

A new Pyrenean hybrid *Cirsium* (Asteraceae) as revealed by morphological and molecular analyses

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A new nothosubspecies *Cirsium* × *vivantii* is described after a molecular and morphological characterization of eight individuals collected in two separate valleys of the French central Pyrenees. Both hypervariable Amplified Fragment Length Polymorphism (AFLP) markers and nuclear rDNA (ITS) and plastid (*trnL-F*, *TRNT-L*) DNA sequences were analysed. The profiles of these hybrid samples were compared to those of 43 individuals belonging to their presumed parental taxa *C. carniolicum* ssp. *rufescens* and *C. palustre*. A total of 133 AFLP bands were scored from three primer-pair combinations. All 130 AFLP bands that amplified in the hybrid samples were present in either *C. carniolicum* ssp. *rufescens*, *C. palustre* or both taxa, supporting the participation of these plant genomes in the resulting hybrids. Several Additive Polymorphic Sites (APS) detected in the ITS sequences of the hybrid samples also confirmed their derived origins from ribotypes of the two parental taxa. The lack of exclusive AFLP markers and the nonconcerted evolution of rDNA polymorphisms towards either of the parental ribotypes indicated their recent origin. Plastid *trnT-L* sequences were used to identify *C. palustre* as the maternal parent of six of these hybrid individuals; either parent could have acted as the plastid genome donor for the other two individuals. The morphological study revealed that all hybrid individuals were morphologically intermediate between their parents showing largely lobed and less spiny basal leaves as in *C. carniolicum* ssp. *rufescens* and decurrent leaf bases and pinkish corollas as in *C. palustre*. © 2007 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2007, 154, 421–434.

ADDITIONAL KEYWORDS: AFLP – Compositae – hybridization – morphology – nrDNA – plastid DNA – thistles.

INTRODUCTION

Cirsium Mill. (thistles) is a large genus of the Asteraceae with c. 250 holartic taxa. Most of this species richness is concentrated in southern Europe and the Caucasus where 60 and seven taxa have been recognized at specific and subspecific taxonomic ranks, respectively (Werner, 1976). Twenty-nine of these have been reported from the Iberian Peninsula (Tala-

vera & Valdés, 1976), and 19 taxa along with seven hybrids from the Pyrenees (Rouy, 1905; Talavera & Valdés, 1976; Guinochet & Vilmorin, 1982; Bolòs & Vigo, 1995; Uribe-Echebarría, 1999; Villar *et al.*, 2001). The genus *Cirsium* apparently exhibits wide interfertility limits (Werner, 1976) which translate into a large number of interspecific and intersubspecific hybrids. The extraordinarily large hybridization potential of the thistles often has been considered a result of the sympatric occurrence of different taxa, the overlap in flowering periods and the close phylo-

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genetic relationships of the parental taxa involved in each hybridization event. Also, molecular cytogenetic studies have correlated larger hybridization potentials between *Cirsium* taxa with their smaller genome sizes (Bures *et al.*, 2004). Therefore, a cline of probabilities of producing hybrids has been detected in the genus: some taxa show a high probability of hybridization, whereas other combinations are apparently very rare or absent in the wild, as documented for other plant genera (Ellstrand, Whitkus & Rieseberg, 1996). Most of the hybrid combinations between taxa of sect. *Cirsium* are easily identified in the field by morphologies intermediate between those of the sympatrically occurring parents (Werner, 1976). Several hybrid taxa have been described based on their morphological intermediateness between their presumed parents but few have been confirmed by molecular means. The identification of hybrid thistles from herbarium specimens represents a more problematic case because the morphological variability of the samples and the loss of morphological attributes of the processed specimens (i.e. flower colour) preclude their correct assignment.

One of the rarest thistle hybrid combinations mentioned in the literature is that produced between the Pyrenean endemic *Cirsium carniolicum* Scop. ssp. *rufescens* (Ramond ex DC.) P. Fourn. and the widespread *C. palustre* (L.) Scop. (Vivant, 1998). However, this presumed hybrid plant was never formally described as a new hybrid taxon. Its intermediate morphology between the morphologies of its putative parents were neither examined through exhaustive morphological study nor confirmed with other means supplementary to morphology. The rarity of hybrid combinations between *C. carniolicum* ssp. *rufescens* and other members of the genus could be attributed to a set of biological and ecological features. This taxon is a narrow endemic from the western and central French Pyrenees where it has been reported so far from just four valleys (Aspe, Ossau, Azun and Luz). It represents the vicariant subspecies of the eastern-alpine endemic *C. carniolicum* ssp. *carniolicum*. Besides its limited distribution range, *C. carniolicum* ssp. *rufescens* is also ecologically specialized, inhabiting calcareous megaphorb communities from middle to high altitudes (1100–1600 m a.s.l.) (Villar & Benito-Alonso, 2003). Because of altitude, its flowering period is expected to be more restricted in duration than in other lowland taxa, and because of its narrow range it is unlikely to overlap in distribution range with many other thistles, therefore reducing the probability of hybridization with other congeners. Furthermore, the presumed chromosome number for this taxon ($2n = 16$, scored for the purportedly diploid type subspecies) is also very uncommon, either within the genus or the section *Cirsium*, where chromosome counts of $2n = 34$

(tetraploids) and $2n = 68$ (octoploids) are the rule. The other presumed parental taxon involved in the hybridization, *C. palustre*, is more widespread in Eurosiberian marshes, wet meadows and woods (Werner, 1976). It shows the frequent tetraploid level of the genus and section ($2n = 34$), which possibly favours a higher potential to produce interspecific hybrids (see the *Index Synonymique de la Flore de France* at <http://www.dijon.inra.fr/flore-france/>, where 13 hybrid combinations involving *C. palustre* have been reported).

