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1   A rare case of a natural contact zone in Morocco between an autopolyploid and an

2   allopolyploid of *C. aspera* with a novel tetraploid block.

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14   **Short title**

15   A natural contact zone with a "tetraploid block"

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24

25   **Abstract**

1   A new contact zone between *C. aspera* and *C. seridis* was found in Morocco.

2   Chromosome counts and flow cytometry showed that both taxa were tetraploid ($4x=44$).

3   Bibliographic research and morphometric analysis established that *C. aspera*

4   corresponds to the autopolyploid *C. aspera* ssp. *gentilii* and *C. seridis* corresponds to

5   the allopolyploid *C. seridis* var. *auriculata*. This contact area was compared with

6   homologous contact zones in Spain formed by the diploid *C. aspera* ssp. *stenophylla*

7   and the tetraploid *C. seridis* ssp. *maritima*. Natural hybrids between the parental species

8   were very frequent in both areas. In Spain, hybrids were triploid (from reduced gametes

9   A and gamete AB) and highly sterile, exerting a 'triploid block'. In Morocco, cytometry

10   showed that hybrids were tetraploid and therefore hypothetically fertile, but all the

11   capitula lacked achenes. The resulting genome of the new tetraploid hybrid (AAAB),

12   through the fusion of reduced gametes AA (from ssp. *gentilii*) and AB (from var.

13   *auriculata*), could explain irregularities in meiosis by formation of aneuploid gametes,

14   and therefore infertility of the hybrid. Moroccan tetraploid hybrids develop, but have the

15   same identical irregularities as the Spanish triploids, due to the odd number of

16   homologous chromosomes, exerting a 'tetraploid block'. The new hybrid is first

17   described as *C. x subdecurrens* nothossp. *paucispinus*. In addition, distribution and

18   ecological traits were analyzed.

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INTRODUCTION

21   Polyploidy, the acquisition of more than two sets of chromosomes, has been

22   suggested as a major driving force in plant evolution (Otto 2007; Soltis & Soltis 2009),

23   and phylogenomic data even indicates ubiquity among them (Jiao *et al*. 2011).

24   Polyploidy can promote changes in genetic diversity, heterozygosity, inbreeding

25   tolerance or adaptability. However, whether polyploidy represents an evolutionary

advantage or disadvantage remains unclear, and mainly depends on taxa (Madlung 2013) and evolutionary moment (Parisod *et al*. 2010). The ecological and evolutionary significance of genome multiplication can be best analyzed in contact zones were related taxa, by polyploidization processes, interact (Lumaret 1988; Bretagnolle & Thompson 1995; Gao *et al*. 2014).

Centaurea L. (Compositae) is a recent and taxonomically intricate genus that presents a high rate of diversification and speciation due to the existence of polyploidy and descending dysploidy, as well as hybridization events (Hellwig 2004; Romaschenko *et al*. 2004). Most analyzed *Centaurea* polyploid contact zones included diploid ($2x$) and tetraploid ($4x$) cytotypes. In some of them, triploid ($3x$), pentaploid ($5x$) or hexaploid ($6x$) cytotypes were observed at very low frequencies (Hardy *et al*. 2000; Koutecký *et al*. 2012; Mráz *et al*. 2012). The allo or autopolyploid origin of the tetraploids cytotypes usually remains controversial, but evidences in the mode of allele inheritance (Hardy *et al*. 2001), divergence of ribotypes (Mráz *et al*. 2012), and even tetraploid phenotypes (Garcia-Jacas *et al*. 2009), have been useful to infer the origin. Concerning this latter approach, if diploid and tetraploid phenotypes are indistinguishable, then the tetraploid is considered a true autopolyploid (Garcia-Jacas *et al*. 2009). If the phenotypes are well differentiated, then other evidence for allo- or autopolyploid origin are needed (Hardy *et al*. 2001; Garcia-Jacas *et al*. 2009; Mráz *et al*. 2012). Sometimes, the tetraploid is clearly morphologically differentiated from the related diploid, and an autopolyploid origin is given (Hardy *et al*. 2000; Koutecký *et al*. 2012).

In addition, formation, ploidy level and fertility of natural hybrids and mating barriers between cytotypes are key issues discussed in these aggregates. The main pattern in *C. weldeniana* Rchb., *C. phrygia* L., *C. stoebe* L., *C. toletana* Boiss. & Reut.*, C. jacea* L. and *C. pseudophrygia* C.A. Mey. complexes was a very low or absent formation of natural hybrids between cytotypes, and in these cases hybrids were mostly tetraploids. Triploid hybrids were very rare or absent (Hardy *et al*. 2000; Hardy *et al*. 2001; Sijak-Yakovev *et al*. 2005; Garcia-Jacas *et al*. 2009; Koutecký *et al*. 2011; Mráz *et al*. 2012; Koutecký *et al*. 2012; Koutecký 2012). These evidences point to a strong natural reproductive isolation among cytotypes.

