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Research

Multiple origins and chromosomal novelty in the allotetraploid *Tragopogon castellanus* (Asteraceae)

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Summary

- *Tragopogon* includes two classic examples of recently formed allopolyploid species in North America: *T. mirus* and *T. miscellus*. Older *Tragopogon* allotetraploids from Eurasia offer ideal taxa for comparing the longer term outcomes of allopolyploidy.
- To help resolve the ancestry of one of these older polyploids, phylogenetic analyses of multiple populations of the allotetraploid *T. castellanus* ($2n = 24$) and its putative diploid parents, *T. crocifolius* and *T. lamottei*, were conducted using sequences from nuclear (internal transcribed spacer, ITS; and alcohol dehydrogenase 1A, *Adh*) and plastid (*trnT-trnL* spacer, *trnL* intron, *trnL-trnF* spacer and *rpl16* intron) loci. Fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) were used to investigate the chromosomal constitution of *T. castellanus*.
- Our data confirm that the widely distributed *T. crocifolius* and the Iberian endemic, *T. lamottei*, are the diploid parents of *T. castellanus*, and that this polyploid formed at least three times.
- One group of populations of *T. castellanus* is distinct in exhibiting two pairs of rearranged chromosomes. These data suggest that some of the chromosomal variants that originate in young polyploids (here, an intergenomic translocation) may become fixed in populations, contributing to novelty in older polyploid lineages. The geographical distributions of the allopolyploids and parents are also complex, with allotetraploid populations being disjunct from one or both of the most closely related diploid parental populations.

Introduction

Tragopogon L. has emerged as a model system for the study of recent polyploidy in natural populations. Intensive study of the New World neoallotetraploids *T. mirus* Ownbey and *T. miscellus* Ownbey has revealed rapid chromosomal and genomic change-catching evolution in the act (Soltis *et al.*, 2012). Losses of homeologs (genes duplicated by polyploidy) and the origins of chromosomal variants (translocations, compensated aneuploidy) in these young allotetraploids have produced arrays of novel genotypes. But what is the fate of the homeolog loss and chromosomal changes observed in these young polyploids after several thousand or more generations? To fill this gap in our understanding of the evolutionary significance of this extensive genetic and chromosomal polymorphism in new polyploids, studies of related and older polyploids are needed.

Dedicated to the memory of Francis Marion Ownbey (1910–1974), Sergei Vasilievich Juzepczuk (1893–1959) and Anton Joseph Kerner Ritter von Marilaun (1831–1898).

Four endemic polyploid taxa of *Tragopogon* are known from Spain and nearby Morocco (Vogt & Oberprieler, 1993; Blanca & Diaz De La Guardia, 1996; Diaz De La Guardia & Blanca, 2004). Here, using genetic and chromosomal data and building on previous results (Mavrodiev *et al.*, 2008), we investigate the ancestry and history of one of them, *T. castellanus* Lereshe & Levier.

Tragopogon castellanus, distributed in the northern half of the Iberian Peninsula (Blanca & Diaz De La Guardia, 1996), was described > 125 yr ago (in Leresche & Levier, 1881), but was documented as a tetraploid ($2n = 24$) only 30 yr ago (Wilson, 1983). On the basis of morphology and cytology, Wilson (1983) suggested that the diploid *T. crocifolius* L. ($2n = 12$) could be one parent of *T. castellanus*. On the basis of morphology and internal transcribed spacer (ITS), external transcribed spacer (ETS) and plastid sequence data, *T. crocifolius* and *T. lamottei* Rouy ($2n = 12$) were proposed as putative parents of *T. castellanus* (Mavrodiev *et al.*, 2008), although sampling of all three species was limited.

As a result of its morphological similarity to *T. crocifolius*, *T. castellanus* has sometimes been considered as a synonym (e.g. Richardson, 1976) or a subspecies (*T. crocifolius* ssp. *badalii* Willk.; Willkomm, 1893; see also Diaz De La Guardia & Blanca, 1990) of this taxon; however, these species have been treated as distinct in more recent studies (e.g. Wilson, 1983; Diaz De La Guardia & Blanca, 1990, 1992; Blanca & Diaz De La Guardia, 1996), but with the inclusion of *T. crocifolius* ssp. *badalii* in *T. castellanus* (Diaz De La Guardia & Blanca, 1990). *Tragopogon crocifolius* is thought to occur throughout the Mediterranean area (Wilson, 1983; Vogt & Oberprieler, 1993), as well as in Central and Northern Europe (Richardson, 1976; Wilson, 1983). However, up to seven species have been previously circumscribed from '*T. crocifolius*', and the taxon is polyphyletic (Mavrodiev *et al.*, 2008, 2012). Only a single collection of *T. crocifolius* has been reported from southwestern Spain (see Blanca & Diaz De La Guardia, 1996), from an area in which *T. castellanus* was not previously found (e.g. Blanca & Diaz De La Guardia, 1996). It is not clear which lineage of *T. crocifolius* occurs in the Iberian Peninsula – including those related to the putative parent of *T. castellanus*. *Tragopogon lamottei* is an Iberian endemic, rediscovered in Spain *c.* 25 yr ago and found primarily in the northern part of the peninsula (Diaz De La Guardia & Blanca, 1988; Blanca & Diaz De La Guardia, 1996).

To test further the proposed parentage of *T. castellanus*, to assess the number of polyploid origins and to characterize the extent of genetic and chromosomal diversity in this allotetraploid, we analysed multiple populations of *T. castellanus* and its proposed parents using DNA sequence data and molecular cytogenetic methods (genomic *in situ* hybridization, GISH; and fluorescence *in situ* hybridization, FISH).

Materials and Methods

Sampling

This study included two rounds of field collecting (Supporting Information Table S1): the first was performed in 2006 by V. N. Suárez-Santiago and the second by D. E. and P. S. Soltis, V. N. Suárez-Santiago and A. Susanna during the summer of 2012. At least two localities of *T. lamottei* and three of *T. castellanus* (including the *locus classicus* of the species, population 3029) were sampled twice (in both 2006 and 2012) (Table S1). We also analysed three samples of *T. crocifolius* collected in Sicily by R. M. Baldini in 2012 (Table S1), and additionally 12 herbarium specimens collected in Italy and deposited in the Museum of Natural History, University of Florence (Florence, Italy, FI), the Herbarium of the Royal Botanic Garden of Madrid (Madrid, Spain, MA), the Herbarium of the University of Rome (La Sapienza, RO) and the Herbarium of the Botanical Institute in Bari, Italy (BI) (Supporting Information Notes S1). Dry leaf tissues were obtained from BI, FI, MA and RO by R. M. Baldini during 2007–2009, together with high-quality images of specimens, whenever possible.

In all, 99 individuals from 59 local populations were analysed: *T. castellanus*, 32 populations; *T. lamottei*, 14 populations; *T. crocifolius*, 13 populations (Tables S1, S2; Notes S1). The GPS coordinates assigned for most populations investigated in Spain are given in Table S1. In many cases, *T. lamottei* and *T. castellanus* occurred at the same locality, and therefore share GPS coordinates, but have different population numbers (Table S1).

Specimen vouchers for the populations listed in Table S1 are deposited at the University of Florida and University of Granada Herbaria (FLAS, GDA).