Dominant molecular markers, such as AFLPs (Vos *et al.*, 1995) or RAPDs (Williams *et al.*, 1990) have proven to be useful tools for deciphering the hybrid origin of many plant taxa by correlation of their observed genomic patterns to those of the parental donors. Some examples of investigated putative hybrids have been confirmed in view of the additive presence of nuclear genomic profiles from both parents into the genomes of the hybrids (De Greef & Triest, 1999; O'Hanlon, Peakall & Briese, 1999; Hardig *et al.*, 2000; Teo *et al.*, 2002; González-Pérez, Caujapé-Castells & Sosa, 2004; Kirk *et al.*, 2004). Additionally, nuclear genome sequences of relatively rapid evolution, such as the Internal Transcribed Spacers of the ribosomal DNA (ITS1 and ITS2), traditionally used for phylogenetic reconstruction, have also been employed to investigate hybridization processes between related taxa (Sang, Crawford & Stuessy, 1995; Whittall *et al.*, 2000). These multiple copy DNA regions have been demonstrated to evolve mostly in a concerted manner that homogenizes most intraindividual ITS copies into a unique sequence (Dover, 1982). However, nucleotide additive patterns, which combine differently inherited parental ribotypes into the hybrid genome, are predicted after a recent hybridization event (Baldwin *et al.*, 1995; Aguilar, Rosselló & Feliner, 1999; Lihova *et al.*, 2004). These additive patterns result in superimposed nucleotide polymorphisms in the DNA sequences (SNAPs; Whittall *et al.*, 2000), whenever the polymorphisms are restricted to parsimony-informative sites, or Additive Polymorphic Sites (APS), whenever the polymorphism occurs at any site of the sequence (Aguilar and Feliner, 2003), providing evidence of hybridization between taxa (Lihova *et al.*, 2004). Hybridization in plants can act in a single or both directions depending on whether each taxon is likely to contribute as the cytoplasm donor during the hybridization event. Bi-directional crosses can occasionally, but not always, affect the morphological features of the resulting hybrids with respect to each of their parents and to the alternative hybrid combination because of cytoplasmic inheritance (Stace, 1987). However, the maternal parent can be identified in views of shared plastid DNA fingerprints with the hybrid.

In this study we have used a combined approach of molecular and morphological studies to investigate the putative hybrid origin of eight individuals that were collected from two Pyrenean valleys while sampling populations of the red thistle *C. carniolicum* ssp. *rufescens*. Their morphological similarity to the hybrid samples mentioned by Vivant (1998), together with the sympatric occurrence of *C. palustre* within two populations of the red thistle, motivated us to presume the existence of potential hybridization events between these two morphologically distinct *Cirsium* taxa. Amplified Fragment Length Polymorphism (AFLP; Vos *et al.*, 1995) and nuclear rDNA (ITS) sequences were analysed to verify additive profiles from both putative parents, whereas plastid (*trnL-F* and *trnT-L*) sequences were analysed to infer the direction of the hybridization events. A set of morphological attributes was also studied in all hybrid samples with respect to the diagnostic traits that separate both parents.

MATERIAL AND METHODS

DNA ISOLATION

Fresh leaves from the eight putative thistle hybrid individuals detected in the field (CV01, France: Azun valley, Arrens, Pène d'Ourey, 7 samples; CV02 France: Ossau valley, Gourette, Plateau de Ley, 1 sample) were collected, immediately dried in silica gel (Chase and Hills, 1991) and stored prior to DNA isolation. Thirty-three samples from 12 populations of *Cirsium carniolicum* ssp. *rufescens* covering the whole distribution range of the taxon (CR01, France: Pas d'Aspe01, 3 samples; CR02, France: Pas d'Aspe02, 1 sample; CR03, France: Maspètres, 3 samples; CR04, France: Ruisseau de Sadum, 3 samples; CR05, France: Pouré de Lamary, 3 samples; CR06, France: Mailh Casoula, 3 samples; CR07, France: Dec de Lhurs, 3 samples; CR08, France: Gorges du Bitet, 3 samples; CR09, France: Gabas, 1 sample; CR11, France: Gourette, 3 samples; CR12, France: Arrens, Pène d'Ourey, 3 samples and CR13, France: Isaby, 3 samples) and ten samples of *C. palustre* from the same locality where the hybrids were more abundant (CP01, France: Arrens, Pène d'Ourey, 10 samples) were also included in the analyses. Leaves were ground to a fine powder in liquid nitrogen. DNA isolation was carried out using the DNeasy Plant Minikit (Qiagen) following the manufacturer's instructions. The samples were diluted to a final concentration of 200 ng μL^{-1} and 1 μL of each sample was gel electrophoresed to confirm DNA quality and quantity.

AFLP PROTOCOL

The AFLP assays were conducted following the manufacturer's protocol (Invitrogen life technologies,

Carisbad, CA, USA) with some modifications. Two-hundred ng of DNA per sample were digested with *EcoRI* and *MseI* enzymes in 6.25 μL digestion reaction volume. Restriction enzymes were inactivated (15 min, 70 °C) prior to ligation with *EcoRI* and *MseI* adapters in a reaction that was carried out in the same tubes. The total digestion-ligation volume (12.5 μL) was diluted in 40 μL of ddH₂O and 2.6 μL of this dilution was used as DNA template for the preselective (i.e. with one nucleotide added after the restriction site of each enzyme) PCR (Vos *et al.*, 1995). This PCR was carried out using the AFLP® Pre-amp Primer mix I (Invitrogen life technologies) and included 30 cycles each of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. Then 6 μL of the preselective PCR was diluted in 94 μL ddH₂O, and a 5 μL aliquot of this dilution was used as DNA template for the selective (i.e. with two additional nucleotides added to those of the preselective primers) PCR. Three primer pair combinations (*EcoRI-AAG/MseI-CTC*; *EcoRI-AGC/MseI-CTA* and *EcoRI-ACC/MseI-CAG*) were selected for the analysis of the whole set of samples: these rendered reproducible and easily scorable banding patterns after the assay of nine primer pair combinations in a subset of three samples of each taxon. The selective PCR program consisted of 12 cycles of touchdown PCR (30 s at 94 °C, 30 s at 65 °C to 56 °C, and 1 min at 72 °C) followed by 23 cycles each of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. PCR products were separated in 6% denaturing polyacrylamide gels at 80 W during two hours and then silver stained (Sanguinetti, Dias-Neto & Simpson, 1994) for band visualization.