In *Centaurea*, crossing experiments have also been carried out artificially to get additional data about reproductive isolation between cytotypes (Hardy *et al*. 2001; Koutecký *et al*. 2011; Koutecký *et al*. 2012). Inter-cytotype crosses (heteroploids) were much less successful than intra-cytotype crosses (homoploid), showing the existence of strong, but not complete, reproductive barriers (Hardy *et al*. 2001; Koutecký *et al*. 2011). The artificial hybrids included triploids (from reduced gametes), tetraploids and pentaploids (involving unreduced and reduced gametes), and hexaploids (from unreduced gametes) (Hardy *et al*. 2001; Spaniel *et al*. 2008; Garcia-Jacas *et al*. 2009; Koutecký *et al*. 2011; Koutecký *et al*. 2012). Furthermore, interspecific homoploid hybridization is frequent in *Centaurea*, and hybrids are often easily recognized due to their intermediate morphology (*e.g.* Koutecký 2007; Blair & Huffbauer 2010; Pisanu *et al*. 2011). In fact, it is usually accepted that the extent of hybridization between particular taxa depends on the ploidy level; taxa of the same ploidy level can cross easily and their hybrids are fertile and capable of backcrossing, whereas taxa differing

1   in ploidy levels hybridize only rarely and their hybrids are sterile (Gardou 1972; Hardy

2   *et al*. 2001; Štepánek & Koutecký 2004).

3

4   Recently, a polyploid complex involving the diploid *C. aspera* L. and the

5   tetraploid *C. seridis* L. has been cited in several contact zones located in Eastern Spain

6   (Garmendia *et al*. 2010; Ferriol *et al*. 2012; Ferriol *et al*. 2014). In contrast with the

7   other reported polyploid complexes in *Centaurea*, highly sterile triploid hybrids

8   between both taxa were very frequent in nature. Ferriol *et al*. (2012) confirmed that the

9   triploid *C. x subdecurrens* Pau represents a true F1 offspring between *C. aspera* and *C.*

10   *seridis*. Genetic analyses showed that backcrossing events and gene flow were very rare

11   or absent, and that genetic diversity was higher in diploid than in tetraploid individuals

12   (Ferriol *et al*. 2012; Ferriol *et al*. 2014). In addition, microsatellite markers suggested an

13   allopolyploid origin of *C. seridis*, being *C. aspera* one of the parental species.

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15   A new Moroccan contact zone is analyzed in the present study. In this contact

16   zone, the same species as those included in the Spanish polyploid complexes are

17   involved. However, ploidy level and infra-specific adscription change: *C. aspera* is

18   represented by *C. aspera* ssp. *gentilii* (Braun-Blanq. & Maire) Dobignard, considered to

19   be a tetraploid (Vogt & Oberprieler 2008), and *C. seridis* is represented by *C. seridis*

20   var. *auriculata* (Bald.) Ball, whose chromosome number is unpublished.

21   Morphologically intermediate individuals between these two taxa occur.

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23   We aimed to analyze how this contact zone is operating and how these closely

24   related taxa interact. The main objectives are:

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(i) to study the distribution and ecological features of the contact zone located in Morocco and compare it with the contact zones located in Spain.

(ii) To clarify the ploidy level of the individual involved in the Moroccan contact zone, including those that morphologically correspond to *C. aspera* ssp. *gentilii*, those that correspond to *C. seridis* var. *auriculata*, and those that correspond to the intermediate forms.

(iii) To establish the morphological differentiation between taxa, both within and between Moroccan and Spanish contact zones. This will allow us to infer the allo or autopolyploid origin of *C. aspera* ssp. *gentilii* and to examine the niche specificity of the phenotypes.

(iv) Finally, to analyze the fertility and origin of the morphologically intermediate individuals in the Moroccan contact zone.

MATERIALS AND METHODS

Study species and study area

Centaurea aspera and *C. seridis* belong to the Seridia (Juss.) Czerep section. Both are polymorphic species with a large number of subspecies and varieties (Ball 1878; Jahandiez & Maire 1934; Tutin *et al*. 1976; Bolòs & Vigo 1995; Mateo & Crespo 2009). They are widespread in Western Mediterranean and Southwestern Morocco (Hellwig 2004), included in the Mediterranean biogeographical sub-region (Médail & Quézel 1999). Both species are insect pollinated, self-incompatible perennial herbs (Bosch *et al*. 1997; Arnelas & Devesa 2011 and pers. orbs.). The distribution of *C. seridis* is usually restricted to the coastline, although it occasionally expands inland, whereas *C. aspera* is more widely distributed (Braun-Blanquet & Maire 1922; Bolòs & Vigo 1995; Mateo & Crespo 2009) (Fig. 1).

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At the coast of Southeastern Spain, *C. aspera* is represented by *C. aspera* ssp. *stenophylla* (Dufour) Nyman. It grows along the littoral, from Castellón to Murcia, and extends inland (Rigual 1984; Alcaraz *et al*. 1985; Bolòs & Vigo 1995; Mateo & Crespo 2009). In this area, *C. seridis* is represented by two subspecies: *C. seridis* ssp. *maritima* (Duf.) Dostál and *C. seridis* ssp. *cruenta* (Willd.) Dostál (Tutin *et al*. 1976; Bolòs & Vigo 1995; Merle *et al*. 2010). *Centaurea seridis* ssp. *maritima* grows from Almería to Castellón on coastal dunes that frequently exhibit a high level of anthropic disturbance (Garmendia *et al*. 2010; Anthos 2014). Contrarily, *C. seridis* ssp. *cruenta* grows inland, along the valley of the Vinalopó River (Alicante) (Bolòs & Vigo 1995; Maestre 1999).