Chromosome counts

For selected individuals representing populations of *T. lamottei* and *T. castellanus* sampled in Spain before 2012, chromosomes were counted at metaphase in root-tip meristem cells taken from germinating seeds. Roots were pretreated with 8-hydroxyquinoline, fixed in ethanol–acetic acid (3 : 1), hydrolysed in 1 M HCl, stained in acetic orcein solution and then flattened for light microscopy (Darlington & La Cour, 1969).

Flow cytometry

The ploidy of silica-dried leaf material collected in the field during 2012 was determined using a modified version of the bead beating method described in Roberts *et al.* (2009). Samples were processed in batches of 24. For each sample, 5 mg of dried *Tragopogon* leaf material and 2 mg of dried *Brassica oleracea* leaf material (as a standard) were placed in a 1.5-ml Eppendorf tube containing two zirconia beads and agitated in a bead mill for 2–3 s. After milling, 500 µl of cold lysis buffer (0.1 M citric acid, 0.5% v/v Triton X-100, 1% w/v PVP-40 (polyvinylpyrrolidone, average molecular weight 40 000)) (Yokoya *et al.*, 2000; Hanson *et al.*, 2005) were added to each sample. After 5 min of incubation on ice and intermittent gentle mixing by hand, each sample was filtered using a 5-ml polystyrene round-bottomed tube with a cell-strainer cap (BD Falcon; Becton Dickinson & Co., Franklin Lakes, NJ, USA). A 140-µl aliquot of filtrate was placed in a new 1.5-ml Eppendorf tube with 1 µl of RNaseA (1 mg ml^{-1}) (Thermo Scientific Molecular Biology, Fisher Scientific, Pittsburgh, PA, USA) and incubated at 37°C for 20 min. Next, 350 µl of propidium iodide (PI) staining solution (0.4 M $NaPO_4$, 10 mM sodium citrate, 25 mM sodium sulfate, 50 µg ml^{-1} PI) were added to each tube of nuclei suspension. After 1 h at room temperature, stained nuclei suspensions were run at 14 µl min^{-1} on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) fitted with a 488-nm laser. Fluorescence measurements were made using the FL2 (585/40 nm) optical filter, capturing 10 000 events and utilizing the FL2-A values for the 2C peak.

Cultivation of plants for GISH and FISH analysis

Plants were grown from field-collected seed to provide root tips for FISH and GISH analysis. Seeds from two to five maternal individuals per population were selected. Germination was carried out by washing seeds in 8% domestic bleach, rinsing in distilled water and placing them in Petri dishes lined with paper

towels moistened with distilled water. Seeds on plates were cold treated at 4°C in the dark for 3 d, and then moved to a bench top close to a window to provide light. Following germination, young plants were grown in a temperature-controlled glasshouse at the University of Florida. Plants were grown in 12.7 cm pots in a mixture of Fafard 52 Mix (Hummert International, Earth City, MO, USA), Quikrete Premium Play Sand (Lowes, Gainesville, FL, USA) and Turface All Sport Granules (John Deere Landscapes, Gainesville, FL, USA).

Fluorescence and genomic *in situ* hybridization

The terminal 2 cm of growing roots were harvested and pretreated in an aqueous solution of 2 mM 8-hydroxyquinoline for 16 h at 4°C. Pretreated roots were then fixed in ice-cold 90% acetic acid for 10 min and transferred to 70% ethanol for −20°C storage, as described by Kato *et al.* (2011). Mitotic chromosome preparations followed Kato *et al.* (2011) using 28 µl of glacial acetic acid for the suspension of digested cells.

DNA probes were made for GISH from total genomic DNA of one individual of *T. crocifolius* and one of *T. lamottei*. DNAs were isolated using a cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987) and treated with RNase. For FISH, probe templates comprised a cloned 1.3-kbp 18S rDNA fragment from *T. dubius* Scop., and complementary oligonucleotides were annealed to make double-stranded DNA (dsDNA) for the centromeric repeat, TPRMBO (Pires *et al.*, 2004), the subtelomeric repeat, TGP7 (Pires *et al.*, 2004), and the interstitial repeat, TTR3 (Chester *et al.*, 2013). We followed the methods of Kato *et al.* (2011) for the labelling of DNA probes by nick translation, using 5 µg of dsDNA as substrate (for details, see Chester *et al.*, 2013). Probes were directly labelled by incorporating one of the following: fluorescein-12-dUTP, cyanine 3-dUTP or cyanine 5-dUTP (Perkin Elmer Inc., Waltham, MA, USA). Nick translation products were purified using a QIAquick Nucleotide Removal Kit (Qiagen Inc., Valencia, CA, USA).

FISH and GISH were conducted following Kato *et al.* (2011). The FISH mixture comprised 350 ng of Cy5-labelled TPRMBO probe, 180 ng of Cy3-labelled TGP7 probe, 300 ng of fluorescein-labelled TTR3 probe, 20 ng of Cy3-labelled 18S rDNA probe, 20 ng of fluorescein-labelled 18S rDNA probe and 700 ng of unlabelled sheared salmon sperm DNA in 0.7 × SSC (300 mM NaCl, 30 mM sodium citrate; pH 7.0). The GISH mixture comprised 400 ng of fluorescein-labelled *T. crocifolius* gDNA, 400 ng of Cy3-labelled *T. lamottei* gDNA and 560 ng of sheared salmon sperm DNA in 0.7 × SSC. Before *in situ* hybridization, slides were UV crosslinked (120 mJ cm^{-2}); the hybridization mixture was then added to the slide, and a coverslip was placed on top. Slides containing the probe mixtures were denatured at 82–83°C for 2 min 30 s and transferred to a sealed humid box for incubation at 55°C for 16 h for FISH, or 36 h for GISH. Following hybridization, slides were washed briefly in 2 × SSC to remove coverslips. Glass coverslips (Corning Inc., Corning, NY, USA) were then mounted using Vectashield containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA). Hybridized chromosome spreads were observed and imaged using a Zeiss Axio Imager.M2 fluorescence microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) with an X-Cite Series 120 Q Lamp (Lumen Dynamics Group Inc., Mississauga, ON, Canada). The brightness and contrast of the captured images were adjusted in AxioVision (version 4.8 Special Edition 64 bit, Carl Zeiss MicroImaging, Inc.) by moving the upper and lower cutoffs in the histogram of signal intensity.

GISH reprobing of FISH-probed slides used the GISH method described above after removing the glass coverslips in 2 × SSC. Karyotypes based on FISH and/or GISH were assembled in Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA) using the merged and coloured TIFF images exported from AxioVision. Chromosome designations A–F for *T. lamottei* and *T. crocifolius* were based on FISH signal distributions (TPRMBO, TGP7, TTR3, TTR15 and 18S rDNA) previously documented for *T. pratensis* L. and *T. dubius*, respectively (Chester *et al.*, 2013).

Amplification, cloning and sequencing

We amplified and sequenced the following DNA regions: two nuclear loci (ITS, alcohol dehydrogenase 1A, *Adh*) and four plastid loci (*trnT-trnL* spacer, *trnL* intron, *trnL-trnF* spacer (all three regions amplified and sequenced as one unit) and the *rpl16* intron). The strategy of amplification and cloning generally followed that described in Mavrodiev *et al.* (2008, 2012), but the number of PCR cycles was decreased from 25–40 to 15 in the case of *Adh* to suppress artefactual recombination events arising during PCR between *Adh* parental copies in *T. castellanus*. ITS copies of *T. lamottei* were previously recovered from individuals of *T. castellanus* in very low numbers by sequencing a large number of clones (Mavrodiev *et al.*, 2008; V. N. Suárez-Santiago *et al.*, unpublished). Because the FISH data show that the *T. lamottei* 35S rDNA contribution has been substantially reduced in all individuals of *T. castellanus* examined (see below), ITS was not cloned in this study for *T. castellanus*. Four plastid regions (*trnT-trnL* spacer, *trnL* intron, *trnL-trnF* spacer and *rpl16* intron) were sequenced, concatenated and analysed as a single contiguous dataset.

