMA, Sneath & Sokal 1973). The goodness of fit
250 of the cluster to the data matrix was calculated using the cophenetic coefficient. The
251 reliability and robustness of the dendrograms were tested by bootstrap analysis with
252 1000 replications to assess branch support using the PHYLIP 3.6 software. Principal
253 Coordinate Analysis (PCoA) was also performed with the molecular data to obtain a
254 graphical representation of the relationship structure of the characterised individuals.
255 The statistical analyses were performed with NTSYS-pc (version 2.0). Genetic
256 distances (Nei 1978) were estimated using POPGENE 32.
257
258

Results

Ploidy level

The 11 individuals that were identified as *C. aspera* ssp. *stenophylla* appeared to be diploid according to flow cytometry, the 12 individuals identified as *C. seridis* ssp. *maritima* were tetraploid, and the 11 individuals identified as *C. x subdecurrens* were triploid, suggesting their hybrid nature. Figure 2 shows the histogram of relative nuclear DNA content of the CRBCs and diploid, triploid, and tetraploid *Centaurea* plants, as well as their morphological features.

Morphology

Classification into three groups was the most consistent and, accordingly, each sampled plant was assigned to its morphotype, which corresponded to taxonomic grouping and ploidy levels: *C. aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima* and *C. x subdecurrens*.

Of the 59 studied characters, only eight leaf characters (RPL, RPW, RNL, LNL, MPL, MPW, MNL, and UNL) did not show significant differences among the morphotypes (see appendix A). The morphotype corresponding to *C. x subdecurrens* displayed intermediate values for almost all the measured characters.

In regards to vegetative characters, rosette leaves were found in *C. x subdecurrens* and *C. seridis* ssp. *maritima* individuals, and significant differences were observed for size and shape. For *C. aspera* ssp. *stenophylla*, no rosette leaves were observed as they dried before the flowering season. However, in winter, when rosette leaves were visible, they were clearly different when compared to the other two morphotypes (pers. obs.). Lower, middle and upper leaves were, in general, smaller in *C. aspera* ssp. *stenophylla* than in *C. seridis* ssp. *maritima*. The *C. x subdecurrens*

leaves were intermediate in size, with the exception of the lower leaves, which were the widest and had the highest number of lobes. All the non-rosette leaves of *C. seridis* ssp. *maritima* were sessile, contrary to *C. aspera* ssp. *stenophylla* and *C. x subdecurrens*, for which only upper leaves were sessile.

Flowering characters were more useful for differentiating the three morphotypes than vegetative characters, which were more variable. Like the leaf measurements, capitulum size was higher in *C. seridis* ssp. *maritima* than in *C. aspera* ssp. *stenophylla*, and was intermediate in *C. x subdecurrens*. However, the triploid individuals had more bracteae (FBN) and outer flowers (FNO) per capitulum than the diploid and tetraploid species. *Centaurea seridis* ssp. *maritima* and *C. x subdecurrens* showed similar values for flower size, but these were higher than those of *C. aspera* ssp. *stenophylla*.

If only vegetative characters had been considered, discriminant analysis would have identified five different morphotypes (Figure 3a). However, when flowering characters were added, three morphotypes emerged (Figure 3b). Therefore, flowering characters are important for clearly discriminating the three taxa, because the variability of leaf characters could create confusion, especially between *C. x subdecurrens* and *C. seridis* ssp. *maritima* or *C. aspera* ssp. *stenophylla*.

The first discriminant function obtained from the standardised coefficients of the discriminant analysis using vegetative and flowering characters accounted for 91.6% of the among-groups variability. Based on both statistical and practical significance, only the first linear discriminant function was considered noteworthy. The coefficients for the first function, which determine how the independent variables are being used to discriminate among morphotypes, were as follows: LNL (1.11), ILL (-1.02), IUL (-0.89), UAL (-0.57), UBT (-0.49), FBN (-0.44), ITL (0.29), and ISL (-0.14). These weights suggest that the best discriminant characters for distinguishing individuals of *C.*

309 *aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima* and *C. x subdecurrens* are the number
310 of lobes of lower leaves (LNL), the length of the petal lobes and petal tubes of the inner
311 flowers (ILL and IUL), and the apical lobe length of the upper leaves (UAL).
312
313 *Genetic structure and diversity using RAPDs*
314 Analysis of the 34 *Centaurea* individuals with 19 RAPD primers identified a
315 total of 129 reproducible fragments. Of these, 126 were polymorphic (97.7%), ranging
316 in size from 290 bp to 2700 bp. The number of fragments detected by an individual
317 primer ranged from 3 (for primers M18 and OPAC12) to 14 (A03), with an average of
318 6.84. Forty percent of the observed RAPD markers were shared by *C. aspera* ssp.
319 *stenophylla* and *C. seridis* ssp. *maritima* (Table II). Genetic variation among individuals
320 in *C. aspera* ssp. *stenophylla* was higher than that examined in *C. seridis* ssp. *maritima*.
321 Out of a total of 124 markers found in *C. aspera* ssp. *stenophylla*, 57 (46%) were
322 present only in this taxon, and 5 of these (9%) were constant. Contrarily, only 67
323 markers were found in *C. seridis* ssp. *maritima*. Nineteen (28%) were present only in
324 this taxon and 8 of these were constant (42%). Among *C. x subdecurrens* plants, 19
325 (40%) of the 98 markers were constant. Three were constant in *C. aspera* ssp.
326 *stenophylla* and completely absent in *C. seridis* ssp. *maritima*, or vice-versa. Of the 57
327 RAPD markers unique to *C. aspera* ssp. *stenophylla*, only 29 (51%) were present in *C. x*
328 *subdecurrens*, and of the 19 markers unique to *C. seridis* ssp. *maritima*, 18 (95%) were
329 also present in *C. x subdecurrens*. Seven *C. x subdecurrens* individuals showed one of
330 the five additional markers that were absent in *C. aspera* ssp. *stenophylla* and *C. seridis*
331 ssp. *maritima*.
332 The cluster analysis produced a cophenetic coefficient of 0.92, indicating a very
333 good fit. The dendrogram grouped the individuals in two major clusters (bootstrap = 64)