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When the distribution areas of *C. aspera* and *C. seridis* overlap, a polyploid hybrid complex arises. Six contact zones involving *C. aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima* and their hybrid *C. x subdecurrens* nothossp. *subdecurrens* Mateo & Crespo have been cited. They were found from northern Castellón to Almería, on coastal sand dunes (El Saler, Santa Pola, Guardamar and Calblanque) and on coastal pebble dunes (Marjal dels Moros and Chilches) (Garmendia *et al*. 2010; Ferriol *et al*. 2012; Ferriol *et al*. 2014). In Sax (inner lands of Alicante), *C. aspera* ssp. *stenophylla* and *C. seridis* ssp. *cruenta* also coexist and hybrid individuals *C. x subdecurrens* nothossp. *oblanceolata* Merle *et al.* were observed (Merle *et al*. 2010; Ferriol *et al*. 2014).

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The ploidy level of the taxa involved in these seven Spanish hybrid complexes has been previously reported (Ferriol *et al*. 2012; Ferriol *et al*. 2014): all individuals belonging to *C. aspera* ssp. *stenophylla* were diploid; those belonging to *C. x*

subdecurrens nothossp. *subdecurrens* and nothossp. *oblanceolata* were triploid; and those belonging to *C. seridis* ssp. *maritima* and ssp. *cruenta* were tetraploid (Table 1). Only in the contact zone located at El Saler, morphometric analyses were conducted, showing that flowering characters clearly discriminate the three taxa (Ferriol *et al*. 2012)

In Morocco, *C. aspera* is represented by a single subspecies: *C. aspera* ssp. *gentilii* (Braun-Blanquet & Maire) Dobignard. This taxon is endemic of South-Western Morocco and Gran Canaria Island (Greuter & Raab-Straube 2007). It is common on coastal sandy soils from Essaouira to Tiznit and it advances inland following the Sous valley on sandy soils reaching the surrounding area of Taroudant (Braun-Blanquet & Maire 1922, and pers. orbs.). It belongs to the association *Bubonio imbricati–Centaureetum gentilii* Peltier on fixed dunes (Médail & Quézel 1999), growing together with *Argania spinosa* Skeels and *Retama raetam* Webb & Berthel. It is also found on slightly sandy cliffs in association with *Argania spinosa* and *Euphorbia beaumierana* Hook.f. & Coss.

In Morocco, at least six varieties of *C. seridis* have been described: var. *auriculata* (Bald.) Ball*,* var. *epapposa* Caballero, var. *calva* Maire & Sauvage, var. *subferox* Pau & Font Quer., var. *pterocaulos* (Pomel) Maire, and var. *oligocentra* Maire (Ball 1878; Caballero 1917; Jahandiez & Maire 1934; Emberger & Maire 1941; González-Bueno 1988). Knowledge about differential characters among these varieties and between these and the Spanish subspecies is scarce. Variety *pterocaulos* and *oligocentra* are currently considered to be synonyms of *Centaurea sphaerocephala* L. Varieties *epapposa* and *subferox* were firstly described and cited in Northeastern

Morocco (Melilla and Axdir respectively), and variety *calva* in the Middle Atlas, around 1500 masl, in the Ifrane Valley. *Centaurea seridis* var. *auriculata* is the only variety that was firstly described and cited in the study area, along the littoral near Mazagan. We found it on semi-fixed coastal sand dunes, usually with evidence of grazing and presence of pathways.

Ecological characterization

The six Spanish and the Moroccan contact zone habitat traits were evaluated (Table 2). Distance to sea, altitude (meters above sea level), slope , and soil texture (pebble dunes, sand dunes and rocky inner mountain) were recorded for each sampled individual and averaged for each location. Local bioclimates were characterized using Rivas-Martínez (2004) indexes. Thermicity (It), ombrothermic (Io), and simple continentality (Ic) indexes, and annual positive precipitation in millimeters (Pp), were calculated from the thermo-pluviometric data of the nearest weather Stations. In addition, anthropogenic disturbance evidences were also recorded: presence of livestock (Liv), crossing trails (Tra), proximity of roads (Roa), pedestrian tourist influx (Tou), or presence of infrastructures (Inf).

Chromosome counts

Chromosomes were photographed and counted at mitotic metaphase in apical root meristems from germinating achenes. Only achenes from clear *C. aspera* and *C. seridis* were studied, because morphologically intermediate individuals were found to be sterile. Mature achenes were collected *in situ* from plants included in the morphometric analyses in the Spanish and Moroccan contact zones. Before germination, achenes were disinfected with diluted NaClO (20' in 0.5% active Cl⁻

solution) and washed three times (5' each time in distilled water). Seeds were

germinated in moistened 9 cm Petri dishes with two layers of sterile filter paper. Petri

dishes were kept at room temperature and natural light. Every 5 days, both the distilled

water and filter paper were changed. One day before collection, always from 9:30 to

10:30 a.m., achenes were watered. Root tips were pre-treated with 8-hydroxyquinoline

at 4ºC during 4-8h, washed twice (5' each time in distilled water), fixed in absolute

ethanol: ferric acetate (3:1) for 24h at 4ºC, washed twice and then conserved in ethanol

70%. Subsequently, root cells were hydrolyzed for 1h at room temperature in 5N HCL,

and washed twice in distilled water before staining. Tips were leaved at least 2h in

Schiff reagent in darkness and then the apical part of the root (only the stained pink end)

was cut and squashed under a cover glass in a drop of aceto-carmine. At least two

achenes per plant and three plants per location were analyzed.