A presence/absence data matrix of all reproducible bands in each individual was compiled. Similarity and distance indices between samples were obtained including Dice's (Dice, 1945) and Jaccard's (Jaccard, 1908) similarity coefficients using NTSys-pc v.2.11a, and the pairwise-distance index (Excoffier, Smouse & Quattro, 1992) using ARLEQUIN v.2000 software (Schneider, Roessli & Excoffier, 2000).

The relationships between samples were visualized with the selected distance matrices by means of Principal Coordinates Analysis (PCO) using NTSys-pc (Rohlf, 2002) and Neighbour-Joining (Saitou and Nei, 1987) clustering analysis using MEGA2 (Kumar *et al.*, 2001).

Nuclear rDNA (ITS) and plastid (*trnL-F*, *trnT*) amplification and sequencing

Both nuclear ITS (ITS1-5.8S-ITS2) and plastid *trnL-F* and *trnT-L* sequences were obtained from five samples each of *C. carniolicum* ssp. *rufescens* and *C. palustre* and from their eight putative sampled hybrids. ITS sequences were amplified using the external primers KRC (forward) (Torrecilla & Catalán, 2002) and ITS4 (reverse) (Hsiao *et al.*, 1995). The PCR cocktail (50 μL)

contained $1 \times Taq$ buffer (Ecogen), 2 mM Cl_2Mg , 0.5 mM each dNTP, 1.25 pmol each of forward and reverse primers, 1.5 units of *Taq* (Ecogen), 20 ng of DNA template and ddH₂O to fill the 50 μ L total volume. The PCR program consisted of a first melting step of 3 min at 94 °C followed by 35 cycles each of 1 min at 94 °C denaturing step, 1 min at 50 °C annealing step, and 1 min at 72 °C extension step. After a further extension step of 7 min at 72 °C the samples were held at 4 °C.

Plastid *trnT-L* and *trnL-F* sequences were amplified using the external primers 'a' (forward) and 'b' (reverse) and 'c' (forward) and 'f' (reverse) of Taberlet *et al.* (1991), respectively. The PCR cocktail (50 μ L) contained $1 \times Taq$ buffer (Ecogen), 5 mM Cl_2Mg , 0.4 mM each dNTP, 1 pmol each of forward and reverse primers, 2.5 units of *Taq* (Ecogen), 20 ng of DNA template and ddH₂O to fill the 50 μ L total volume. The PCR program consisted of a first melting step of 1 min 94 °C followed by 30 cycles each of 15 s at 94 °C denaturing step, 30 s at 45 °C annealing step, and 1 min at 72 °C extension step. After a further elongation step of 7 min at 72 °C the samples were held at 4 °C.

PCRs were checked for positive amplifications on agarose gels and then products were purified using PCR Purification Kit (Qiagen). The cleaned products were subsequently sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) v. 3.1 and electrophoresed in an ABI PRISM 3700 automated sequencer. Forward and reverse sequences of each amplified region in each sample were obtained. The two chromatograms were compared, assembled and corrected where necessary using SEQUENCHER (GeneCodes corp., MI, USA), thus establishing the consensus sequence of the sample. IUPAC ambiguity codes were used for coding polymorphic positions. Identification of co-occurring ITS ribotypes in the hybrids was inferred by subtraction (Clark, 1990) using the *C. carniolicum* ssp. *rufescens* and *C. palustre* sequences as reference. We followed the recommendations of Aguilar *et al.* (1999) and considered a site as polymorphic whenever more than one peak occurred at a given site of the electropherogram in both forward and reverse strands and when the weakest fluorescence signal reached at least 25% of

the strongest. All nuclear and plastid sequences have been deposited in GenBank.

MORPHOLOGICAL DATA

A set of 24 morphological traits that served to identify and to distinguish *C. carniolicum* ssp. *rufescens* from *C. palustre* were analysed in the 40 sampled individuals from both parents and in the eight putative hybrid samples in order to confirm the intermediacy of these morphological features on the hybrids. The studied characters were the general indumentum, the shape of the basal leaves, the length of decurrent bases of the stem leaves, the spinescence of the leaves, the number and disposition of the capitula, the degree of spinescence and the presence or absence of apical callus in the involucre bracts, and the colour of the corollas (see Table 4).

RESULTS

AFLP ANALYSES

One hundred and thirty-three bands were scored from the three primer combinations amplified. These primers amplified 103 and 89 AFLP bands in *Cirsium carniolicum* ssp. *rufescens* and in *C. palustre*, respectively. Forty-four (33.08%) bands were exclusive to *C. carniolicum* ssp. *rufescens* and 30 (22.56%) to *C. palustre*, whereas 59 (44.36%) AFLP bands were shared between the two *Cirsium* taxa (Table 1). The same AFLP combinations of primers amplified 130 bands in the eight putative hybrid samples. All AFLP bands present in these eight samples were also present in either *C. carniolicum* ssp. *rufescens*, *C. palustre* or in both taxa (results not shown). The three bands not detected in the putative hybrids corresponded to low frequency bands exclusive of *C. carniolicum* ssp. *rufescens*. The combination of the 133 AFLP bands allowed the identification of a unique AFLP phenotype for each of the 33 samples studied of *C. carniolicum* ssp. *rufescens* and for each of the eight hybrid accessions, whereas only two different AFLP phenotypes were scored for the ten studied samples of *C. palustre*.