(Figure 4). Cluster I included the individuals belonging to *C. aspera* ssp. *stenophylla*. Within this cluster, only individual pairs clustered together with high bootstrap values. Cluster II included the individuals belonging to *C. seridis* ssp. *maritima* and *C. x subdecurrens*. Three subclusters within cluster II were observed. Clusters II.1 and II.2 were composed of triploid individuals. In contrast, cluster II.3 included all the individuals representative of *C. seridis* ssp. *maritima*. Furthermore, one *C. seridis* ssp. *maritima* plant appeared as the most distant individual within cluster II. Despite this clear grouping, only subclusters II.1 and II.3 were significantly consistent, as were the relationships among the individuals composing them, as supported by high bootstrap values. In contrast, the position in the dendrogram of cluster II.2 and the more distant individual appeared with low bootstrap values.

These results were corroborated by Principal Coordinates Analysis, which represents the distribution of the different accessions according to the two principal axes of variation (Figure 5). On the basis of the first coordinate, which accounted for 30.8 % of the total variation, the individuals were grouped according to ploidy level. *Centaurea aspera* ssp. *stenophylla* individuals grouped together in the right extremity of the first coordinate, while *C. seridis* ssp. *maritima* individuals grouped in the left extremity. Finally, the *C. x subdecurrens* individuals appeared in an intermediate position according to this first coordinate, although it was considerably closer to *C. seridis* ssp. *maritima* than to *C. aspera* ssp. *stenophylla*. The second coordinate, which accounted for 9.9% of the total variation, did not furnish additional information, except that it showed a higher dispersion of individuals within *C. seridis* ssp. *maritima* and *C. x subdecurrens* than within *C. aspera* ssp. *stenophylla*.

According to these analyses, the genetic distances (Nei, 1978) among ploidy levels showed that *C. x subdecurrens* is genetically more similar to *C. seridis* ssp.

359 *maritima* (0.0778) than to *C. aspera* ssp. *stenophylla* (0.1835). As expected, the greatest
360 distance was between *C. seridis* ssp. *maritima* and *C. aspera* ssp. *stenophylla* (0.3058).
361
362 *Genetic structure and diversity within and among populations using TBPs*
363 Using TBPs, a total of 18 scorable amplification products were obtained, ranging
364 from 220 to 950 bp. Sixteen of the 18 fragments were polymorphic among all the
365 individuals. Differences in genetic diversity between *C. aspera* ssp. *stenophylla* and *C.*
366 *seridis* ssp. *maritima* were more dramatic when using TBPs than when using RAPDs.
367 Twenty-one percent of the TBP markers observed were shared by the two species
368 (Table II). As the results obtained with RAPD, genetic variation among individuals in
369 *C. aspera* ssp. *stenophylla* was higher than that observed in *C. seridis* ssp. *maritima*. Of
370 the 15 markers found in *C. aspera* ssp. *stenophylla*, 11 (73%) were present only in this
371 taxon, all of which were polymorphic. Contrarily, of the 7 markers found in *C. seridis*
372 ssp. *maritima*, 3 (43%) were present only in this taxon and none of them were
373 polymorphic. Among *C. x subdecurrens* plants, 5 (45%) of the 11 markers were
374 constant. Three were constant in *C. aspera* ssp. *stenophylla* and completely absent in *C.*
375 *seridis* ssp. *maritima*, or vice-versa. Of the 11 TBP markers unique to *C. aspera* ssp.
376 *stenophylla*, only 4 (36%) were present in *C. x subdecurrens*, and all 3 that were unique
377 to *C. seridis* ssp. *maritima* were also present in *C. x subdecurrens*. No unique markers
378 were detected in the triploid individuals.
379 The results of the genetic structure obtained with RAPD markers are supported
380 by the dendrogram (not shown) and by the PCoA (Figure 5) derived from the TBP data.
381 In spite of the paucity of markers, both analyses clearly separated *C. aspera* ssp.
382 *stenophylla* individuals on the one hand and *C. seridis* ssp. *maritima* individuals on the
383 other. The *C. x subdecurrens* individuals were grouped in an intermediate position in

384 the PCoA diagram, although considerably closer to — and even intermingled with — *C.*
385 *seridis* ssp. *maritima*, than to *C. aspera* ssp. *stenophylla*. Accordingly, the genetic
386 distances (Nei, 1978) between ploidy levels showed that *C. x subdecurrens* is
387 genetically more similar to *C. seridis* ssp. *maritima* (0.0266) than to *C. aspera* ssp.
388 *stenophylla* (0.5401). The greatest distance between *C. seridis* ssp. *maritima* and *C.*
389 *aspera* ssp. *stenophylla* was 0.5490.
390
391

392 **Discussion**

393 Polyploidy and naturally occurring hybrids are well-known phenomena in
394 *Centaurea s.l.* (Hellwig 1994; Suárez-Santiago et al. 2007). In sections *Chamaecyanus*
395 and *Acrocentron*, three and four ploidy levels were found, respectively, and fertile
396 hybrids between the two sections have been reported (Fernández Casas & Susanna
397 1986; Font et al. 2008). Diploid and tetraploid cytotypes were also found within *C.*
398 *phrygia* and *C. jacea*, in the section *Jacea – Lepteranthus* (Hardy et al. 2000; Koutecky
399 2007). In this section, taxa of the same ploidy level can cross easily and their hybrids
400 are fertile, whereas taxa differing in ploidy level hybridise only rarely, and their hybrids
401 are almost sterile (Koutecky 2007). In section *Acrolophus*, *C. stoebe* includes diploid
402 and tetraploid cytotypes (Spaniel et al. 2008). Similarly, although polyploid taxa exist,
403 only hybrids between species with the same chromosome number have been reported in
404 the section *Willkommia* (Blanca 1981).