Flow cytometry of individuals

Sixty-two individuals were sampled at Zaouiat el Kourati (Morocco) for flow

cytometry. Twenty of them were initially identified as *C. aspera* ssp. *gentilii*, 21 as *C.

seridis* var. *auriculata* and 21 were morphologically intermediate. Leaf samples were

sent from the study area by rapid refrigerated transport (approx. 12 hours) to the Flow

Cytometry Area of the Instituto Valenciano de Investigaciones Agrarias (IVIA). Ploidy

level of Spanish individuals was analyzed in previous studies (Garmendia *et al*. 2010,

Ferriol *et al*. 2012, Ferriol *et al*. 2014).

The methodology described by Aleza *et al*. (2009) was applied. Each sample

consisted of a small piece of leaf (~ 0.5 mm^2) collected from the plant to be analyzed

together with a similar leaf piece taken from a diploid control plant (*C. aspera* ssp.

stenophylla). Samples were chopped together using a razor blade in the presence of a nuclei isolation solution (High Resolution DNA Kit Type P, solution A; Partec®, Münster, Germany). Nuclei were filtered through a 30-µm nylon filter and stained with a DAPI solution (4,6-diamine-2-phenylindol) (High Resolution DNA Kit Type P, solution B; Partec®). Following a 5-min incubation period, stained samples were run in a CyFlow® Ploidy Analyzer (Partec®) flow cytometer equipped with optical parameters for the detection of DAPI fluorescence. The DNA fluorochrome DAPI was excited by the UV-LED at 365 nm. Histograms were analyzed using the CyView software (Partec®), which determines peak position, coefficient of variation (CV), arithmetic mean and median of the samples.

Morphometric analysis

In addition to the previous morphological characterization of *C. aspera* ssp. *stenophylla*, *C. seridis* ssp. *maritima* and *C. x subdecurrens* nothossp. *subdecurrens* growing in El Saler, in the other six Spanish contact zones, 100 individuals were sampled in order to perform morphological analysis (Table 1). In the Moroccan contact zone 24 individuals were collected to perform the same analyses. The morphological traits were evaluated from fresh flowering complete individuals (with caulinar leaves, stems, and capitula) in the field. At least one voucher specimen for each location and taxon was collected for the Herbarium of the Universitat Politècnica de València (VALA). Geographic coordinates were recorded for each individual using G.P.S. (Garmin Oregon 300).

Thirty-nine quantitative variables were evaluated: 9 corresponded to reproductive traits and 30 to vegetative traits (Table 3). The basal leaves from all

studied taxa dried off just before the flowering season and therefore they were not analyzed. Upper leaves were usually entire and sessile, and therefore variables from petioles and lobe number were dismissed. None of the morphologically intermediate individuals and only a few of the measured individuals from *C. aspera* and *C. seridis* had achenes. Consequently the measures from the achenes were not used for multivariate analyses.

In order to compare the Spanish and Moroccan contact zone, data from the Spanish locations were put together, including Sax. Therefore, *C. seridis* in the Spanish complexes included ssp. *maritima* from coastal localities, as well as ssp. *cruenta* from Sax. Similarly, *C. x subdecurrens* in the Spanish localities included nothossp. *subdecurrens* from the coastal localities, and nothossp. *oblanceolata* from Sax.

Normality Shapiro-Wilk tests (Royston 1982), and descriptive statistical measures (mean, standard deviation and error, etc.) were computed, as well as Pearson correlation tests between all pairs of variables. Length of involucre (IL) and upper leaves lobe (UAL), were log transformed to meat the normality requirement. ANOVAs and post-hoc Tukey HSD comparisons were calculated for all the variables, both among the Moroccan taxa and between Moroccan and Spanish contact zone. Bonferroni correction (Dunn 1961) was applied to the ANOVAs significance in order to correct the effect of several repeated analyses (Table 4 and supplementary material: Table S1 and Table S2).

Linear discriminant analysis (LDA), which attempts to maximize differences among *a priori* defined groups, was employed to test the discriminating power of

individual characters, both among the Moroccan taxa and between Moroccan and Spanish complexes. Variables were selected among those that were normal, non-correlated and resulted in significant differences in the previous ANOVAs (Table 4).

To test the accuracy of the discriminant model, a parametric method and a non-parametric k-nearest neighbor algorithm with similar design of cross validation were performed. All analyses were carried out using R (R Core Team 2013), with the following extra libraries: Plotrix library (Lemon 2006), which was used to calculate standard errors, and MASS and class libraries (Venables & Ripley 2002), which were used for discriminant and k-nearest neighbor analyses, respectively.

RESULTS

Distribution and ecological traits of Moroccan and Spanish taxa

The ecology of *C. seridis* and *C. aspera* is very similar in Spain and Morocco. *Centaurea seridis* prefers coastal areas (it is a dune specialist), but it may occasionally appears inland, settling on sandy soils. *Centaurea aspera* has a wider distribution that includes coastal and widespread inland areas. It usually prefers loose sandy soils but can occur on any type of soil. When the distribution area of these two taxa overlaps, both in Spain and Morocco a contact zone with intermediate forms can arise. In the study area of Morocco, *C. seridis* var. *auriculata* was distributed northern, from Safi to Essaouira. Contrarily C. *aspera* ssp. *gentilii* was distributed from Zaouiat to the south (Fig.1). Thus, from Safi to Tiznit we only found one contact zone close to Zaouiat el Kourati (Fig.1 and Table 1). In Spain the overlap area is much larger than in Morocco, therefore more contact zones were found (Garmendia *et al*. 2010). Nevertheless, we cannot rule

1   out the emergence of new contact areas in Morocco, since only part of the territory has

2   been prospected.