Table 1. Number and percentages of monomorphic and polymorphic AFLP bands obtained from three selective primer-pairs combinations in the studied *Cirsium* taxa. Private bands among the scored ones are indicated in brackets. *N*, sample size

	<i>N</i>	Monomorphic	Polymorphic	Total
<i>Cirsium carniolicum</i> ssp. <i>rufescens</i>	33	79 (76.70%) [28(35.44%)]	24 (23.30%) [16(66.67%)]	103
<i>Cirsium palustre</i>	10	88 (98.88%) [29(32.95%)]	1 (1.12%) [1(100%)]	89
<i>Cirsium</i> \times <i>vivantii</i>	8	113 (86.92%)	17 (13.08%)	130

The PCO (Fig. 1) analysis revealed the genetic intermediateness of the eight hybrid samples with respect to those of the parental taxa. In the two dimensional PCO plot the samples analysed clustered into three separate groups along the first axis that accumulated 75.26% of the variance. The samples of *C. carniolicum* ssp. *rufescens* clustered closely into the positive extreme of axis I, those of *C. palustre* clustered oppositely into the negative, and the eight hybrid samples clustered intermediately between these two groups. A similar result was obtained in the NJ tree constructed with the Euclidean distance matrix between AFLP phenotypes (Results not shown). In this tree the genetic distances between samples of the same parental taxon were considerably shorter than between both taxa that clustered at larger distances. The hybrid samples again clustered intermediately.

Nuclear rDNA (ITS) and plastid (trnL-F, trnT-L) sequences

The ITS primers produced an amplicon of 647 bp in both parental taxa and the putative hybrids. Twenty-nine polymorphic sites (4.48% of the total) were found of which 17 (58.62%) corresponded to the ITS1 and 12 (41.38%) to the ITS2, respectively (Table 2). Both parental taxa and the hybrids conserved 5.8S nrDNA. The hybrid accessions showed additive polymorphisms at the 29 sites where both parents differed in sequence.

The plastid *trnL-F* primers amplified a region of 857 nucleotides in all 18 samples studied. Sequence variation was restricted to two variable sites, 149 and 503 of the consensus assemblage. The two detected *trnL-F* haplotypes were shared between individuals of both parental taxa and the putative hybrid samples (Table 3). The *trnT-L* intergenic spacer ranged from 497 to 511 nucleotides in *C. carniolicum* ssp. *rufescens* and from 501 to 512 nucleotides in *C. palustre*, summarizing a consensus sequence of 512 nucleotides. Twenty variable sites (3.91% of the total) were identified in the 18 sequences studied (Table 3), distinguishing five different haplotypes. Three haplotypes (I, II and IV) were exclusive of *C. carniolicum* ssp. *rufescens*, one haplotype (V) was restricted to *C. palustre*, and the remaining haplotype (III) was shared between both taxa. The concatenation of the two chloroplast sequenced regions did not revealed additional haplotypes. One of the two haplotypes detected in the hybrid accessions (haplotype V) was present in six samples whereas the second haplotype (haplotype III) was present in the other two samples.

MORPHOLOGY

Each of the two presumed parents of the hybrid thistle accessions was characterized by a set of unique morphological traits. *Cirsium carniolicum* ssp. *rufescens* had large shallowly lobed basal leaves, sessile to

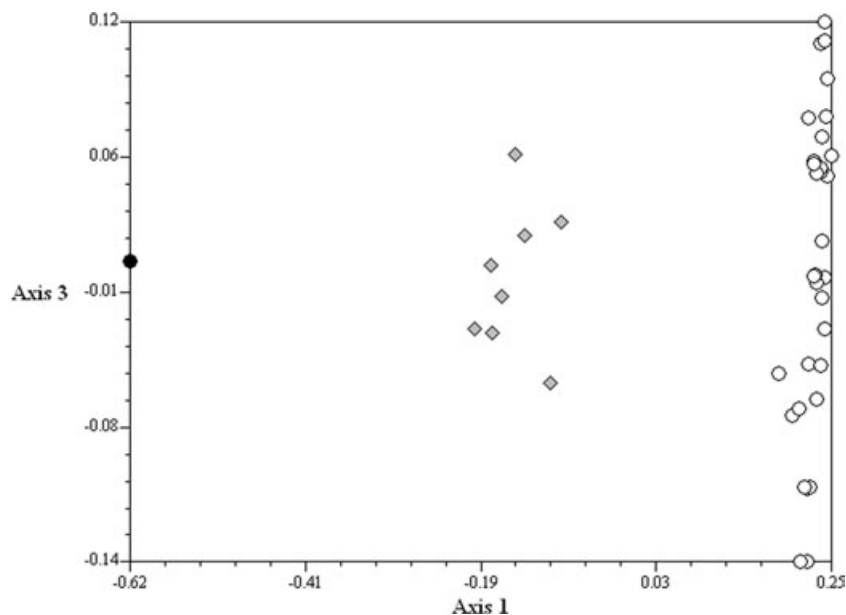


Figure 1. Two-dimensional PCO plot of the 51 samples of *Cirsium* studied. The Dice similarity matrix was used to compute the eigenvectors and eigenvalues. The first and third axes accumulated the 75.26% and 2.48% of the total variance, respectively. ○ = *C. carniolicum* ssp. *rufescens*; ● = *C. palustre*; ◆ = hybrid samples.