405 The difficulty of hybridisation between diploid and tetraploid *Centaurea*
406 individuals is probably due to triploid seed abortion stemming from failure of the
407 endosperm (Ramsey & Schemske 1998). With rare exceptions, such as a few triploid
408 individuals detected in *C. phrygia* (Koutecky 2007), no triploid plants have been
409 observed in this genus. This finding contrasts with other taxa, such as *Chamerion*
410 *angustifolium* (L.) Holub. (Onagraceae) (Husband & Schemske 2000), *Betula*
411 (Betulaceae) (Thorsson et al. 2001), *Dactylorhiza* (Orchidaceae) (Aagard et al. 2005),
412 and *Dianthus broteri* Boiss. & Reut. (Balao et al. 2009) (Caryophyllaceae), where
413 triploids show different degrees of abundance, viability, fertility, and ability to act as a
414 genetic bridge between both parents.

415 In this study, the exceptional existence of a polyploid complex in *Centaurea* in
416 which the diploid *C. aspera* ssp. *stenophylla* and the tetraploid *C. seridis* ssp. *maritima*

417 occur in sympatry along with triploid individuals (*C. x subdecurrens*) is reported. The
418 ploidy level of *C. x subdecurrens* was successfully assessed by flow cytometry. A
419 significant proportion of RAPD and TBP markers were species-specific, so they clearly
420 discriminated *C. aspera* ssp. *stenophylla* and *C. seridis* ssp. *maritima* and confirmed
421 that these taxa are the parents of the triploid hybrid individuals. Accurate identification
422 of hybrid genealogies is a prerequisite in studies of diploid-polyploid hybrid zones in
423 order to assess the possibility of gene flow across ploidy levels. True F1 hybrids should
424 possess all the markers that are constant in one parent and absent in the other as well as
425 those that are constant in both parents. Caraway et al. (2001) estimated that in F1
426 hybrids, about 95% of the constant RAPD markers should be present, and in F2 hybrids
427 about 75%. In intermediate percentages, the origin of hybrids is uncertain. In our study,
428 using RAPDs, the triploid individuals showed a percentage of constant markers ranging
429 from 77% to 93%. However, using TBPs, these percentages ranged from 83% to 100%.
430 Moreover, hybrid individuals were highly or totally sterile triploids, as all of them
431 showed aborted achenes. These observations suggest that triploid individuals constitute
432 the F1 generation and that backcrosses with any of the parents are very rare or absent.
433 This hypothesis does not contradict the fact that the distances between the taxa (or
434 ploidy levels) and the number of markers common to the parents and hybrids showed
435 closer genetic similarity between *C. x subdecurrens* and *C. seridis* ssp. *maritima* than *C.*
436 *aspera* ssp. *stenophylla*. This is probably due to the fusion of a 2n gamete from *C.*
437 *seridis* ssp. *maritima* and an n gamete from *C. aspera* ssp. *stenophylla* at the origin of a
438 triploid hybrid individual, and not to the occurrence of backcrossing events. In this
439 scenario, the existence of a few hybrid individuals that appeared genetically
440 intermingled among the *C. seridis* ssp. *maritima* individuals, forming a continuum, may

441 be due to the combination of alleles, as each triploid individual corresponds to an
442 independent hybridisation event.

443 Morphologically, vegetative characters displayed high variability and could not
444 clearly discriminate the different taxa. This has lead to several taxonomic interpretations
445 based on the wide morphological range that showed several intermediate forms (Crespo
446 & Mateo 1990, Mateo & Crespo 2009). A large amount of morphological intermediate
447 forms resulting from hybridisation between individuals with different ploidy levels has
448 also been reported in other *Centaurea* species, such as *C. phrygia s.l.* (Koutecky 2007),
449 *C. toletana* Boiss. & Reut., and *C. saxifraga* Coincy (García-Jacas et al. 2009).

450 When flowering characters were considered, the taxa could be clearly
451 differentiated. This result, along with that of the discriminant analysis, agree with other
452 morphological studies of diploid and tetraploid individuals in the genus *Centaurea*,
453 where the characters that best discriminate cytotypes were mostly flowering and fruiting
454 ones, just as in *C. jacea* (Hardy et al. 2000), *C. phrygia* (Koutecky 2007), and *C. stoebe*
455 (Spaniel et al. 2008).

456 According to the morphological classification, genetic grouping of the
457 individuals separated the three ploidy levels. The genetic variability was higher in the
458 diploid *C. aspera* ssp. *stenophylla* than in the tetraploid *C. seridis* ssp. *maritima*, and the
459 triploid hybrids showed intermediate values. This was confirmed by the number of
460 polymorphic loci and by the branch lengths in the cluster analysis using both marker
461 types, although this pattern was more evident when using TBPs than when using
462 RAPDs. These results contrast those found in *C. jacea*, where tetraploid individuals
463 displayed a higher genetic diversity, probably due to the presence of several rare alleles
464 and the existence of a unidirectional gene flow from the diploid to the tetraploid
465 individuals (Hardy and Vekemans 2001). However, in our study, gene flow may be very

466 limited or inexistent, as the percentage of unique markers of *C. aspera* ssp. *stenophylla*
467 and *C. seridis* ssp. *maritima* was high (46% and 28%, respectively, using RAPDs, and
468 73% and 43%, respectively, using TBPs), and backcrossing events are probably rare or
469 inexistent, as discussed previously.