3

4   The 8 contact zones appeared to have similar ecological features (Table 2). All

5   were thermo-mediterranean (except for Sax), being Zaouiat el Kourati the warmest (It=

6   446), but close to the rest (El Saler, It= 395). Ombrothermic belt varied from upper

7   semiarid (Guardamar del Segura, Io= 1.3) to low dry (El Saler, Io= 2.5). Zaouiat el

8   Kourati highlighted as hyper oceanic (Ic= 7.5) while the other locations were oceanic

9   (Ic= 14.5 to 16.7). Positive annual rainfall varied from 271 to 536 mm, being 330 mm in

10   Zaouiat el Kourati. All locations had strong evidence of disturbance. In Zaouiat el

11   Kourati grazing was one of the most significant disturbances, while Spanish polyploid

12   complexes were mainly disturbed by tourism, but also grazing in Marjal dels Moros and

13   Chilches. Sandy soils and semi-fixed coastal dunes were the usual habitats in both

14   countries, but in Spain contact zones also appeared in pebble coastal dunes (Chilches

15   and Marjal dels Moros) and inland mountainous slopes (Sax).

16

17   *Chromosome counts*

18   *C. aspera* and *C. seridis* chromosomes were counted. In Spain *C. aspera* ssp.

19   *stenophylla* had 22 chromosomes ($2x=22$) (Fig. 2, A-B), and C. *seridis* ssp. *maritima*

20   had 44 chromosomes ($4x=44$) (Fig. 2, C-D). In Morocco, *C. aspera* ssp. *gentilii* and *C.*

21   *seridis* var. *auriculata* had 44 chromosomes ($4x=44$) (Fig. 2, E-H). No chromosome

22   counts were performed to the morphologically intermediate individuals because none of

23   them produced achenes.

24

25   *Flow cytometry*

Ploidy levels were estimated using flow cytometry (Fig. 3). Diploid ($2x=22$), triploid ($3x=33$) and tetraploid ($4x=44$) individuals were identified among the studied populations (Fig. 3). Spanish taxa were previously reported (Table 1). All the 62 individuals analyzed but one, included in the Moroccan contact zone, were tetraploids, independently from the taxonomical adscription (*C. aspera* ssp. *gentilii*, *C. seridis* var. *auriculata*, and the morphologically intermediate individuals between both species) (Table 1). However, although populations of *C. aspera* ssp. *gentilii* and *C. seridis* var. *auriculata* were homogeneous with respect to ploidy level for this location, the morphologically intermediate individuals included one triploid and twenty tetraploids.

Morphological analysis of Moroccan taxa

ANOVAs, after Bonferroni correction, showed that the variables that best separated the three Moroccan taxa were the flowering traits: involucre maximum width (IW), involucre proportional roundness (IR), spine maximum length (SL) and number of interior flowers (NI); and the vegetative traits: medium leaves internodal length (MI), upper leaves leaf roundness (UP), upper leaves apical lobe width (UAW) and medium leaves apical lobe width (MAW) (Table 4).

Although high correlation values were observed between several pairs of variables within taxon, these correlations disappeared when analyzed among taxa. For *C. seridis* var. *auriculata*, three of the four flowering variables were highly correlated: IW (R=0.95, p=0.0011), IR (R=0.97, p=0.00036) and SL (R=0.93, p=0.0026). For *C. aspera* ssp. *gentilii* this correlation was only maintained between IW and IR ((R=0.85, p=0.008) and there also was a high correlation between IW and NI ((R=0.92, p=0.0011) For the intermediate individuals, high correlation values were found between SL and IR

1   (R=0.80,p=0.018), SL and IW (R=0.84, p=0.0089), and IW and NI (R=0.78, p=0.024).

2   Therefore, all these flowering variables were useful for the multivariate analysis among

3   Moroccan taxa.

4

5   UW and UAW were highly correlated both within and among taxa and

6   consequently, UW was removed in subsequent analyses. There were only a few

7   significant correlations between the selected vegetative variables. The correlation

8   between MI and MAW (R=0.85, p=0.008) was significant for *C. aspera* ssp. *gentilii*,

9   but not for the other taxa.

10

11   The discriminant analysis clearly separated *C. aspera*, *C. seridis* and the

12   intermediate individuals from Moroccan contact zone (Fig. 4). The model obtained was:

13   lda1 = 0.06033 IW +0.0256 IR +0.4488 SL +0.1495 NI +0.7828 MI +0.9288 UP

14   +0.5456 UAW +0.2061 MAW, with a trace proportion of 0.6479, and lda2 = -5.206 IW

15   +1.398 IR +4.416 SL +1.996 NI -0.5246 MI -0.5646 UP -1.927 UAW +0.5005 MAW,

16   with a trace proportion of 0.3521. Nevertheless there was some overlapping,

17   highlighting the proximity between phenotypes. The apparent error rate was 0, the cross

18   validated error rate was 0.091 and the k-nearest neighbor cross validated error rate was

19   0.273.