Table 3. Summary of nucleotide site variation of the plastid *trnT-L* and *trnL-F* regions of *Cirsium carniolicum* ssp. *rufescens*, *C. palustre* and their hybrids defining five haplotypes. For each taxon sample the population code and the GenBank accession numbers are indicated (see Material and methods)

Chloroplast region	<i>trnT-L</i>										<i>trnL-F</i>												
	57	58	59	60	104	184	185	186	187	188	189	190	214	221	222	223	224	225	235	272	149	503	Haplotype
<i>C. carniolicum</i> ssp. <i>rufescens</i>																							
CR01, DQ875425, DQ875407	-	-	-	-	T	-	-	-	-	-	-	-	A	-	-	-	-	A	T	G	C	C	I
CR06, DQ875426, DQ875408	C	T	A	T	G	T	T	T	T	T	-	-	T	T	T	T	-	T	T	T	T	T	II
CR11, DQ875427, DQ875409	C	T	A	T	G	T	T	T	T	T	-	-	T	T	T	T	-	T	T	T	T	T	II
CR12, DQ875429, DQ875410	C	T	A	T	G	-	-	-	-	-	-	-	A	-	-	-	-	A	T	G	C	C	III
CR13, DQ875428, DQ875411	C	T	A	T	G	T	T	T	T	T	-	-	T	T	T	-	-	A	A	T	T	T	IV
<i>C. palustre</i>																							
CP01, DQ875430, DQ875412	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CP01, DQ875431, DQ875413	C	T	A	T	G	-	-	-	-	-	-	-	A	-	-	-	-	A	T	G	C	C	III
CP01, DQ875432, DQ875414	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CP01, DQ875433, DQ875415	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CP01, DQ875434, DQ875416	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
<i>C. × vibrantii</i>																							
CV01, DQ875435, DQ875417	C	T	A	T	G	-	-	-	-	-	-	-	A	-	-	-	-	A	T	G	C	C	III
CV01, DQ875436, DQ875418	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CV01, DQ875437, DQ875419	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CV01, DQ875438, DQ875420	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CV01, DQ875439, DQ875421	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CV01, DQ875440, DQ875422	C	T	A	T	G	-	-	-	-	-	-	-	A	-	-	-	-	A	T	G	C	C	III
CV01, DQ875441, DQ875423	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V
CV02, DQ875442, DQ875424	C	T	A	T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V

Table 4. Comparison of selected morphological traits between *Cirsium palustre*, *C. carniolicum* ssp. *rufescens* and their hybrid

	<i>Cirsium carniolicum</i> ssp. <i>rufescens</i>	<i>Cirsium</i> × <i>vivantii</i>	<i>C. palustre</i>
Indument	Villous. Upper part of stems densely covered by reddish-brown pluricellular trichomes. Leaves with similar indument	Villous. Upper part of stems densely covered with white-hyaline pluricellular trichomes. Leaves with similar indumenta of whitish pluricellular trichomes turning brownish in the upper leaves.	Arachnoid. Leaves and stems densely covered by white pluricellular trichomes
Stem	Not alate, harmless	Partially alate and prickly	Alate, prickly
Leaves	<i>Basal</i> Ovate, lobulate, petiolate, prickly	Ovate to ovate-lanceolate, lobulate, petiolate, prickly	Lanceolate to linear-lanceolate, alate-petiolate, pinnately lobed to pinnatisect, prickly
	Length × width of limb (cm) Length × width of petioles (cm) Length/diameter of spines (mm)	16–32 × 12–14 14.5–17 1–2/0.1–0.3	6–27.5(33.5) × 2.5–10.1 (1.7)2–7.1(9) 2–10/0.3–0.5
	<i>Middle</i> Length × width (mm) Length/diameter of spines (mm)	Slightly auriculate to shortly decurrent 11.5–24.5 × 1.5–10 1–5/0.1–0.3	Longley decurrent, prickly (3.1)4.3–19.7 × 0.3–4.6 (2)3–13/0.3–0.5
	<i>Upper</i> Number Length × width (mm) Length of spines (mm)	Linear-lanceolate 6–7 (2.5)3.7–5.7 × 0.2–0.7 1–11	Linear-lanceolate, prickly 0–1 0.8–4.4 × 0.1–0.2 (1)3–7
Capitula	Number per branch Diameter (cm) Height (cm) Involutral bracts	1–2(3) 1.2–2.5 1.5–1.8 Patent, without apical callus, lanceolate, unarmed; outer and middle bracts with patent distal portion, with spinose apex, inner bracts with scariosus apex	Erect, with apical callus; outermost and middle bracts oblong, with spinose apex, inner bracts lanceolate with a scariosus mucro
	Outer involuclral bract length (mm) Middle involuclral bract length (mm) Inner involuclral bract length (mm)	4–7 6–9(12) 12–18 Yellow with pink lobes 12.5–19	(3.4)4–6.6 5–7.2 (9.2)1 L-15 Pink 10.8–15.8
Corolla	Colour Length (mm)	4–5 13–14	(3)3.2–4 (9)10–11.2(12.4)
Achenes	Length (mm) Length of pappus		