Phylogenetic analyses

Maximum likelihood (ML) analyses of the ITS, *Adh* and plastid datasets were conducted separately using RAxML (Stamatakis, 2014) following the strategy described in Mavrodiev *et al.* (2008, 2012) using sequence data from Mavrodiev *et al.* (2008) (plastid sequences) and Mavrodiev *et al.* (2012) (ITS and *Adh* sequences), with the addition of new sequences for samples of *T. castellanus*, *T. crocifolius* and *T. lamottei*. The ITS matrix included sequence data for most species of *Tragopogon* (Mavrodiev *et al.*, 2012).

For ITS, only direct ('raw') sequences were included in the analyses (as noted above, cloning was conducted earlier for *T. castellanus*). For *Adh*, both direct sequences (from *T. lamottei*) and clones (from *T. crocifolius*, *T. castellanus* and *T. lamottei*) were included in the analyses (individuals were selected based on

the analyses of ITS and plastid sequence data; for the individuals cloned, see Table S1). For cloned samples, single nucleotide polymorphisms (SNPs) observed in single clones were excluded as possible artefacts of amplification and/or sequencing following Mavrodiev *et al.* (2012).

Results

Ploidy

Ploidy was estimated via flow cytometry of field-collected or dried leaf tissues, or by chromosome counts of plants grown from seed (Table S1). Of the 99 individuals sampled, ploidy was obtained for almost all recently collected samples (77 of 87). Chromosomes were counted for 28 individual plants. We consistently found *T. castellanus* to be tetraploid with counts of $2n = 4x = 24$, and all *T. lamottei* and *T. crocifolius* to be diploid with counts of $2n = 2x = 12$.

ITS sequences

The total number of positions in the ITS alignment was 727; 13 SNPs and two single nucleotide insertions/deletions differentiated *T. crocifolius* and *T. lamottei* (Table S2). Populations of *T. lamottei* had one of two ITS variants that differed by a single SNP (157) (Table S2). These two variants formed a clade (86% ML bootstrap (BS)) that was sister to *T. porrifolius* ssp. *porrifolius* (58% ML BS) (Fig. 1c), in agreement with previous results (e.g. Mavrodiev *et al.*, 2008).

All *T. castellanus* individuals had identical ITS sequences. *Tragopogon castellanus* was sister to samples of *T. crocifolius* from central Italy (Lazio, Pescara) (1065A, 1071A), southern Italy (park La Sila, Calabria) (1096A) and Sicily (3606, 3608 and 3609) (72% ML BS) (Fig. 1b). These *T. crocifolius* sequences differed from the ITS sequences of *T. castellanus* at only two positions (558, 614) (Tables S2, S5). None of the ITS sequences of nine additional collections of *T. crocifolius* (Notes S1; Table S5) appeared to be as closely related to the ITS sequences of *T. castellanus* (Supporting Information Fig. S2).

Plastid sequences

The ML tree based on four plastid regions (*trnT-trnL* spacer, *trnL* intron, *trnL-trnF* spacer and *rpl16* intron) was largely unresolved (data not shown), but visual comparison of insertions and deletions, together with nucleotide substitutions of *T. castellanus*, *T. crocifolius* and *T. lamottei*, was informative for resolving the parentage of *T. castellanus* (for a summary, see Table S3). In the *rpl16* intron, *T. crocifolius* and *T. lamottei* were differentiated by two or three indels and from zero to five SNPs (Table S3). *Tragopogon lamottei* individuals fell into one of two major haplotype groups (see later). With one exception, the sequences of *T. castellanus* were identical, or very similar to, the plastid sequences of *T. lamottei*, and most of the *T. castellanus* individuals could therefore be placed in one of the two *T. lamottei* haplotype groups. The *T. lamottei*-1 and *T. lamottei*-2 haplotype groups differed by five SNPs (positions 58, 61, 62, 65 and 715) and a 6-bp deletion (869–874) (Table S3).

The *T. lamottei*-1 group comprised 18 *T. lamottei* individuals and 24 *T. castellanus* individuals (Table S1), all with identical sequences. The *T. lamottei*-1 haplotype differed from *T. crocifolius* (3606, 3608, 3609) by two indels (171–189 and 869–874 bp). The *T. lamottei*-2 group comprised 35 *T. castellanus* individuals and five *T. lamottei* individuals (Table S3). Four individuals of *T. lamottei* from the *T. lamottei*-2 haplotype group differed from the other *T. lamottei*-2 individuals by indels at positions 248–253, 852–864 and 597, and by an SNP at position 715. These results therefore further differentiated *T. lamottei*-2 into subgroups *T. lamottei*-2a and *T. lamottei*-2b (Table S3).

Haplotype group C, referred later as a separate origin, comprised a single individual of *T. castellanus* (population CAST-MON) and several samples of *T. crocifolius* (3606, 3608, 3609) (Table S1). In the *rpl16* intron, the plants of haplotype C differed from *T. lamottei* by two or three indels and from zero to five SNPs (Table S3).

The other plastid regions were less informative. Within the *trnL* intron, a single SNP (position 345) was present. This was an A in *T. crocifolius* (3606, 3608, 3609) and individuals of *T. lamottei* and *T. castellanus* from the *T. lamottei*-1 group, whereas individuals of *T. castellanus* and *T. lamottei* from the *T. lamottei*-2 group had a T. One *T. lamottei* individual (3569, population 3031) from the *T. lamottei*-2 haplotype group differed from all other individuals by a 10-bp insertion (positions 1120–1130) in the *trnL-trnF* spacer.

Adh sequences

We sequenced *c.* 850 bp of the *Adh* gene spanning four introns and four exons, sampling individuals to capture all of the variation uncovered by ITS and plastid sequencing, and the variation uncovered by karyotyping (see later). For *Adh* sequencing, sampling comprised one individual of *T. crocifolius* (3606), 12 individuals of *T. lamottei* (including individuals from haplotype groups *T. lamottei*-1 and *T. lamottei*-2) and 13 individuals of *T. castellanus* (including individuals from haplotype groups *T. lamottei*-1 and *T. lamottei*-2) (Table S1).

For *T. lamottei*, direct sequencing of 10 individuals and cloning of two individuals recovered identical *Adh* sequences (Table S4). Only a single sequence type was recovered from the diploid individual of *T. crocifolius* (Table S4; Fig. S1). The *Adh* sequences of *T. crocifolius* and *T. lamottei* differed from each other by 29 or 30 SNPs and four or five indels (Table S4).