470 The existence of triploid hybrids that act as a reproductive barrier and the lower
471 genetic diversity in tetraploid than in diploid individuals may reflect the occurrence of a
472 minority cytotype exclusion effect. In the long term, this may result in niche
473 differentiation and spatial segregation among cytotypes within contact zones (Levin
474 1975). In dune fields, local adaptation to different environmental factors, such as soil
475 and microclimatic characteristics, can occur on a scale of a few metres (Linhart & Grant
476 1996). In the coastal dune fields of eastern Spain, diploid *C. aspera* ssp. *stenophylla*
477 grows in the inland dunes (Bolos 1967; Costa & Mansanet 1981), whereas tetraploid *C.*
478 *seridis* ssp. *maritima* grows in the dunes nearest the sea (Rivas-Goday & Rigual 1958;
479 Rigual 1972, Pérez 1997). However, in the coastal dunes of El Saler, *C. aspera* ssp.
480 *stenophylla* coexists with *C. seridis* ssp. *maritima*, leading the intermediate forms
481 corresponding to *C. x subdecurrens* to arise. This coexistence may be due to the fact
482 that the dunes of El Saler display a high level of nitrophily and disturbance, due to
483 human use and urbanisation (Costa & Mansanet 1981). In disrupted habitats, hybrids
484 are particularly common because disturbance can, on the one hand, alter parental
485 species distribution, thereby increasing their contact and creating new mating
486 opportunities, and, on the other hand, provide novel or open environments in which
487 hybrids are able to establish themselves (McKinnon 2005; Thorsson et al. 2007; Blanca
488 1984).

489

490 **Aknowledgements**

491 This work is posthumously dedicated to Antonio Samo Lumbreras, to whom we are
492 very grateful for all his help. This study was sponsored by the Valencian Government
493 (Research Project GVPRE/2008/130) and the Polytechnic University of Valencia
494 (Research Project Ref. 3241).

References

Aagaard SMD, Sastad SM, Greilhuber J, Moen A. 2005. A secondary hybrid zone between diploid *Dactylorhiza incarnata* ssp. *cruenta* and allotetraploid *D. lapponica* (Orchidaceae). Heredity 94: 488-496.

Balao F, Casimiro-Soriguer R, Talavera M, Herrera J, Talavera S. 2009. Distribution and diversity of cytotypes in *Dianthus broteri* as evidenced by genome size variations. Ann Bot 104(5): 965-973.

Bardini M, Lee D, Donini P, Mariani A, Giani S, Toschi M, Lowe C, Breviario D. 2004. Tubulin-based polymorphism (TBP): a new tool, based on functionally relevant sequences, to assess genetic diversity in plant species. Genome 47: 281-291.

Breviario D, Morello L, Manca A, Giani S. 2008. The importante of being an intron. By wild…type tubulin genes. In: Blume YB, Baird WV, Yemets AI, Breviario D, editors. The plant cytoskeleton: a key tool for agro-biotechnology. Dordrecht: Springer. p 199-218.

Bennet MD. 2004. Perspectives on polyploidy in plants – ancient and neo. Biol J Linn Soc 82: 411–423.

Blanca G. 1981. Origen, evolución y endemismo en la sección *Willkommia* G. Blanca (género *Centaurea* L.). Actas III Congr OPTIMA. Anales Jard Bot Madrid 37(2): 607-618.

Blanca G. 1984. Sobre algunas centaureas del Sur de España. Lazaroa 6: 169-174.

Bolòs O. 1967. Comunidades vegetales de las comarca próximas al litoral situadas entre los los rios Llobregat y Segura. Mem. R. Acad. Cienc. Art. Barcelona 38(1).

Bolòs O, Vigo J. 1995. Flora dels Països Catalans. Vol. III. Barcelona: Barcino.

Con formato: Español (España, internacional)

Caraway V, Carr GD, Morden CW. 2001. Assessment of hybridization and introgression in lava-colonizing Hawaiian *Dubautia* (Asteraceae: Madiinae) using RAPD markers. Am J Bot 88(9): 1688-1694.

Cires E, Cuesta C, Peredo EL, Revilla MA, Fernández Prieto JA. 2009. Genome size variation and morphological differentiation within *Ranunculus parnassifolius* group (Ranunculaceae) from calcareous screes in the Northwest of Spain. Plant Syst Evol 281: 193-208.

Comai L. 2005. The advantages and disadvantages of being polyploid. Nat Rev Genet 6: 836-846.

Conte L, Cottia C, Cristofolini G. 2007. Molecular evidence for hybrid origin of *Quercus crenata* Lam. (Fagaceae) from *Q. cerris* L. and *Q. suber* L. Plant Biosyst 141(2): 181-193.

Coyne JA, Orr HA. 2004. Speciation. Sunderland: Sinauer Associates.

Costa M, Mansanet J. 1981. Los ecosistemas dunares levantinos: la dehesa de la Albufera de Valencia. Actas III Congr OPTIMA. Anales Jard Bot Madrid 37(2): 277-299.

Crespo MB, Mateo G. 1990. Novelties on taxonomy and nomenclature of Spanish vascular hybrids. Collectanea Botanica 18: 93-98.

Cueto M, Blanca G. 1986. Números cromosomáticos de plantas occidentales 392-402. Anales Jard Bot Madrid 43(2): 403-409.

D'Andrea L, Felber F, Guadagnuolo R. 2008. Hybridization rates between lettuce (*Lactuca sativa*) and its wild relative (*L. serriola*) under field conditions. Environ Biosaf Res 7: 61-71.