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21   *Morphological comparison of the Moroccan and Spanish taxa*

22   After Bonferroni correction of the ANOVAs, the variables that best separated *C.*

23   *aspera* ssp. *stenophylla* and *C. aspera* ssp. *gentilii* were the lengths of the capitulum

24   (CL), involucre (IL), upper leave (UL), and upper leave lobe (UAL), which were

25   significantly larger in *C. aspera* ssp. *gentilii* (Table S2). As expected, there was a high

correlation between CL and IL and between UL and UAL in both subspecies (R > 0.84,

p < 0.004). Therefore only IL and UAL, were selected for the discriminant analysis.

The number of spines (NS) was lower in the Moroccan *C. seridis* var. *auriculata* (5-7) than in the Spanish *C. seridis* ssp. *maritima* (7-11). The plant height (HP), and the proportion of the internodes occupied by decurrence in upper (UDP) and medium leaves (MDP), were significantly lower in *C. seridis* var. *auriculata*, whereas the upper leaves and apical lobe lengths (UL and UAL respectively), as well as the stalk width of the medium leaves (MSW), were higher. UL and UAL were highly correlated and therefore UAL was chosen for the discriminant analysis. Surprisingly, the number of spines per bract (NS) and UAL were also correlated in both *C. seridis* taxa, saying that the plants with more involucral spines, also had longer upper leaves (R=0.96, p=0.0005 in Morocco and R=0.48, p=0.0034 in Spain) (Table S1). Therefore, the selected variables for the multivariate analysis were ln(IL), ln(UAL), NS, UDP, MDP and ln(UAL). Some of them were log transformed to meet normality requirements.

In the discriminant analysis using morphological data from both Spanish and Moroccan taxa the first axis separated *C. aspera* from *C. seridis* in both countries, being *C. x subdecurrens* and the morphologically intermediate Moroccan individuals in a central position between *C. aspera* and *C. seridis*. The second axis separated Spanish individuals from those from Morocco (Fig. 5).

The models for the first and second axes were lda1 = -0.6362 CL +0.805 ln(IL) - 0.87 IW +0.8417 IR +0.4022 SL -0.1966 NS +0.09341 NI +0.6504 MI +0.2972 UDP +1.197 MDP +0.1054 UP -0.5905 ln(UAL) +0.1872 UAW, and lda2 = -2.624 CL

+2.835 ln(IL) -1.092 IW -0.5919 IR +1.016 SL -0.1082 NS +0.0656 NI +0.5478 MI +0.6082 UDP -0.6518 MDP -0.6325 UP -0.3002 ln(UAL) -0.2768 UAW, with a trace proportions of 0.6150 and 0.2982 respectively.

DISCUSSION

Autopolyploid and allopolyploid origin of parentals

The classification of polyploids into the two major categories of autopolyploids and allopolyploids is still debated (Clausen *et al*. 1945; Ramsey & Schemske 1998; Parisod *et al*. 2010; Mlinarec *et al*. 2012). Autopolyploids are traditionally considered to arise within a single species by doubling of structurally similar, homologous genomes (AAAA), and show multivalent formation during meiosis. In contrast, allopolyploids arise via interspecific hybridization and subsequent doubling of nonhomologous genomes (AABB). They form bivalents during meiosis, as nonhomologous chromosomes do not pair (Jackson 1982; Ramsey & Schemske 2002; Parisod *et al*. 2010). This classification is now considered over simplistic, as it draws a somewhat arbitrary division through a continuum of degrees of divergence between parents involved in crossing and polyploid formation (Stebbins 1971; Abbott *et al*. 2013).

Indistinguishable field phenotypes have been frequently used as a diagnostic trait to differentiate autopolyploids from allopolyploids (Hardy *et al*. 2000; Soltis *et al*. 2007; Garcia-Jacas *et al*. 2009). In fact, many studies showed that in both synthetic and natural polyploids, interspecific hybridization is to trigger substantial reorganization of the gene expression network, while genome doubling reforms it to a state similar to that of its diploid progenitors (Rieseberg 2001; Riddle & Birchler 2003; Hegarty *et al*. 2006; Parisod *et al*. 2010; Xu *et al*. 2012).

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In this case, *C. aspera* ssp. *stenophylla* resulted to be diploid ($2x=22$) and *C. aspera* ssp. *gentilii* resulted to be tetraploid ($4x=44$) (Fig. 2), according with other published data (Vogt & Oberprieler 2008). Phenotypes of *C. aspera* ssp. *stenophylla* and ssp. *gentilii* were indistinguishable in the field, despite the small differences showed by the morphometric analysis. Consequently, we can consider *C. aspera* ssp. *gentilii*, a true autopolyploid of the diploid *C. aspera sensu lato* (AA).

Centaurea seridis ssp. *maritima* ($4x$) and ssp. *cruenta* ($4x$), have already been pointed out as allopolyploids (AABB), being *C. aspera* ($2x$) (AA) one of the parentals (Ferriol *et al*. 2014). *Centaurea seridis* var. *auriculata* was also tetraploid ($4x$) (Fig. 2) and most likely an allopolyploid, considering that its general phenotype coincided with *C. seridis s. l.* (including subspecies and varieties).