Across all accessions of *T. castellanus*, two distinct *Adh* sequence types were consistently recovered, with one being *T. crocifolius*-like and the other being *T. lamottei*-like. The *T. crocifolius*-like *Adh* copy obtained from *T. castellanus* differed from the *Adh* sequence of diploid *T. crocifolius* at only a single site (450) (Table S4). The *T. lamottei*-like *Adh* copies of *T. castellanus* (variants I and II, Table S4) differed from the *Adh* sequences of *T. lamottei* by an SNP at position 200 and a 6-bp

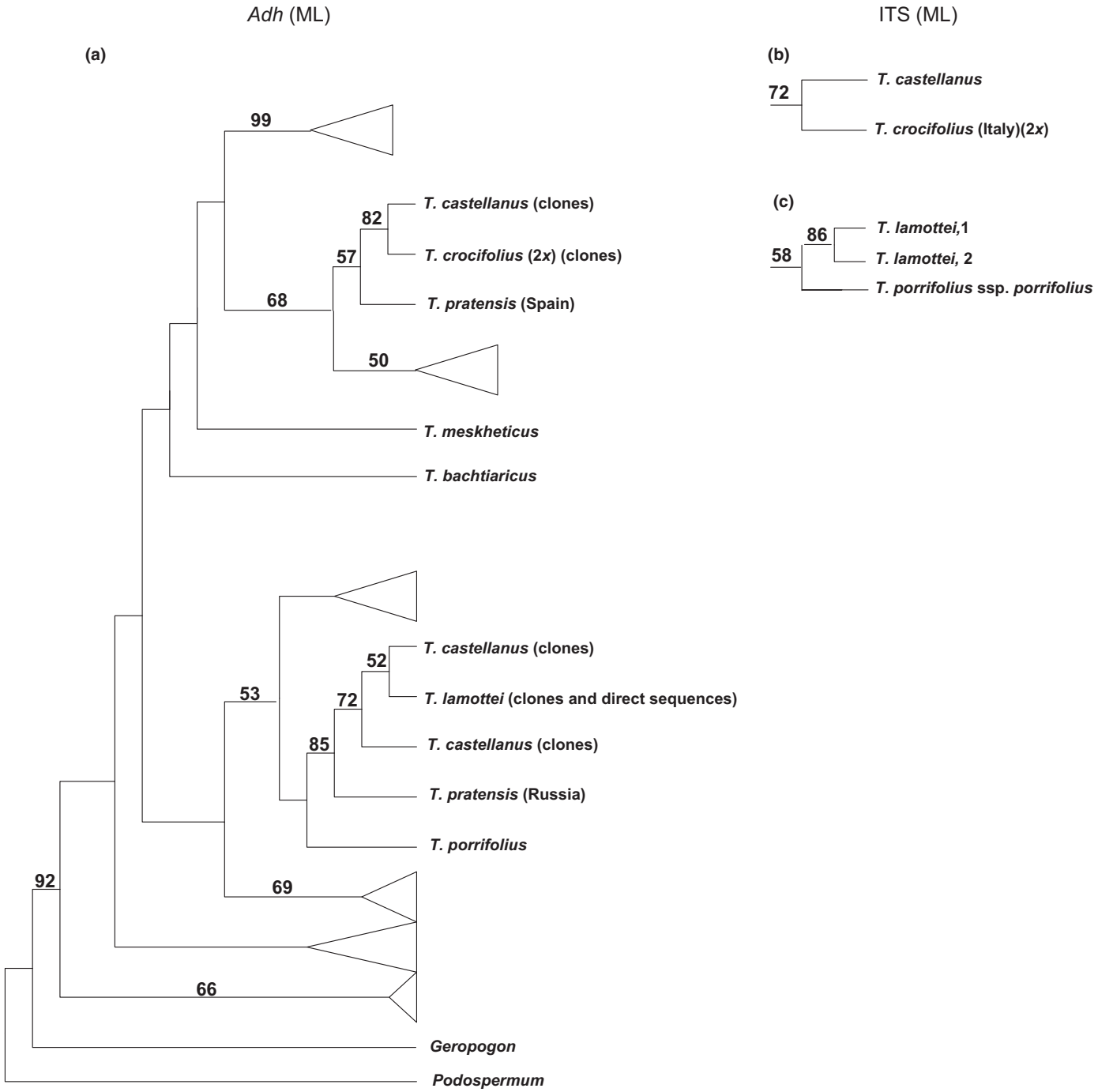

Fig. 1 (a) Simplified most probable topology recovered from a maximum likelihood (ML) analysis of alcohol dehydrogenase 1A gene (*Adh*) sequence data showing positions of clones and/or direct sequences of *Tragopogon castellanus*, *T. crocifolius* and *T. lamottei*. (b, c) Selected clades from most probable topology recovered from an ML analysis of internal transcribed spacers (ITS) one and two plus 5.8S ribosomal RNA gene (ITS) sequence data, showing positions of direct sequences of *T. castellanus*, *T. crocifolius* and *T. lamottei*. See Supporting Information Figs S1 and S2 for the details.

indel in *Adh* intron seven, positions 615–621 (Table S4). Furthermore, some were obtained from the same individual, 3627, which was from the type population of *T. castellanus* (Spain, province Burgos, Alar del Rey; ALAR), which possessed variants I and II. In the ML analysis, the *T. crocifolius*-like *Adh* copy was placed as sister to diploid *T. crocifolius* (82% ML BS), and the *T. lamottei*-like copy was placed in a clade together with *T. lamottei* and *T. pratensis* (Russia) (85% ML BS) (Figs 1, S2; Table S4).

GenBank accession numbers for new sequence data used in this study are: *Adh*, KJ666771–KJ666877; *rpl16*, KJ666878–KJ666961; ITS, KJ666962–KJ667046.

Chromosome analysis

Mitotic FISH karyotyping was conducted on the diploids *T. crocifolius* and *T. lamottei* (Fig. 2). One FISH probe (18S rDNA) hybridized to the nucleolar organizing regions, that is, the 35S rDNA arrays, and the other probes hybridized to *Tragopogon* tandem repeat sequences. The probes were applied as a mixture containing the 18S rDNA, centromeric repeat (TPRMBO), subtelomeric repeat (TGP7) and interstitial repeat (TTR3) (Fig. 2). All four probes hybridized to the genomes of the *Tragopogon* taxa tested. To check FISH karyotyping of *T. crocifolius*, which lacked FISH signals on the three smallest chromosomes, the TTR15 probe was used. This probe was applied to chromosomes of *T. crocifolius*, the closely related *T. pratensis* (from Spain) (Fig. S3) and two *T. castellanus* individuals (Fig. S4).

GISH was applied to *T. castellanus* individuals from the *T. lamottei*-1 and *T. lamottei*-2 haplotype groups (Figs 3,4). All were found to be allotetraploids, with one subgenome predominantly hybridized by *T. lamottei* gDNA and the other subgenome predominantly hybridized by *T. crocifolius* gDNA. Two distinct *T. castellanus* karyotypes were observed, with one being a nonrecombined karyotype and the other having an intergenomic translocation (Figs 3,4).