Dolezel J, Gohde W. 1995. Sex determination in dioecious plants *Melandrium album* and *M. rubrum* using high-resolution flow cytometry. Cytometry 19: 103-106.

Con formato: Inglés (Estados Unidos)

Con formato: Español (España, internacional)

Con formato: Francés (Francia)

Con formato: Español (España, internacional)

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Droogenbroeck B, Kyndt T, Romeijn-Peeters E, Thuyne W, Goetghebeur P, Romero-Motochi JP, Gheysen G. 2006. Evidence of natural hybridization and introgression between *Vasconcellea* species (Caricaceae) from Southern Ecuador revealed by chloroplast, mitochondrial and nuclear DNA Markers. Ann Bot 97(5): 793-805.

Ducarme V, Vrancken J, Wesselingh RA. 2010. Hybridization in annual plants: patterns and dynamics during a four-year study in mixed *Rhinanthus* populations. Folia Geobot (in press).

Fernández-Casas FJ, Susanna A. 1986. Monografía de la sección *Chamaecyanus* Willkomm del género *Centaurea* L. Treb. Inst. Bot. Barcelona 10: 1-174.

Font, M, Vallès J, Susanna A, García-Jacas N. 2008. Auto- and allopolyploidy in *Centaurea* sect. *Acrocentron s. l.* (Asteraceae, Cardueae): karyotype and fluorochrome banding pattern analyses. Collect Bot 27: 7-18.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 200: 1049-1051.

García-Jacas N, Soltis PS, Font M, Soltis DE, Vilatersana R, Susanna A. 2009. The polyploid series of *Centaurea toletana*: Glacial migrations and introgression revealed by nrDNA and cpDNA sequence analyzes. Mol Phylogen Evol 52(2): 377-394.

Hardy OJ, Vanderhoeven S, de Loose M, Meerts P. 2000. Ecological, morphological and allozymic differentiation between diploid and tetraploid knapweeds (*Centaurea jacea*) from a contact zone in the Belgian Ardennes. New Phytol 146(2): 281-290.

Con formato: Español (España, internacional)

Con formato: Francés (Francia)

Hardy OJ, Vekemans X. 2001. Patterns of allozyme variation in diploid and tetraploid Centaurea jacea at different spatial scales. Evolution 55(5): 943–954.

Hellwig FH. 1994. Chromosomenzahlen aus der Tribu Cardueae (Compositae). Willdenowia 24: 219–248.

Hellwig FH. 2004. Centaureinae (Asteraceae) in the Mediterranean – history of ecogeographical radiation. Plant Syst Evol 246: 137-162.

Husband BC. 2004. The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biol J Linn Soc 82(4): 537–546.

Husband BC, Schemske DW. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. J Ecol 88: 689-701.

Koutecky P. 2007. Morphological and ploidy level variation of *Centaurea phrygia* agg. (Asteraceae) in the Czech Republic, Slovakia and Ukraine. Folia Geobot 42: 77-102.

Levin DA. 1975. Minority cytotype exclusion in local plant populations. Taxon 24: 35-43.

Linhart YB, Grant MC. 1996. Evolutionary significance of local genetic differentiation in plants. Ann Rev Ecol Syst 27: 237-277.

MacQueen JB. 1967. Some methods for classification and analysis of multivariate observations. Proceedings of 5-th Berkeley Symposium on Mathematical Statistics and Probability. Berkeley: University of California Press.

McKinnon G. 2005. Reticulate evolution in higher plants. In: Henry RJ, editor. Plant diversity and evolution: genotypic and phenotypic variation in higher plants. Wallingford: CABI. p 81-96.

Mateo G, Crespo MB, Nebot JR. 1987. Fragmenta chorologica occidentalia, 928-950. Anales Jard. Bot. Madrid 44(1): 155-157.

Con formato: Español (España, internacional)

Mateo G, Crespo MB. 2003. Manual para la determinación de la flora valenciana. 3d ed. Monografías de Flora Montibérica 4. Valencia: Moliner-40.

Mateo G, Crespo MB. 2009. Sobre algunos híbridos ibéricos del género *Centaurea* L. (Compositae). Flora Montib 41: 28-34.

Merle H, Garmendia A, Ferriol M. 2010. Nuevo híbrido del género *Centaurea* L. (Compositae) sección *Seridia* (Juss.) Czerep. Flora Montib 44: 66-71.

Nei M. 1978 Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:5 83-590.

Nei M, Li W. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 79: 5269-5273.

Osborn TC. 2004. The contribution of polyploidy to variation in *Brassica* species. Physiol Plantarum 121: 531-536.

Otto F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Crissman HA, Darzynkiewicz Z, editors. Methods in cell biology (vol. 33). New York: Academic Press. p 105-110.

Parra R, Valdés B, Álvarez A, Villareal J, Roldán J. 1998. Números cromosómicos para la flora española. 793-805. Lagascalia 20: 302-308.

Pau C. 1898. Noticia de algunas plantas curiosas o nuevas. Actas Soc. Esp. Hist. Nat. 27: 196-200.

Pellicer J, Garnatje T, Hidalgo O, Tagashira N, Vallès J, Kondo K. 2010. Do polyploids require proportionally less rDNA loci than their corresponding diploids? Examples from *Artemisia* subgenera *Absinthium* and *Artemisia* (Asteraceae, Anthemideae). Plant Biosyst 144(4): 841-848.

Con formato: Español (España, internacional)

Con formato: Español (España, internacional)

Pérez Badia MR. 1997. Flora vascular y vegetación de la comarca de la Marina Alta (Alicante). Colección Técnica 16. Instituto de Cultura Juan Gil Albert. Alicante: Diputación Provincial.

Petit C, Bretagnolle F, Felber F. 1999. Evolutionary consequences of diploid – polyploid hybrid zones in wild species. TREE 14(8): 306-311.