The morphologically intermediate Moroccan individuals appeared in the discriminant analysis in a equidistant position between *C. aspera* ssp. *gentilii* and *C. seridis* var. *auriculata* (Fig. 4). The phenotype of this taxon also coincided with *C. x subdecurrens* (Fig. 5), previously confirmed as a triploid F1 hybrid between *C. aspera* ssp. *stenophylla* and *C. seridis* ssp. *maritima* (Ferriol *et al*. 2012). In addition, all these individuals were sterile. These evidences allow us to consider those individuals as hybrids between *C. aspera* ssp. *gentilii* and *C. seridis* var. *auriculata*.

It is exceptional to find a contact zone between an auto and an allotetraploid originated from the same parental (*C. aspera*), growing in sympatry, and generating sterile hybrids. To our knowledge, this mechanism has not been previously reported.

Putative evolutionary advantages of auto or allopolyploids have been long discussed (Parisod *et al*. 2010). For a long time, autopolyploids were believed to suffer from several evolutionary disadvantages when compared with allopolyploids, and this led Clausen *et al*. (1945) and Stebbins (1971) to propose that autopolyploids are rare and represent evolutionary dead-ends. However, *C. seridis* (allopolyploid) and *C. aspera* ssp. *gentilii* (autopolyploid) occupy large areas and are both well established. Therefore, nothing suggests that allo or autopolyploid strategy *per se* gives advantages, according with recent studies (Parisod *et al*. 2010).

Triploid / tetraploid block

The term 'triploid block' has been defined as the difficulty of obtaining viable triploid seeds by diploid-tetraploid and tetraploid-diploid crosses (Marks 1966). Triploid embryos may die because of abnormal endosperm development, due to non-viable ploidies of an embryo and/or its associated endosperm (Satina *et al*. 1938; Marks 1966, Ramsey & Schemske 1998). However, in the Spanish contact zones, the triploid block is taking place later, during gamete formation by the well developed triploid.

Ferriol *et al*. (2012) found that the diploid *C. aspera* ssp. *stenophylla* (AA), and the allotetraploid *C. seridis* ssp. *maritima* or ssp. *cruenta* (AABB) formed sterile triploid hybrids *C x subdecurrens* (AAB, from reduced gamete A and AB, respectively). These triploids (AAB) developed vigorously but were sterile, probably due to asymmetric segregation of alleles during meiosis that leaded to a high frequency of aneuploid gametes (Darlington 1937; Ramsey & Schemskey 1998).

In the Moroccan contact zone, parentals are presumably the autotetraploid *C. aspera* ssp. *gentilii* (AAAA), and the allotetraploid *C. seridis* var. *auriculata* (AABB). Like in the Spanish contact zones, natural hybrids between these two taxa are very frequent, but their ploidy level was mostly tetraploid with only one triploid (discussed after).

Generally, in *Centaurea*, hybrids resulting from crosses of taxa with the same ploidy level are fertile and capable of backcrossing, whereas hybrids resulting from crosses of taxa with different ploidy level are rare and sterile (Štepánek & Koutecký 2004). Consequently, these tetraploid hybrids were supposed to be fertile. However, we observed no capitula with achenes. This sterility could be explained by the formation of aneuploid gametes by the tetraploid hybrid with a genome (AAAB), formed through the fusion of the reduced gamete AA (from ssp. *gentilii*) and AB (from var. *auriculata*). When compared with the Spanish triploids, these tetraploid hybrids developed also vigorously, but have the same identical meiotic irregularities, due to their odd number of homologous chromosomes. Consequently this tetraploid hybrid may be acting as a block, exerting the same role as the Spanish triploids. Such a model of "tetraploid block" has not been previously described.

One triploid in the tetraploid hybrid Moroccan population.

Although, individuals of *C. aspera ssp. gentilii* and *C. seridis var. auriculata* in Zaouiat el Kourati were all tetraploids, the hybrid population included one triploid individual and 20 tetraploids. Preliminary studies suggested that Moroccan populations of *C. aspera* ssp. *gentilii* may contain very few diploid individuals (data not showed). Probably, a diploid *C. aspera* ssp. *gentilii* individual unsampled at Zaouiat el Kourati

would explain the formation of the triploid hybrid within the Moroccan complex. Triploid (3*x)*, pentaploid (5*x*) and hexaploid (6*x*) cytotypes have been observed at very low frequencies in other *Centaurea* complexes (Hardy *et al*. 2000; Koutecký *et al*. 2012; Mráz *et al*. 2012)

Morphological proximity and ecological consequences in Centaurea contact areas

Morphologically, the linear discriminant analysis showed that the three taxa involved in the Spanish complexes were more differentiated than those in the Moroccan contact zone (Fig. 5). In Spain, an exclusion effect of the minor tetraploid cytotype could be acting due to the presence of a triploid block (Ferriol *et al*. 2012). However, spatial segregation and habitat specificity could counteract this cytotype minority effect and promote the survival of the two ploidy levels in mixed populations (Ferriol *et al*. 2012; Ferriol *et al*. 2014).

The morphological similarity of the three taxa was higher in Morocco than in Spain and appears as a continuum (Fig. 5). Particularly, *C. seridis* var. *auriculata* is the most similar *C. seridis* taxon to *C. aspera*, since it only has five spines in the involucral bracts (var. *maritima* has 11) and not very large capitula. Niche specificity is correlated with phenotype variations that are adapted to the specific requirements for each habitat (Sultan 1987; Scheiner 1993). This morphological continuum may indicate the possibility of less habitat specificity and therefore a most direct competition among taxa.