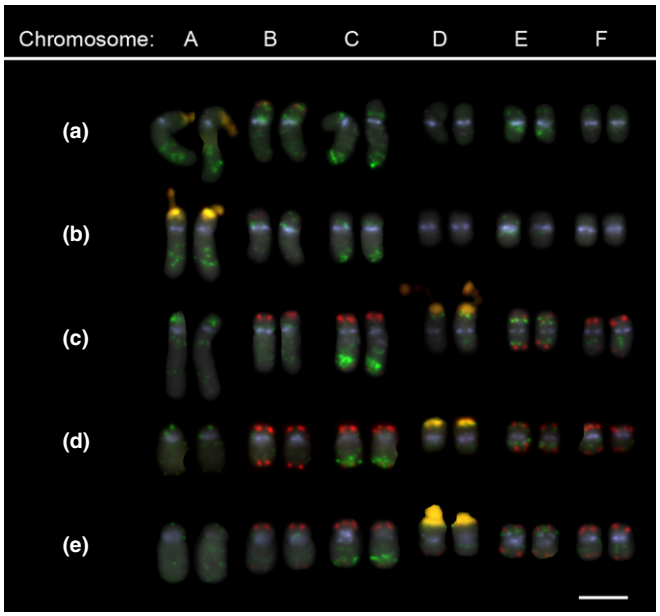

Fig. 2 Mitotic karyotypes of diploid progenitors of *Tragopogon castellanus* based on fluorescence *in situ* hybridization (FISH). (a, b) *T. crocifolius* and (c–e) *T. lamottei*. FISH probes were as follows: centromeric *Tragopogon pratensis* tandemly repetitive sequence MboI-rich (TPRMBO; lilac); subtelomeric *Tragopogon porrifolius* subtelomeric repeat-7 (TGP7; red); interstitial *Tragopogon* tandem repeat-3 (TTR3; green); and 18S rDNA (yellow/orange). Chromosomes, arranged by type (A–F), were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; grey). Parental accession numbers: (a) A-5; (b) A-4; (c) 3040-2/3576; (d) 3049-6/3582; and (e) 3031-19; see Supporting Information Table S1. Bar, 5 μm.

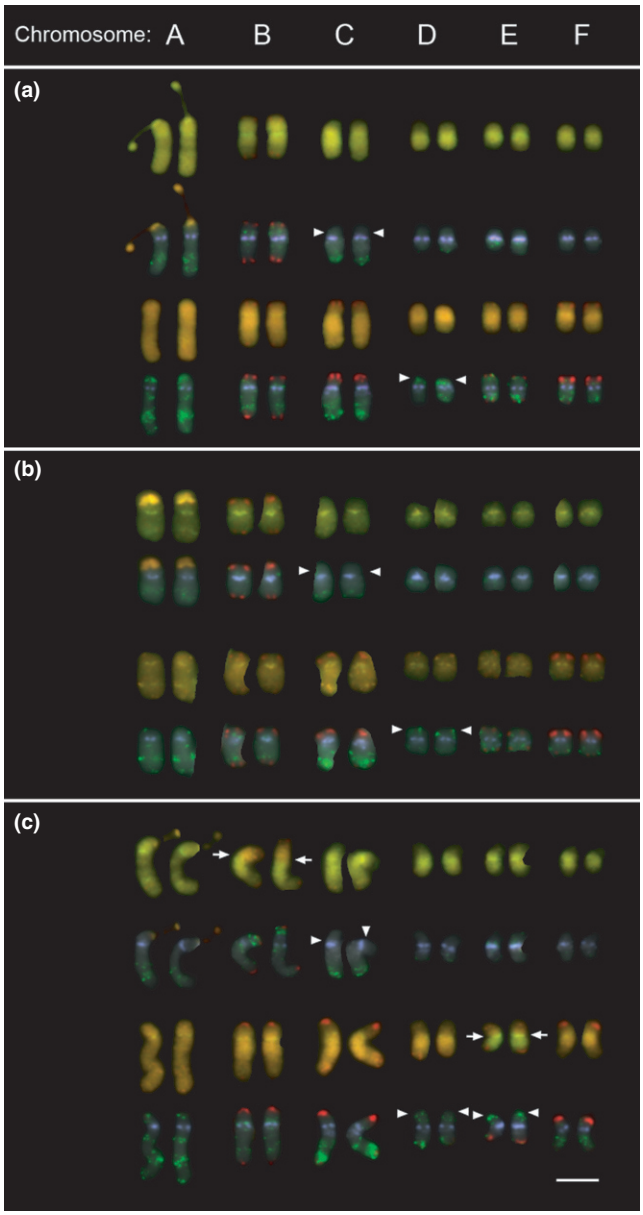

Fig. 3 Mitotic karyotypes of *Tragopogon castellanus* based on sequential fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH). Chromosomes, arranged by type (A–F), were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; grey) and are shown twice, with GISH signals and FISH signals, above and below, respectively. Karyotypes are shown for three individuals (*T. castellanus* types A, B2 and B1 shown in a, b and c, respectively). Mitotic chromosomes were first subjected to FISH (above) and then to GISH (below). FISH probes were as follows: centromeric *T. pratensis* tandemly repetitive sequence MboI-rich (TPRMBO; lilac); subtelomeric *T. porrifolius* subtelomeric repeat-7 (TGP7; red); interstitial *Tragopogon* tandem repeat-3 (TTR3; green); and 18S rDNA (yellow/orange). GISH reprobing of the same chromosome preparations using total genomic DNA of *T. crocifolius* (green) and *T. lamottei* (red). Arrows indicate the positions of translocation breakpoints. Arrowheads indicate absent TTR3 signals compared with *T. crocifolius*. Parental accession numbers: (a) 3024-11/3537; (b) 3035-6/3552; and (c) 3053-2/3599. Bar, 5 μm.

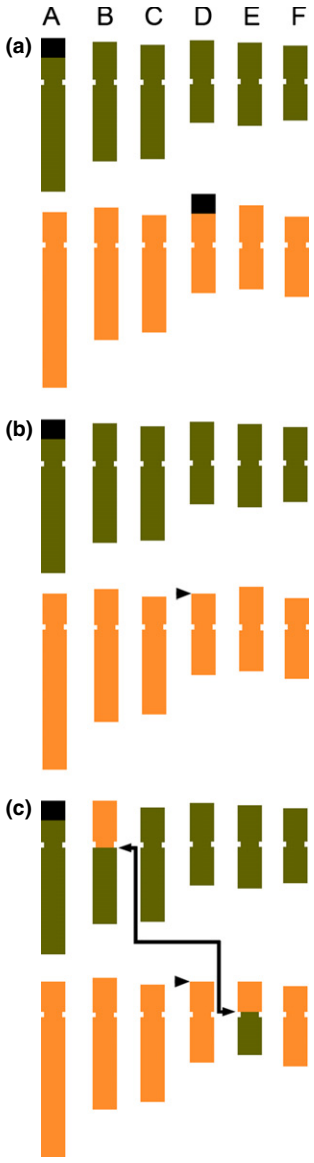

Fig. 4 Idiograms of *Tragopogon castellanus*. Hypothetical chromosome composition for an allotetraploid derived from *T. crocifolius* and *T. lamottei* shown in (a). Observed chromosome compositions are shown in (b) for *T. castellanus* types A and B2, and in (c) for *T. castellanus* type B1. Chromosomes are arranged by type (A–F) with colors indicating chromatin of *T. crocifolius* origin (green) and *T. lamottei* origin (orange). Blocks in black represent 35S rDNA sites. Arrowheads indicate the missing 35S rDNA sites. Arrows indicate the position of translocation breakpoints.

All *T. castellanus* individuals with a *T. lamottei*-1 plastid haplotype were found to have a nonrecombined karyotype, and are referred to as 'type A' *T. castellanus* (Fig. 3a,b). By contrast, GISH revealed *T. castellanus* individuals with the *T. lamottei*-2 plastid haplotype to have either a nonrecombined karyotype ('B2', Fig. 3b) or a recombined karyotype B1 (Fig. 3c). All B1 individuals were observed to be morphologically different from B2 individuals (with B1 individuals appearing more like *T. crocifolius*), indicating a possible link between the chromosomal alteration and morphology.