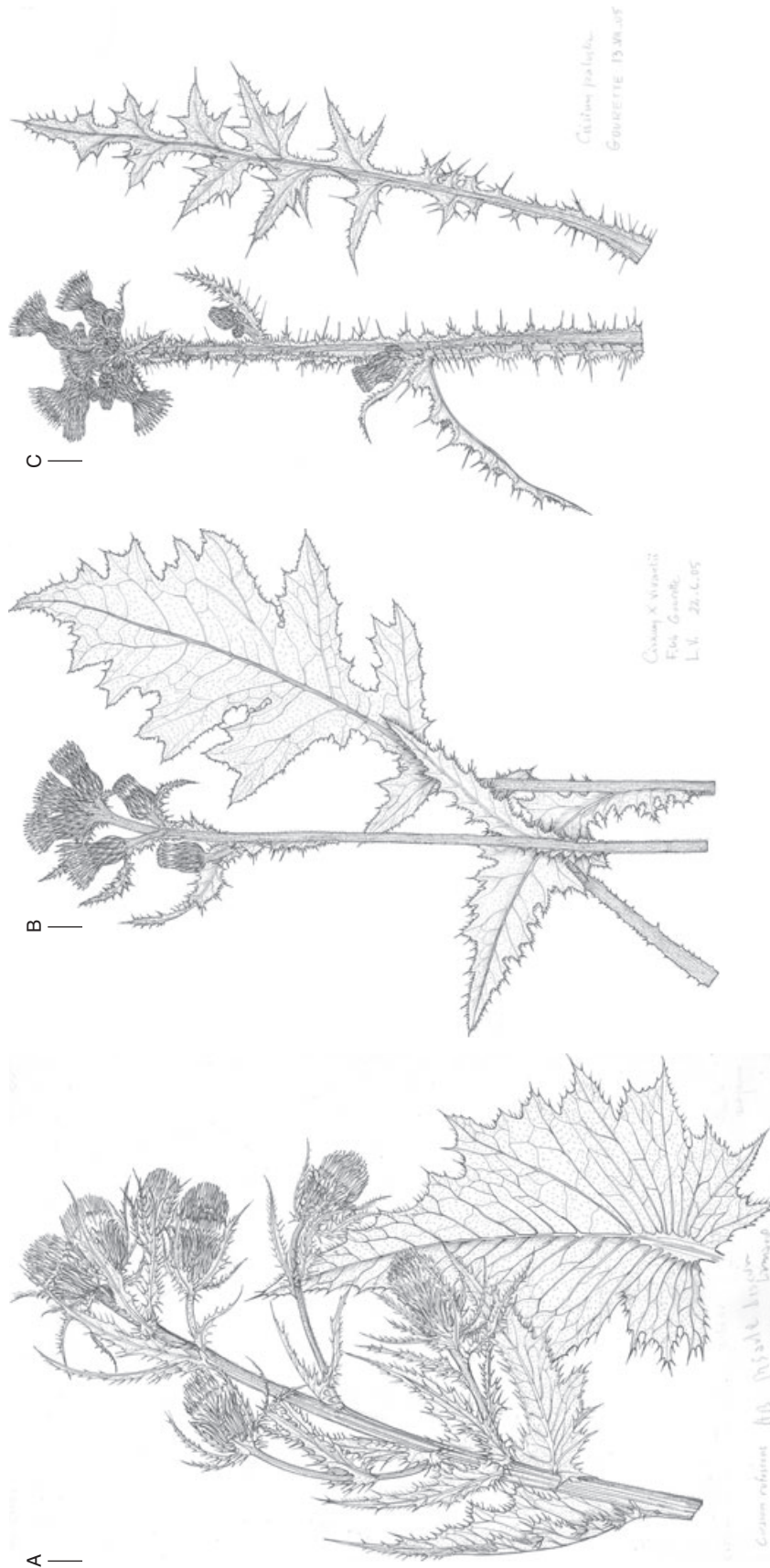


Figure 2. Habit of *Cirsium carniolicum* ssp. *rufescens* (A, France, Aspe valley, Lescun *M Saule* herb pers s/n), *Cirsium palustre* (C, France, Ossau valley, Gourette JACA R280827) and their hybrid, *Cirsium x vivanii* (B, France, Ossau valley, Gourette JACA R280825) from the HOLOTYPE. Scale bars = 1 cm.

other two hybrids was ambiguous since haplotype III was shared by *C. carniolicum* ssp. *rufescens* and *C. palustre*. As a consequence of our study a new hybrid *Cirsium* is described here.

CIRSIIUM × VIVANTII VILLAR, SEGARRA-MORAGUES, LÓPEZ, PÉREZ-COLLAZOS AND CATALÁN, **NOTHOSSP. NOVA** [*CIRSIIUM CARNIOLICUM* SCOP. SSP. *RUFESCENS* (RAMOND EX DC.) P. FOURN. × *CIRSIIUM PALUSTRE* (L.) SCOP.]

Holotype: France, Pyrénées-Atlantiques: nr. Gourette, Plateau de Ley, 30TYN1760, 1210 m a.s.l., 22.vi.2005, L Villar (JACA R280825). *Illustration*: Fig. 2B).

Diagnosis: A *Cirsio carniolico* ssp. *rufescensi* caulis pro parte alatis, foliis saturate viridibus, basalibus profundius pinnatifidis et longius petiolatis, capitulis breviter tantum pedunculatis vel sessilibus et corollis apice roseis differt; autem a *C. palustri* caule non prorsus alato, foliis basalibus minus profunde pinnatifidis et brevius petiolatis atque spinis praeditis mitibus nec vulnerantibus, capitulis semper sessilibus et corollis, apice excepto, luteis differt.

Description: Perennial, 80–100 cm high, pubescent. STEMS partially prickly winged. LEAVES dark green, flat, pubescent with multicellular trichomes on both sides, basal leaves 16–32 cm long × 12–14 cm wide, ovate-lanceolate, petiolate, irregularly pinnate to pinnatisect, with slightly prickly fimbriate margin; leaf spines small (1–2 mm) greenish or brownish; petioles 14.5–17 cm long; middle and upper leaves 11.5–24.5 cm long × 1.5–10 cm wide, sessile, auriculate to

shortly decurrent, pinnatifid and prickly; spines 1–5 mm long; upper leaves narrowly lanceolate and longer than capitula, capitula 1–2(3) per branch, 1.2–2.5 cm in diameter, subsessile to shortly pedunculate; involucre bracts lanceolate, without apical callus; the inner row with scarioso apex. COROLLA 12–19 mm long, yellow with pink lobes. ACHENES 4–5 mm long with plumose pappus of 13–14 mm. Flowering period: June to August.

Etymology: Dedicated to our friend J. Vivant, French botanist from Orthez, who discovered and mentioned for the first time the hybrid we have studied and described here.

Distribution: *Cirsium × vivantii* is known only from two French central Pyrenean valleys (Azun and Ossau) where it coexists with its parents (Fig. 3). Because of the specialized ecology of both parents the new hybrid was found only in two low altitude populations of *C. carniolicum* ssp. *rufescens* that grew in close proximity to permanent water flows occupied by *C. palustre*. This rare hybrid taxon grows at 1190 m a.s.l. on limestone soils, in megaphorb communities located on shady and wet areas at the foot of the calcareous cliffs together with other western Pyrenean endemics such as *Aconitum variegatum* L. ssp. *pyrenaicum* Vivant and Delay and *Thalictrum macrocarpum* Gren.