Program ANTHOS. 2011. Sistema de información sobre las plantas de España. Ministerio de Medio Ambiente / Fundación Biodiversidad / Real jardín Botánico, CSIC. http://www.anthos.es.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploidy formation in flowering plants. Ann Rev Ecol Syst 29: 467-501.

Repplinger M, Johannesen J, Seitz A, Comes HP. 2007. Morphological and molecular evidence for hybridization and introgression in Central European *Arctium* (Asteraceae). Plant Syst Evol 268: 75-95.

Rigual A. 1972. Flora y vegetación de la provincia de Alicante. Alicante: Instituto de Estudios Alicantinos.

Rivas-Goday S, Rigual A. 1958. Algunas asociaciones de la provincia de Alicante.; Anales Inst Bot Cavanilles 16: 533-548.

Romaschenko K, Ertugrul K, Susanna A, García-Jacas N, Uysal T, Arslan, E., 2004. New chromosome counts in the *Centaurea Jacea* Group (Asteraceae, cardueae) and some related taxa. Bot J Linn Soc 145: 345-352.

Seo MN, Sanso AM, Xifreda CC. 2010. Chromosome numbers and meiotic behaviour in South American species of *Hybanthus* Jacq. and *Anchietea* A. St.-Hil. (Violaceae). Plant Biosyst 144(2): 340-347.

Sneath PHA, Sokal RR. 1973. Numerical taxonomy. San Francisco: Freeman and Co.

Con formato: Inglés (Estados Unidos)

Soltis DE, Albert VA, Leebens-Mack J, Bell CD, Paterson AH, Zheng C, Sankoff D, de Pamphilis CW, Wall PK, Soltis PS. 2009. Polyploidy and angiosperm diversification. Am J Bot 96(1): 336-348.

Sozen E, Ozaydin B. 2010. A study of genetic variation in endemic plant *Centaurea wiedemanniana* by using RAPD markers. Ekoloji 77: 1-8.

Spaniel S, Marhold K, Hodalova I, Lihova J. 2008. Diploid and tetraploid cytotypes of *Centaurea stoebe* (Asteraceae) in Central Europe: morphological differentiation and cytotype distribution patterns. Folia Geobot 43: 131-158.

Stuessy TF, Weiss-Scheeweiss H, Keil DJ. 2004. Diploid and polyploid cytotype distribution in *Melampodium cinereum* and *M. leucanthum* (Asteraceae, Heliantheae). Am J Bot 91(6): 889-898.

Suárez-Santiago VN, Salinas MJ, García-Jacas N, Soltis PS, Soltis DE, Blanca G. 2007. Evolution by reticulation of the *Acrolophu*s subgroup (*Centaurea* L., Compositae) in the occidental Mediterranean: origin and diversification of the section *Willkommia* Blanca. Mol Phylogen Evol 43: 156-172.

Susanna A. 1988. *Centaurea x valentina* Rouy. In: Fernández-Casas J, editor. Asientos para una flora occidental 9. Fontqueria 18: 51.

Thorsson ATH, Salmela E, Anamthawat-Jonsson K. 2001. Morphological, cytogenetic, and molecular evidence for introgressive hybridization in birch. J Hered 92(5): 404-408.

Thorsson ATH, Palsson S, Sigurgeisson A, Anamthawat-Jonsson K. 2007. Morphological variation among *Betula nana* (diploid), *B. pubescens* (tetraploid) and their triploid hybrids in Iceland. Ann Bot 99: 1183-1193.

Con formato: Español (España, internacional)

Con formato: Español (España, internacional)

Yildirim N, Sunar S, Agar G, Bozari S, Aksakal O. 2009. Biochemical and molecular characterization of some *Centaurea* species growing in the Eastern Anatolia region of Turkey. Biochem Genet 47:850–859.

Table I. Measured morphological characters and abbreviations of the *Centaurea* individuals.

Leaf characters		Flowering characters	
RTL	Rosette leaf: total length	FCD	Fresh capitulum diameter
RBL	Rosette leaf: blade length	FCL	Fresh capitulum length
RAL	Rosette leaf: apical lobe length	FNS	Fresh capitulum: number of bracteal spines
RPL	Rosette leaf: petiole length	FLS	Fresh capitulum: length of the longer spine
RAW	Rosette leaf: apical lobe width	FBN	Fresh capitulum: number of bracteae
RTW	Rosette leaf: total width	FNO	Fresh capitulum: number of outer flowers
RPW	Rosette leaf: petiole width	FNI	Fresh capitulum: number of inner flowers
RNL	Rosette leaf: number of lobes	DCD	Dry capitulum diameter
RBT	Rosette leaf: blade thickness	DCL	Dry capitulum length
RNT	Rosette leaf: nerve thickness	DNS	Dry capitulum: number of bracteal spines
LTL	Lower leaf: total length	DLS	Dry capitulum: length of the longer spine
LBL	Lower leaf: blade length	DBN	Dry capitulum: number of bracteae
LAL	Lower leaf: apical lobe length	ITL	Inner (fertile) flowers: total length
LPL	Lower leaf: petiole length	IUL	Inner (fertile) flowers: tube length
LAW	Lower leaf: apical lobe width	ILL	Inner (fertile) flowers: petal lobes length
LTW	Lower leaf: total width	IAL	Inner (fertile) flowers: anther length
LPW	Lower leaf: petiole width	IPL	Inner (fertile) flowers: pistil length
LNL	Lower leaf: number of lobes	ISL	Inner (fertile) flowers: stigma length
LBT	Lower leaf: blade thickness	OTL	Outer (infertile) flowers: total length
LNT	Lower leaf: nerve thickness	OUL	Outer (infertile) flowers: tube length
MTL	Middle leaf: total length	OPL	Outer (infertile) flowers: petal length
MBL	Middle leaf: blade length		
MAL	Middle leaf: apical lobe length		
MPL	Middle leaf: petiole length		
MAW	Middle leaf: apical lobe width		
MTW	Middle leaf: total width		
MPW	Middle leaf: petiole width		
MNL	Middle leaf: number of lobes		
MBT	Middle leaf: blade thickness		
MNT	Middle leaf: nerve thickness		
UTL	Upper leaf: total length		
UBL	Upper leaf: blade length		
UAL	Upper leaf: apical lobe length		
UAW	Upper leaf: apical lobe width		
UTW	Upper leaf: total width		
UNL	Upper leaf: number of lobes		
UBT	Upper leaf: blade thickness		
UNT	Upper leaf: nerve thickness		