FISH and GISH reprobing was conducted to identify subgenomes and to compare repeat distributions in *T. castellanus* with those in *T. lamottei* and *T. crocifolius*. Most FISH signals in *T. castellanus* (A, B1, B2) matched those observed at the corresponding sites in the genomes of *T. lamottei* and *T. crocifolius* (Figs 2–4). One exception to parental additivity in all *T. castellanus* plants was the absence of a 35S rDNA site in the *T. lamottei* subgenome that was expected to be present on the short arm of chromosome D. The other consistent difference was a TTR3 signal on the short arm of chromosome C from *T. lamottei*, which was present in the FISH-karyotyped *T. crocifolius* (Fig. 2), but absent in all *T. castellanus* (A, B1 and B2) (Figs 3, S4). FISH with TTR3 to the closely related Spanish *T. pratensis* (Mavrodiev *et al.*, 2012; E. V. Mavrodiev *et al.*, unpublished) showed a better match with regard to chromosome C, but exhibited differences on chromosome B (Fig. S3).

The intergenomic translocation characterizing type B1 *T. castellanus* appears to involve putatively nonhomeologous chromosomes, namely B from *T. crocifolius* and E from *T. lamottei*. Therefore, one chromosome of *T. castellanus* now comprises the long arms of E from *T. crocifolius* and B from *T. lamottei*, and the other comprises the reciprocal. However, the breakpoints in both chromosomes are in close proximity to the centromere (Fig. 3), and so the recombination event may have occurred between homologous centromeric sequences (e.g. TPRMBO). One other common change was that the short arm of E of *T. lamottei* origin lacked the subtelomeric repeat (TGP7) signals expected on the basis of *T. lamottei* karyotyping (Fig. 2).

Geographical distributions

Diploid *T. crocifolius* has been reported from France, Italy, Morocco and southwestern Spain (the single collection: Cordova, Carretera de Trassierra, río Guadalmellatillo, 5-V-1982, M. J. Díaez, I. Fernandez, SEV 90758) (e.g. Wilson, 1983; Vogt & Oberprieler, 1993; Blanca & Diaz De La Guardia, 1996). As a result of the wide polyphyly of *T. crocifolius* (Fig. S1; Table S5; Notes S1; see also Mavrodiev *et al.*, 2008, 2012) and its unstable taxonomy, it is not clear which Spanish lineage of *T. crocifolius* was investigated by previous authors (reviewed in Blanca & Diaz De La Guardia, 1996). During recent rounds of field collecting (2006 and 2012), we were unable to find or confirm diploid *T. crocifolius* in Spain.

The geographical distributions of *T. lamottei* and *T. castellanus* A, B1, B2 and C plants (Tables 1, S1) are summarized in Figs 5 and 6. B1 plants of *T. castellanus*, seven populations of *T. castellanus* B2 and the single population of *T. castellanus* C (Table S1) occur in the northern part of the Spanish province of Aragón (Fig. 5), in the central Pyrenees, in the vicinity in which most *T. lamottei* populations of the *T. lamottei*-1 haplotype group occur (Figs 5, 6). By contrast, *T. castellanus* A populations, as well as the *T. lamottei*-2 haplotype group, occupy a different geographical area that mostly corresponds to the Cantabrian provinces of northern Spain (Navarra, La Rioja, northern and northeastern Castilla y León, south of Cantabria to southeastern Galicia), as well as the southern part of Aragón (Figs 5, 6).

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Table 1 General summary of results for ploidy levels, karyotypes, sequence comparisons and morphology of *Tragopogon castellanus*, *T. crocifolius* and *T. lamottei*

Species	Parents/origins	Ploidy	Karyotype (FISH/GISH[1])	Plastid (*rpl16*)	ITS	*Adh*		Habit
T. crocifolius (Italy, Sicily)	Paternal parent (origins A and B)	2*x*	*T. crocifolius*	*T. crocifolius*		*T. crocifolius*		*T. crocifolius*
T. castellanus	Origin A	4*x*	*c.* (*T. crocifolius* + *T. lamottei*)	*T. lamottei*, 1				*T. castellanus*
T. castellanus	Origin B1	4*x*	*c.* (*T. crocifolius* + *T. lamottei*)	Similar to *T. lamottei*, 2	*T. crocifolius*	*T. crocifolius* - Like clones	*T. lamottei*-Like clones	*T. crocifolius*
T. castellanus	Origin B2 (presumable)	4*x*	*c.* (*T. crocifolius* + *T. lamottei*)	Similar *to T. lamottei*, 2 *T. lamottei*, 1				*T. castellanus*
T. lamottei	Maternal parent (origins A and B)	2*x*	*T. lamottei*		*T. lamottei*		*T. lamottei*	*T. lamottei*
T. castellanus	Origin C (reciprocal to origins A and B)	4*x*	No data	*T. lamottei*, 2 *T. crocifolius*	*T. crocifolius*	*T. crocifolius*-like clones	*T. lamottei*-like clones	*T. crocifolius*

Adh, alcohol dehydrogenase 1A gene; *c.*, approximately; FISH, fluorescence *in situ* hybridization; GISH, genomic *in situ* hybridization; ITS, internal transcribed spacer.

Discussion

The origins of *T. castellanus* – evidence from molecular data and karyotyping

Sequence data in combination with the results of FISH and GISH confirmed that *T. castellanus* is derived from the diploid taxa *T. crocifolius* (3606, 3608, 3609) and *T. lamottei*, as proposed earlier on the basis of more limited sampling of populations and genes (Mavrodiev *et al.*, 2008), and that this polyploid formed at least three times. In two cases, *T. lamottei* was the maternal parent (Mavrodiev *et al.*, 2008). *Tragopogon crocifolius* was the maternal parent only in the case of population CASTMON (Tables 1, S1; Fig. 5). This population represents a separate origin (C) of *T. castellanus* and requires further investigation.

GISH and FISH also confirmed that *T. castellanus* is an allotetraploid. The cytogenetic data provide strong support for one parent being *T. lamottei*; the chromosomes of *T. lamottei* appear unchanged in *T. castellanus*, except for the loss of the parental 35S rDNA locus. The other parent of the allotetraploid is most likely *T. crocifolius*, although one of the interstitial FISH signals was absent in *T. castellanus* compared with the *T. crocifolius* that was sampled. Possible explanations for the missing signal are that the exact parental genotype was not sampled, or that a loss or gain occurred following allotetraploid formation.

The ITS and *Adh* sequence data (Fig. 1; Tables S2, S4) are consistent with only a single origin of *T. castellanus*. By contrast, plastid sequence data suggest a minimum of three origins of *T. castellanus* (*T. castellanus* A, B and C) (Tables 1, S3).

Although distinguishable by karyotype and gross morphology, *T. castellanus* B1 and B2 could not be differentiated on the basis of the genetic markers used here. Therefore, the chromosomal rearrangements observed in *T. castellanus* B1 may have arisen following a single origin of B. Alternatively, B1 and B2 may represent independent origins, but the sequence data employed were unable to resolve them.