New Moroccan hybrid taxonomic adscription

Centaurea aspera ssp. *gentilii* differs from ssp. *stenophylla* mainly in their ploidy level and their distinct geographical distributions (Morocco and Spain respectively), therefore it can be treated as autonomous taxa at least at infraspecific level. *Centaurea seridis* var. *auriculata* differs widely from *C. seridis* ssp. *maritima* or ssp. *cruenta* as supported by morphological data. The use of distinct taxonomic categories at infraspecific level (variety or subspecies) is due to the different authors criteria (Dostál and Ball) but not based on the magnitude of the divergences. Therefore, the rare tetraploid hybrid between *C. aspera* ssp. *gentilii* and *C. seridis* var. *auriculata* is described, named and cited for the first time in the present study:

Centaurea x subdecurrens Pau in proceedings Soc. Esp. Hist. Nat. 27: 198. 1898 (*C. aspera x C. seridis*) **nothossp. *paucispinus*** Ferriol M, Merle H & Garmendia A, nothossp. nov. (*C. aspera* ssp. *gentilii x C. seridis* var. *auriculata*).

Holotypus: Drier (Morocco): Zaouiat el Kourati, near the road between Essaouira to Safí (R301), N31 42.837 W9 38.402, 63 m, semi-fixed coastal dune, inter parentes, 24-VI-2011, *Leg. et Det. Merle H, Ferriol M & Garmendia A* (VALA 9519).

The nothossp. *paucispinus* differs from other nothossp. of *C. x subdecurrens* in the geographical distribution and in the modal number of spines of the involucral bracts, 5 (3-7) in nothossp. *paucispinus*, and 7 (5-9) in nothossp. *oblanceolata* and nothossp. *subdecurrens*. Nothosubspecies *paucispinus* displays intermediate morphological characters between parentals, and sometimes it is difficult to differentiate them. Complete sterility of the capitula and a strong hybrid vigor are differential traits.

Many times in genus *Centaurea* it has been emphasized that the morphological variability of hybrid is enormous, especially with respect to the key determination character, the shape of appendages of involucral bracts (Vanderhoeven *et al*. 2002; Koutecký *et al*. 2011). In this case, noting the normal distribution and standard deviation of taxa in the discriminant analysis, the morphological variability of both hybrids (Spanish and Moroccan) is similar to the variability of its parent (Fig. 5).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. ANOVAs of the morphometric variables among *Centaurea aspera*

Table S2. ANOVAs of the morphometric variables comparing *Centaurea seridis*

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8   following hybridisation and genome doubling in trigenomic allohexaploid

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1   Figure legends

2

3   Fig. 1. Geographical distribution of the studied taxa. Dots represent the observed

4   individuals (own data) and the bibliography citations data. The colored or white areas

5   represent the approximate distribution of the taxa according to all the data available.

6

7   Fig. 2. Metaphase of root-tip mitoses. A-D: Spanish contact zone; A-B: *Centaurea*

8   *aspera* ssp. *stenophylla* ($2x=22$); C-D: *C. seridis* ssp. *maritima* ($2x=44$). E-H: Moroccan

9   contact zone; E-F: *C. aspera* ssp. *gentilii* ($2x=44$); G-H: *C. seridis* var. *auriculata*

10   ($2x=44$).

11

12   Fig. 3. Histograms of the relative fluorescence (DAPI staining) obtained through flow

13   cytometry. A-C: Spanish contact zone; A: *Centaurea aspera* ssp. *stenophylla* diploid $2x$

14   (left); B: hybrid triploid $3x$ (middle); C: *C. seridis* ssp. *maritima* tetraploid $4x$ (right); D-

15   F: Moroccan contact zone; D: *C. aspera* ssp. *gentilii* tetraploid $4x$ (left); E: hybrid

16   tetraploid $4x$ (middle); F: *C. seridis* var. *auriculata* tetraploid $4x$ (right). In the figures,

17   2C, 3C and 4C represent the relative amount of DNA in each peak (all analyses were

18   performed with 2n controls).

19

20   Fig. 4. Discriminant analysis plot for the first two axes (LD1, LD2) between the three

21   Moroccan taxa. Each letter represents an individual plant. G: *Centaurea aspera* ssp.

22   *gentilii*; A: *C. seridis* var. *auriculata*; X: *C. x subdecurrens* nothossp. *paucispinus*.

23   Discriminant analysis is decomposed in histograms for each species in the LD1: *C.*

24   *aspera* is represented by light grey, *C. seridis* is represented by dark grey, and the

25   hybrid is represented by dotted diagrams.

1

2   Fig. 5. Discriminant analysis plot for the first two axes (LD1, LD2) between the

3   Spanish and Moroccan taxa. Each letter represents an individual plant. G: *Centaurea*

4   *aspera* ssp. *gentilii*; A: *C. seridis* var. *auriculata*; X: the hybrid between the

5   aforementioned taxa (*C. x subdecurrens* nothossp. *paucispinus*); S: *C. seridis* in Spain

6   (ssp. *maritima* and ssp. *cruenta*) ; T: *C. aspera* ssp. *stenophylla*; H: *C.* x *subdecurrens*

7   in Spain (nothosubsp. *subdecurrens* and   nothosubsp. *oblanceolata*): Discriminant

8   analysis is decomposed in histograms for each species in the Spanish and Moroccan

9   hybrid complexes for LD1: *C. aspera* is represented by light grey, *C. seridis* is

10   represented by dark grey, and the hybrid is represented by dotted diagrams.

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