Table II. Summary of the RAPD and TBP markers observed in *Centaurea aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima* and *C. x subdecurrens*. A total of 129 reproducible fragments were identified among all the individuals sampled.

RAPD / TBP markers			
	Centaurea aspera ssp. *stenophylla*	*C. seridis* ssp. *maritima*	*C. x subdecurrens*
Total number	124 / 15	67 / 7	98 / 11
Constant markers	11 / 2	25 / 6	19 / 5
Polymorphic markers	113 / 13	42 / 1	79 / 6
Shared by *C. aspera* and *C. seridis*	51 / 4	51 / 4	-
Constant in *C. aspera* and *C. seridis*	3 / 2	3 / 2	-
Unique to species / hybrid	57 / 11	19 / 3	5 / 0
Constant in species / hybrid	5 / 0	8 / 3	0 / 0
Polymorphic in species / hybrid	52 / 11	11 / 0	5 / 0
Constant in hybrid			
Polymorphic in *C. aspera* and/or *C. seridis*	-	-	13 / 0
Constant in *C. aspera* / absent in *C. seridis* or vice-versa	-	-	3 / 3

Figure 1. Study area. *Centaurea aspera* ssp. *stenophylla* individuals are represented by crosses, *C. seridis* ssp. *maritima* by filled circles, and *C. x subdecurrens* by open squares.

Figure 2. Morphological features and flow cytometric profiles of propidium iodide (PI)-stained nuclei of diploid (a), triploid (b), and tetraploid (c) *Centaurea* individuals (peak D). Chicken red blood cells (CRBCs) were used as an internal standard (peaks B, C, and next). The mean channel number (PI fluorescence), DNA index [DI = mean channel number of sample (peak D) / mean channel number of diploid reference standard (peak B)], and coefficient of variation value (CV, %) of each peak are also given.

Figure 3. Discriminant analysis using the morphotypes obtained from the classification analysis: a) vegetative characters; b) flowering characters. *Centaurea aspera subsp. stenophylla* individuals are represented by crosses, *C. seridis ssp. maritima* by filled circles, and *C. x subdecurrens* by open squares. Open triangles represent the group centroids.

Figure 4. Dendrogram showing relationships among 34 individuals of *C. aspera* ssp. *stenophylla*, *C. seridis ssp. maritima*, and *C. x subdecurrens* using RAPD markers based on DICE distance and UPGMA. Bootstrap values over 50 are indicated and are based on 1000 re-samplings of the data set. A: *Centaurea aspera subsp. stenophylla*, S: *C. seridis ssp. maritima*, H: *C. x subdecurrens*, O: outgroup.

Figure 5. Diagram showing relationships among 34 individuals of *C. aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima*, and *C. x subdecurrens* based on Principal

Coordinates Analysis using a) RAPDs, and b) TBPs. *Centaurea aspera* ssp. *stenophylla* individuals are represented by crosses, *C. seridis* ssp. *maritima* by filled circles, and *C. x subdecurrens* by open squares.

Complete addresses of the authors:

[1] Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46021 Valencia, Spain

[2] Departamento de Biología Aplicada, Universidad Miguel Hernández, Crta. De Beniel s/n, 03312 Orihuela (Alicante), Spain

[3] Departamento de Ecosistemas Agroforestales, Universidad Politécnica de Valencia, Camino de Vera s/n, 46021 Valencia, Spain

Con formato: Español (España, internacional)

Appendix A. Measured morphological traits of the *Centaurea* individuals. ANOVAs and PostHoc (L.S.D.) analyses for *Centaurea aspera subsp. stenophylla*, *C. seridis ssp. maritima*, and *C. x subdecurrens*. Different letters represent significant differences between morphotypes ($p < 0.05$).