HYBRIDIZATION ANALYSIS

Although hybridization is a large extended phenomenon within most of the angiosperms (Stebbins, 1950; Grant, 1981; Stace, 1987), the ages of the newly arisen

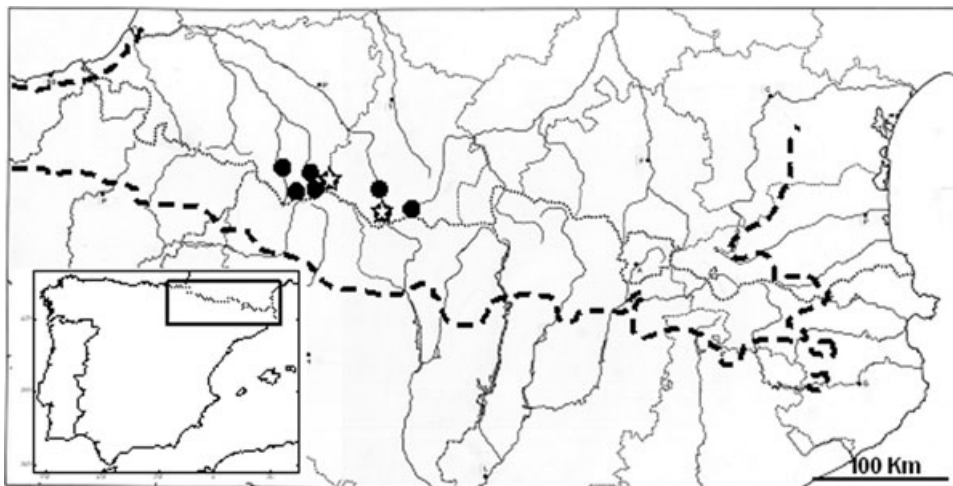


Figure 3. Area of distribution of *Cirsium carniolicum* ssp. *rufescens* (●), *C. palustre* (---), and *Cirsium × vivantii* (☆).

hybrid taxa vary greatly between old hybrid lineages and more recent crosses depending on several evolutionary, biological, geological and climatic parameters (Soltis & Soltis, 1993, 2000; King & Ferris, 2000). The Pyrenees have been recognized as a secondary contact zone for hybrid swarms of several plant and animal species at different Tertiary and Quaternary times (Hewitt, 1996, 2000; Segarra-Moragues & Catalán, 2005). Several molecular and cytogenetic features point towards the recent origin of *Cirsium* × *vivantii*. The absence of exclusive AFLP bands in the hybrid samples with respect to those of *C. carniolicum* ssp. *rufescens* and *C. palustre* indicates that these taxa were involved in the crosses that originated the hybrids and that the hybridization events likely occurred in relatively recent times. Also, the expected biased concerted evolution of the nuclear rDNA sequences towards one of the parental genomes, characteristic of more ancient hybrids (Aguilar *et al.*, 1999), did not occur within the sampled individuals of *C. × vivantii* which showed equal number of APS from both parents. The scarcity of the hybrid individuals detected so far in the field and the lack of large and stable hybrid populations further support the idea of the recentness of those sporadic crosses. This could be the consequence of unfertile triploid individuals, resulting from the cross of putative diploid (*C. carniolicum* ssp. *rufescens*) and tetraploid (*C. palustre*) parents, that have not yet evolved into amphiploids.

Our study demonstrates that the direction of the hybridization events has followed mostly a unidirectional pathway involving *C. palustre* as the maternal parent in six out of the eight (75%) hybrid accessions analysed. However, it is also evident from our data that the hybridization between these two thistles has occurred at least twice, given the two plastid haplotypes found in these samples. Bidirectional hybridization could not be completely ruled out, because plastid haplotype III was shared between two (25%) of the hybrid accessions and both parental taxa. Morphological features examined across these two hybrid specimens did not permit the preferential direction of the crosses to be differentiated.

Because of the reduced number of *Cirsium* × *vivantii* individuals found, this taxon should be considered as naturally very rare and included in National catalogues of endangered taxa and Red Lists as Critically Endangered (CR). Despite this rarity, the conservation of *C. × vivantii* may not pose serious problems. Current populations of *C. × vivantii* could benefit from conservation policies devoted to its coexisting parent *C. carniolicum* ssp. *rufescens*, as the protection of populations of the latter would indirectly protect those of the hybrid. Also, they co-occur within the area of the National Park of the Pyrénées where the collection of

species are regulated (Villar, 1999). Because its range is restricted to two close Pyrenean valleys, further conservation efforts should focus on providing precise population censuses within known populations and at exploring other potential sites where this taxon could have passed undetected.

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APPENDIX

Representative specimens examined

Cirsium palustre

FRANCE: ARIÈGE: Ax-les-Termes, Col de Pradelle, Turbera-manantial, 1500 m a.s.l., 31TDH23, 16.vi.1993, *P Montserrat and F Cernoch* (JACA 56993); Mendive, Behorlegui, regatos turbosos, 1100 m a.s.l., 30TXN5773, 3.vii.1998, *L Villar et al.* (JACA 110398); Mendive, Behorlegui, helechal-pasto con *Geranium endressii*, 1000 m a.s.l., 30TXN5775, 12.vii.1979, *P Montserrat and L Villar* (JACA 195479); Aspe valley, Urdos, Sansanet, megaforbios, 1400 m a.s.l., 30TXN9940, 10.vii.1977, *P Montserrat and L Villar* (JACA 82477); Ossau valley, Gourette, Plateau de Ley, orillas arroyo húmedo, megaforbios, 1210 m a.s.l., 30TYN1760, 13.vii.2005, *L Villar and A Lanaspá* (JACA R280828).