Our observations show that the chromosomes of the allotetraploids are still close to the sum of their parents in terms of content, except for the lost 35S rDNA locus originating from *T. lamottei*. The chromosomal rearrangement in *T. castellanus* B1 appears to have arisen through a nonhomeologous intergenomic translocation. Therefore, as a result of the translocations being in a reciprocal state, the genetic contributions of the diploids are expected to be additive in these regions. Nonhomeologous translocations are much less common than homeologous translocations in young allopolyploid *Tragopogon* populations (*T. mirus* and *T. miscellus*) (Chester *et al.*, 2012, Chester *et al.*, 2014). The chromosomal alteration also raises the question as to whether it may provide a barrier to gene flow between type B1 vs A and B2 *T. castellanus*. No apparent hybrids were detected in *T. castellanus* that combined the B1 and B2 karyotypes. If the morphologically distinct *T. castellanus* B1 is consistently chromosomally distinct from A and B2, this might be another source of evidence for the recognition of B1 as a distinct species.

The intergenomic translocation in *T. castellanus* B1 presumably originated early after the origin of this allotetraploid type because it is fixed in multiple populations. Our results demonstrate that chromosomal novelty of the type observed shortly post-polyploidization in the recent allotetraploids *T. mirus* and *T. miscellus* (Chester *et al.* 2012; Chester *et al.*, in press) can become fixed in a population.

Taxonomy

The taxonomy of the Spanish species of *Tragopogon* is extremely difficult even after comprehensive treatments (Diaz De La Guardia & Blanca, 1988, 1990, 1992, 2004; Blanca & Diaz De La Guardia, 1996; Blanca & De la Guardia, 1997; Suarez-Santiago *et al.*, 2011). For example, in the context of the present study, the

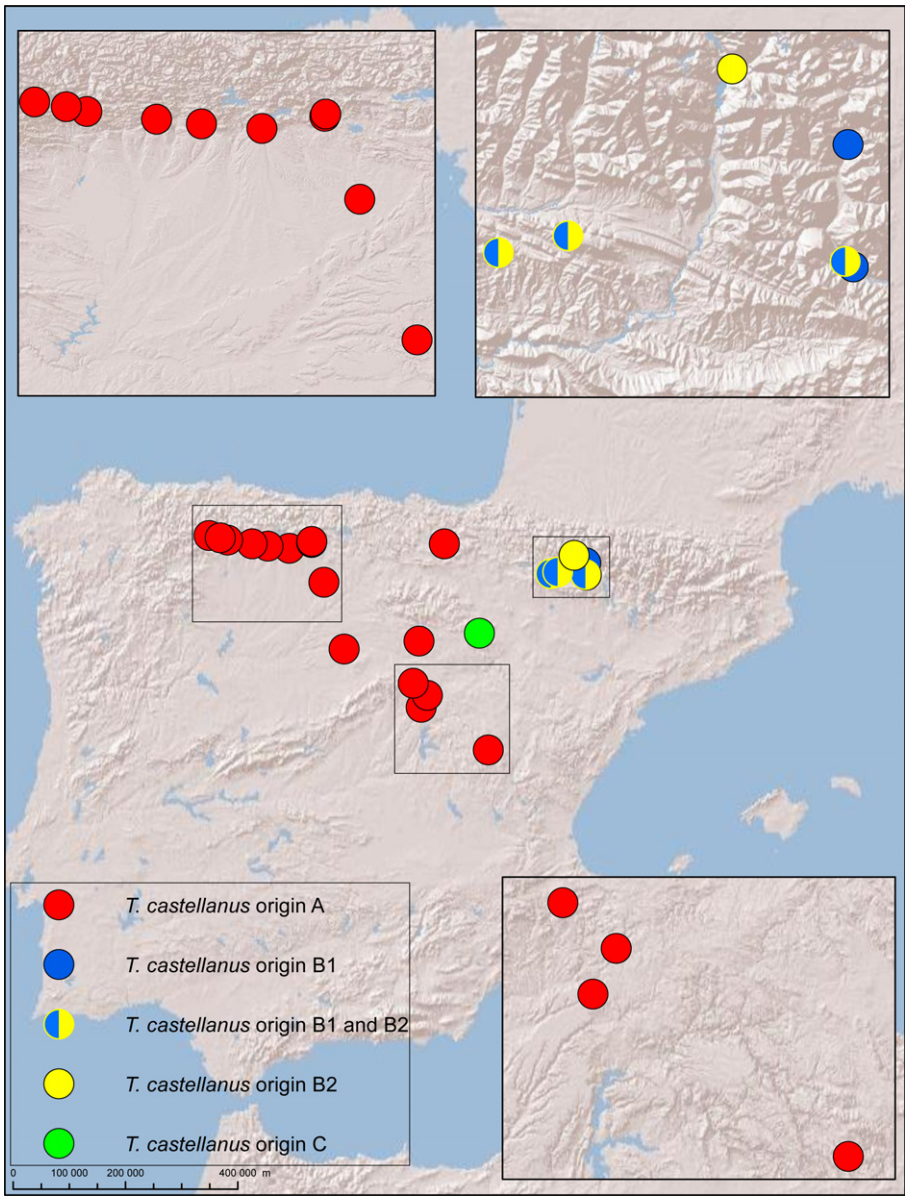

Fig. 5 Distribution of *Tragopogon castellanus*. Map generated using ArcMap 10 and locality data for collections used in this study (Supporting Information Table S1).

circumscription of the binomials '*T. bombycinus* Gredilla', '*T. pratensis* L.', '*T. villosus* L.' or '*T. flaviflorus* (Willk.) Willk.' still remains unclear (see also notes of Diaz De La Guardia & Blanca (1992) and Blanca & Diaz De La Guardia (1996) on *T. villosus*). This taxonomic uncertainty is partly understandable in the light of recent molecular studies showing that *Tragopogon* is a young, rapidly evolving genus (Bell *et al.*, 2012; Mavrodiev *et al.*, 2012) with hybridization and polyploidy adding to the complexity (e.g. Mavrodiev *et al.*, 2008, 2012; Suarez-Santiago *et al.*, 2011).

Geographical distributions

Based on herbarium records, initially we assumed that *T. castellanus* co-occurs with its two parents only in the Pyrenees. However, our data reveal that herbarium specimens identified as '*T. crocifolius*' from this area are actually *T. castellanus* with a unique karyotype (B1) or unique plastid haplotype (origin C) (Fig. 5). Indeed, despite extensive fieldwork, we did not find *T. crocifolius* in Spain. Moreover, both *T. castellanus* B1 and B2 often co-occur in the Pyrenees with one parental species, *T. lamottei* (Figs 5, 6), but the *T. lamottei* sampled from the Pyrenees is not actually the genotype that contributed to the formation of *T. castellanus* B1 and B2 in that region – that parental genotype was restricted to northwestern Spain, genetically distinct and geographically well separated from plants in the Pyrenees (Fig. 6). Plants from the haplotype group *T. lamottei*-1 (the proposed maternal parent for *T. castellanus* origin A from Cantabria) are nearly absent in northern and northwestern Spain outside of Aragón. Hence, the allotetraploids are not growing in close proximity to the proposed maternal genotypes from which they appear to have been formed (reviewed in Juzepczuk, 1939; Stebbins, 1950). This picture is generally different from that which is observed in the northwestern USA, where the parental