Trait	p	*C. aspera subsp. stenophylla*		*C. x subdecurrens*		*C. seridis ssp. maritima*	
Leaf characters							
RTL	<0.001	No data		195.25 ± 57.03	a	296.00 ± 7.57	b
RBL	<0.001	No data		169.25 ± 53.47	a	269.63 ± 9.35	b
RAL	<0.001	No data		61.00 ± 24.53	a	91.00 ± 3.08	b
RPL	0.17	No data		26.25 ± 15.22	-	26.38 ± 6.86	-
RAW	<0.001	No data		35.25 ± 16.03	a	68.75 ± 2.42	b
RTW	<0.001	No data		43.25 ± 11.41	a	78.25 ± 4.89	b
RPW	0.23	No data		3.75 ± 2.46	-	3.83 ± 1.20	-
RNL	0.07	No data		9.00 ± 1.08	-	6.75 ± 0.29	-
RBT	<0.001	No data		0.64 ± 0.25	a	0.93 ± 0.05	b
RNT	<0.001	No data		1.85 ± 0.84	a	3.24 ± 0.08	b
LTL	<0.001	77.04 ± 3.35	a	187.00 ± 14.05	b	180.50 ± 0.29	b
LBL	<0.001	73.20 ± 1.75	a	156.83 ± 5.14	b	194.50 ± 5.48	c
LAL	<0.001	16.85 ± 1.25	a	44.83 ± 3.73	b	80.00 ± 2.89	c
LPL	0.01	3.84 ± 2.17	-	31.33 ± 8.81	-	No data	
LAW	<0.001	6.40 ± 0.75	a	33.17 ± 1.76	b	45.50 ± 2.02	c
LTW	<0.001	20.81 ± 1.43	a	56.50 ± 1.70	c	45.50 ± 2.02	b
LPW	0.02	0.65 ± 0.35	-	3.19 ± 0.86	-	No data	
LNL	0.07	8.18 ± 0.62	-	9.33 ± 0.36	-	6.50 ± 0.87	-
LBT	<0.001	0.27 ± 0.02	a	0.64 ± 0.02	b	0.62 ± 0.12	b
LNT	<0.001	0.65 ± 0.05	a	2.15 ± 0.10	b	2.65 ± 0.23	c
MTL	<0.001	58.87± 2.57	a	108.75 ± 11.86	b	179.38 ± 19.59	c
MBL	<0.001	57.54 ±2.83	a	108.75 ± 11.67	b	187.13 ± 18.42	c
MAL	<0.001	17.42 ± 2.21	a	38.38 ± 5.64	b	65.63 ± 5.05	c
MPL	0.15	1.34 ± 1.34	-	5.13 ± 3.10	-	No data	
MAW	<0.001	4.30 ± 0.32	a	25.75 ± 4.27	b	39.13 ± 2.08	c
MTW	<0.001	16.20 ± 1.06	a	37.25 ± 4.43	b	52.63 ± 5.35	c
MPW	0.13	0.14 ± 0.14	-	0.61 ± 0.39	-	No data	
MNL	0.29	6.73 ± 0.74	-	9.13 ± 0.48	-	7.63 ± 1.37	-
MBT	<0.001	0.29 ± 0.02	a	0.54 ± 0.07	b	0.74 ± 0.04	c
MNT	<0.001	0.72 ± 0.06	a	1.53 ± 0.23	b	2.40 ± 0.07	c
UTL	<0.001	38.85 ± 1.86	a	51.63 ± 4.79	b	71.38 ± 4.57	c
UBL	<0.001	38.85 ± 1.86	a	58.50 ± 5.60	b	91.88 ± 3.86	c
UAL	<0.001	13.21 ± 2.93	a	27.38 ± 4.34	b	43.63 ± 2.10	c
UAW	<0.001	3.84 ± 1.98	a	13.63 ± 3.02	b	22.50 ± 2.23	c
UTW	<0.001	6.05 ± 0.83	a	17.38 ± 2.41	b	25.13 ± 1.34	c
UNL	0.19	2.27 ± 0.60	-	5.13 ± 0.79	-	5.63 ± 1.13	-
UBT	<0.001	0.30 ± 0.04	a	0.54 ± 0.07	b	0.72 ± 0.05	c

UNT	<0.001	0.57 ± 0.06	a	1.12 ± 0.17	b	1.62 ± 0.11	c
Flowering characters							
FCD	<0.001	6.83 ± 0.46	a	14.96 ± 0.70	b	16.74 ± 0.44	c
FCL	<0.001	11.31 ± 0.27	a	16.90 ± 0.35	b	20.00 ± 0.49	c
FNS	<0.001	3.00 ± 0.00	a	7.50 ± 0.29	b	8.50 ± 0.38	c
FLS	<0.001	2.43 ± 0.15	a	6.99 ± 0.41	b	9.19 ± 0.39	c
FBN	<0.001	41.55 ± 1.32	a	65.25 ± 1.24	c	55.00 ± 1.38	b
FNO	<0.001	12.18 ± 0.95	a	18.25 ± 0.22	c	15.50 ± 0.70	b
FNI	<0.001	18.00 ± 2.59	a	49.50 ± 3.18	b	61.25 ± 1.06	c
DCD	<0.001	7.33 ± 0.33	a	15.20 ± 0.44	b	18.89 ± 0.35	c
DCL	<0.001	11.56 ± 0.29	a	16.23 ± 0.34	b	18.07 ± 0.52	c
DNS	<0.001	3.00 ± 0.00	a	7.00 ± 0.00	b	9.50 ± 0.26	c
DLS	<0.001	2.82 ± 0.15	a	8.04 ± 0.48	b	9.54 ± 0.24	c
DBN	<0.001	41.64 ± 2.07	a	70.13 ± 0.89	b	64.38 ± 3.00	b
ITL	<0.001	19.01 ± 0.63	a	24.69 ± 0.41	b	25.03 ± 0.43	b
IUL	<0.001	12.62 ± 0.33	a	17.75 ± 0.45	b	20.08 ± 0.39	c
ILL	<0.001	3.66 ± 0.11	a	4.93 ± 0.18	b	4.84 ± 0.22	b
IAL	<0.001	6.31 ± 0.22	a	7.56 ± 0.39	b	8.68 ± 0.11	c
IPL	<0.001	18.42 ± 0.99	a	26.27 ± 0.28	b	27.39 ± 0.91	b
ISL	<0.001	1.97 ± 0.06	a	2.41 ± 0.06	b	2.60 ± 0.02	c
OTL	<0.001	18.07 ± 0.75	a	31.39 ± 1.03	b	34.48 ± 1.39	b
OUL	<0.001	12.36 ± 0.40	a	22.59 ± 0.85	b	24.18 ± 0.81	b
OPL	<0.001	5.71 ± 0.54	a	8.80 ± 0.27	b	10.30 ± 0.72	b