SPAIN: HUESCA: Biescas, Barranco del Asieso, fuente en abetal, 1450 m a.s.l., 30TYN2327, 13.vii.1978, *L Villar* (JACA 200978); Bielsa, Turberas bajando del Ibón, 1900 m a.s.l., 31TBH6934, 26.vii.1993, *J A Sesé* (JACA 119693); Benasque, Subida a Remuñe, suelo ácido, 1700 m a.s.l., 31TCH0328, 17.ix.1994, *J V Ferrández* (JACA 286994); Hecho, Bajo Ibón de Lacherito, suelo permotriásico húmedo, 1700 m a.s.l., 30TXN8749, 3.ix.1972, *L Villar* (JACA 10170872); Hecho, El Barcal, manantiales en morrenas, 1330 m a.s.l., 30TXN9146, 22.viii.1993, *P Montserrat* (JACA 182793); Hoz de Jaca, bosque mixto-hayedo al pie de la Peña de Hoz, 1460 m a.s.l., 30TYN2028, 22.vi.1986, *L Villar et al.* (JACA 504986); Jaca, Astún, hacia Ibón de Escalar, orillas arroyo en permotriás, 1780 m a.s.l., 30TYN0443, *J A Sesé and J*

Cirsium carniolicum ssp. *rufescens*

FRANCE: Ansó-Urdos, Pas d'Aspe, roquedos y megaforbios, 1600 m a.s.l., 30TXN9940, 16.vii.1796, *L Villar* (JACA 10013076); Ansó-Urdos megaforbio en pie de roquedo calizo, Bosque de las Hayas, 1550–1600 m a.s.l., 30TXN9940, 25.viii.1991, *D Gómez and P Castro* (JACA 253391); Aspe valley, Urdos, megaforbio en pie de roquedo calizo, Bosque de las Hayas, 1700 m a.s.l., 30TXN9939, 8.viii.1996, *D Gómez and A Lanaspá* (JACA 104696); Aspe valley, Urdos, Pas d'Aspe-Sansanet, megaforbio en terreno calizo, 1550 m a.s.l., 30TXN9940, 10.vii.1977, *P Montserrat and L Villar* (JACA 80977); Aspe valley, Borce, Espelunguère, glera caliza al pie de roquedo, 1650 m a.s.l., 30TXN9742, 6.x.1995, *P Montserrat, L Villar and J Molero* (JACA 294595); Aspe valley, Borce, Espelunguère, pie de roquedo calizo al N, 1700 m a.s.l., 30TXN9642, 13.viii.2002, *L Villar, J L Benito and P Rodríguez* (JACA R270010); Aspe valley, Borce, Pène d'Udapat, megaforbio en rellano de roquedo calizo al N, 1400 m a.s.l., 30TXN9571, 8.viii.2000, *L Villar and F Cassou* (JACA 246300); Aspe valley, Etsaut, Bco. de Sadum, megaforbio sombrío, 1400 m a.s.l., 30TYN0053, 16.viii.2000; *L Villar and F Cassou*

(JACA 248300); Aspe valley, Accous, hacia el Pic Bergon, megaforbio en pedriza caliza, 1400 m a.s.l., 30TYN0060, 26.ix.2000, *L Villar et al.* (JACA 253000); Aspe valley, Escot, Mail Casoula, megaforbios en terreno calizo, c. 1100 m a.s.l., 30TXN9971, 20.vii.1999, *L Villar et al.* (JACA 135699); Ossau valley, Laruns, Gorges du Bitet, 1200 m a.s.l., 30TYN0657, hayedo húmedo-megaforbio, 3.viii.1972, *P Montserrat and L Villar* (JACA 523672); Ossau valley, Gabas, Bioux, hayedo-abetal, megaforbios, c. 1150 m a.s.l., 30TYN0859, 6.viii.1980, *P Montserrat and L Villar* (JACA 343980); Azun valley, Arrens, Pène d'Ourey, rellano de roca caliza, 1100 m a.s.l., 30TYN2557, 4.ix.2001, *L Villar* (JACA R265595); Azun valley, Arrens, Pène d'Ourey, avellanar húmedo, 1090 m a.s.l., 30TYN2658, 13.vii.2005, *L Villar and A Lanaspá* (JACA R280827); Luz valley, Pierrefitte-Nestalas, Villelongue, l'Isaby, rocher calcaire, Cascade de Parpich, 1120 m a.s.l., 30TYN4359, 18.ix.2002, *F Cassou* (JACA 270236).

Cirsium × vivanii

FRANCE: Azun valley, Arrens, Pène d'Ourey, avellanar húmedo al pie de roquedo, 1090 m a.s.l., 30TYN2658, 13.vii.2005, *L Villar and A Lanaspá* (JACA R280826); Azun valley, Arrens, Pène d'Ourey, avellanar húmedo, 30TYN2658, 1090 m a.s.l., 19.viii.2002, *L Villar and M Saule* (JACA R270033); *ibidem* (R 270034); Azun valley, Arrens, Pène d'Ourey, avellanar húmedo, 30TYN2658, 1090 m a.s.l., 22.vii.2003, *D Fallour* (JACA R280830); Ossau valley, Gourette, Plateau de Ley, megaforbios en orilla de arroyo, 1210 m a.s.l., 30TYN1760, 22.vi.2005, *L Villar* (JACA R280825 HOLOTYPUS); Ossau valley, Gourette, Plateau de Ley, megaforbios en orilla de arroyo, 1210 m a.s.l., 30TYN1760, 13.vii.2005, *L Villar and A Lanaspá* (JACA R280831); Ossau valley, Gourette, Plateau de Ley, megaforbios en orilla de arroyo, 1210 m a.s.l., 30TYN1760, 22.vii.2003, *J G Segarra and J. V. Andrés* (JACA R280829).