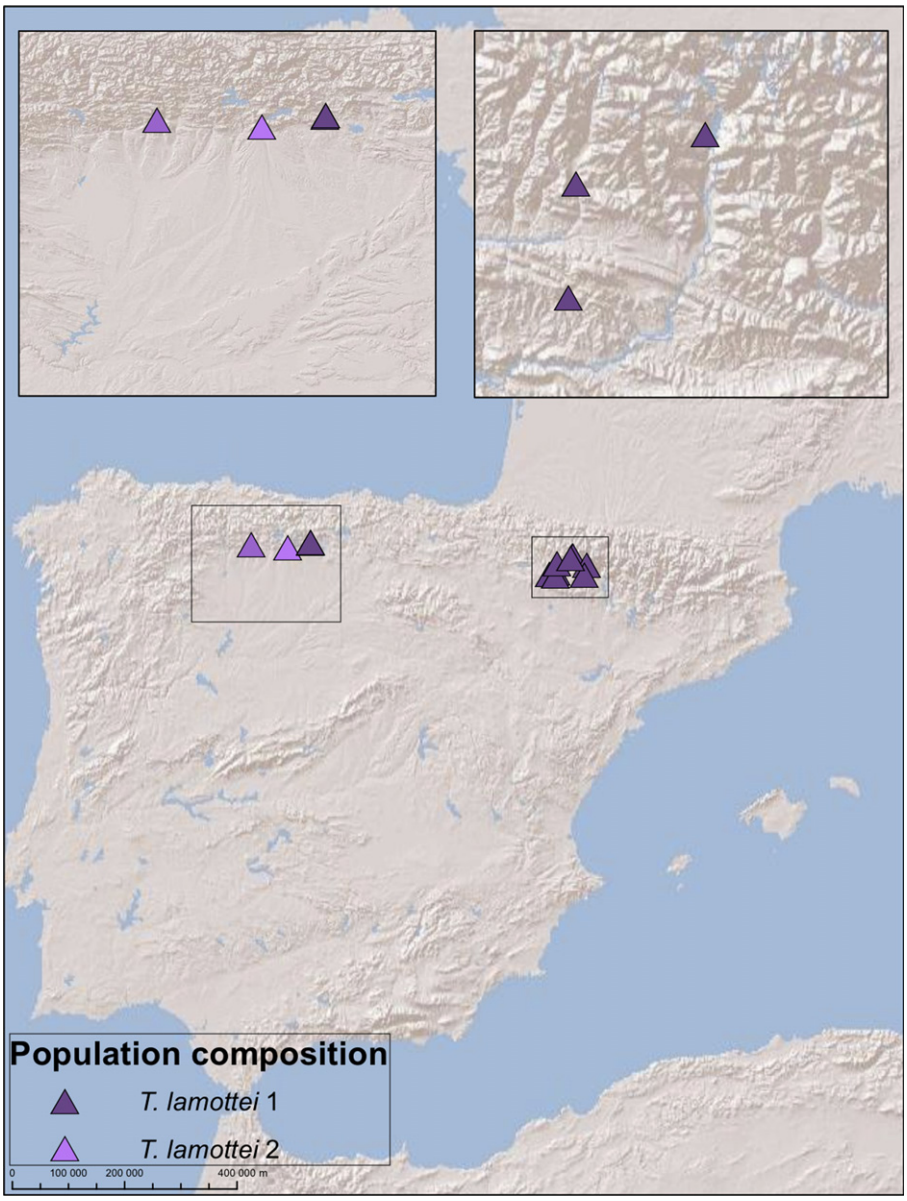

Fig. 6 Distribution of *Tragopogon lamottei*. Map generated using ArcMap 10 and locality data for collections used in this study (Supporting Information Table S1).

genotypes are often in the same area as their young allotetraploid derivatives (*T. mirus* and *T. miscellus*) (Soltis *et al.*, 2012; but see Roose & Gottlieb, 1976; Soltis & Soltis, 1991). Together with the apparent absence of diploid *T. crocifolius* in northern Spain (or perhaps the whole Iberian Peninsula), these data indicate that *T. castellanus* did not form recently and *what might appear to be a straightforward occurrence of parents and their polyploid derivative is actually much more complex*. For example, rather than indicating a recent formation of *T. castellanus* in the Pyrenees, the data reveal that genotypes have migrated, perhaps even becoming extinct in Spain (*T. crocifolius*), producing a complex evolutionary mosaic (see Stebbins, 1950).

Tragopogon castellanus is likely to be older than the recently formed New World allotetraploids *T. mirus* and *T. miscellus* which are estimated to be *c.* 40 generations old (e.g. Soltis & Soltis, 1991; Soltis *et al.*, 2012). However, the exact age of the Spanish polyploid still remains unclear. *Tragopogon castellanus* may have originated before the Last Glacial Maximum (LGM), and therefore its present-day distribution, and the distribution of its parents, could be a result of demographic changes during the ice ages of the Quaternary period (the last 2.6 million yr; Webb & Bartlein, 1992). The Iberian Peninsula was one of the most important Pleistocene glacial refugia in Europe, and it has been shown that several Iberian refugia existed for a range of flora and fauna (cf. Gomez & Lunt, 2006; Garcia-Jacas *et al.*, 2009; Nieto Feliner, 2011). The internal complexity of the Iberian Peninsula as a glacial refugium could have supported a high degree of genetic diversity for many species throughout the Pleistocene. Various migrations caused by glaciation, for example, *Centaurea* (Suarez-Santiago *et al.*, 2007; Garcia-Jacas *et al.*, 2009) and *Armeria* (Fuertes Aguilar *et al.*, 2011), together with the *in situ* survival of populations in microrefugia, could explain the genetic

complexity observed for *T. castellanus* and it parents. The timeframe proposed here for *Tragopogon* in Spain agrees with that proposed for *Centaurea* (Barres *et al.*, 2013).

Conclusions

Tragopogon crocifolius and the Iberian endemic, *T. lamottei*, are the parents of *T. castellanus*, which has formed at least three times. One morphologically distinct group of populations is differentiated from other *T. castellanus* in exhibiting two pairs of rearranged chromosomes, suggesting that some of the chromosomal variants that originate in young polyploids (here, an intergenomic translocation) may become fixed in populations, contributing to novelty in older polyploid lineages. The geographical distributions of *T. castellanus* and its parental taxa are complex, with allotetraploid populations being disjunct from one or both of the most similar diploid parental populations.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Maximum likelihood (ML) tree resulting from the analysis of *Tragopogon* alcohol dehydrogenase 1A (*Adh*) data matrix.

Fig. S2 Maximum likelihood (ML) tree resulting from the analysis of *Tragopogon* internal transcribed spacer (ITS) sequences.

Fig. S3 Mitotic karyotypes of the diploids *Tragopogon pratensis* and *T. crocifolius* based on fluorescence *in situ* hybridization (FISH).

Fig. S4 Mitotic karyotypes of *Tragopogon castellanus* based on sequential fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH).

Notes S1 Additional herbarium collections of *Tragopogon crocifolius* included in the study.

Table S1 Detailed summary of field collections of *Tragopogon castellanus*, *T. crocifolius* and *T. lamottei* used in this study, including ID, population numbers/abbreviations, GPS coordinates, ploidy level and summary of sequence data

Table S2 Summary of internal transcribed spacer (ITS) sequence comparisons for *Tragopogon castellanus*, *T. crocifolius* and *T. lamottei*

Table S3 Summary of *rpl16* sequence comparisons for *Tragopogon castellanus*, *T. crocifolius* and *T. lamottei*

Table S4 Summary of alcohol dehydrogenase 1A (*Adh*) sequence comparisons and cloning data for *Tragopogon castellanus*, *T. crocifolius* and *T. lamottei*

Table S5 Summary of internal transcribed spacer (ITS) sequence comparisons for all herbarium samples of *Tragopogon crocifolius*

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