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REGULAR PAPER

Effects of fragmentation and population size on the genetic diversity of *Centaurea cyanus* (Asteraceae) populations

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Aims – *Centaurea cyanus* L. is a patrimonial species emblematic of the flora associated with cereal crops, but it is currently declining in Western Europe because of agricultural intensification. The decrease in both the area and connectivity of populations could affect the genetic structure and variability of the populations, and in turn their potential to survive.
Methods – We studied the genetic variability of ten large and five small *C. cyanus* populations from two contrasted French regions using seven nuclear isozyme markers, and of twelve Western European populations using eighteen chloroplast markers.
Results – The analysis of leaf isozymes revealed that all populations, except one, had a deficit of heterozygous individuals (average Fis = 0.81), which could confirm the presence of a proportion of self-compatible plants. The level of variability was high. Genetic diversity was not lower in small than in large populations, and there was no reduced genetic diversity in the region where *C. cyanus* populations are rare. There was low but significant differentiation among populations (Fst = 0.068), especially between small and large populations, but not between regions. In contrast, no polymorphism was found among the 371 chloroplast DNA sequences analysed (i.e. around 8–10% of the chloroplast genome).
Conclusions – The observed pattern of variability could reflect the first effects of the rarefaction and fragmentation of previously abundant, widely distributed and poorly structured *C. cyanus* populations, on the reproduction regime and onset of genetic heterogeneity among populations.

Key words – Allozyme, cornflower, population genetic structure, self-incompatibility, weed, chloroplast.

INTRODUCTION

The diversity of the weed flora has decreased drastically over the past few decades. This results from an increased fertilizer use, more efficient weed seed sieving in crop seed lots, and herbicide use (Sutcliffe & Kay 2000, Baessler & Klotz 2006, Hyvönen & Huusela-Veistola 2008). A number of weed species are currently endangered in Europe, albeit with different status according to regions (Storkey et al. 2012). Fragmentation and the small size of populations generally lead to a loss of genetic diversity within populations and increased differentiation between them (Aguilar et al. 2006). However, genetic structures have been studied for very few rare weed species associated to crops (Brütting et al. 2012) so that it is not yet clear how this trend affects the agrosystem. *Centaurea cyanus*, an iconic arable field-dependent species, could be a good candidate to investigate further the consequences of plant rarefaction in the agrosystem because it is now declining in Western Europe while it was previously widespread.

Centaurea cyanus L. (synonym of *Cyanus segetum* Hill, cornflower), is considered in some places as a species of societal heritage, worth preserving (Tranchard 1993). Cornflower is a diploid Asteraceae that emerges mostly in autumn and goes through winter as a rosette in winter cereals and winter oilseed rape. It is an allogamous species displaying a self-incompatibility system (Svensson & Wigren 1984, Bellanger et al. 2014) and it is very attractive to insects (Roscoe & Irvin 2010). Its beautiful, blue heads flower up to crop harvest within the canopy of the crop and are pollinated mainly by Hymenoptera (Svensson & Wigren 1984). This weed species is thought to be an anthropochorous weed that arrived in Europe in Neolithic times with cereals (Rösch 1998). It was widespread throughout Europe sixty years ago. While it is considered an invasive weed in North America (Muth & Pigliucci 2006) and a troublesome weed in Eastern Europe, a marked regression is now being observed in Western Europe (Baron 1989, Pichot 1991, Sutcliffe & Kay 2000, Baessler & Klotz 2006). Regression has been estimated to be similar in other crop weeds, which have decreased by ca. 90% from

1950 to 1970 in central France (Aymonin 1976) and by ca. 70% in the UK during the last half century (Wilson 2007).

A recent census in the Poitou-Charentes, a Western French region, indicated the presence of *C. cyanus* in only 0.4% of the fields (Bellanger et al. 2012). Because of this low frequency and the presence of small populations, we here tested the following hypotheses: (1) genetic diversity is reduced in small versus large populations; (2) genetic diversity is lower in Poitou-Charentes than in Burgundy, an Eastern French region where *C. cyanus* is still present in 9% of the fields; (3) genetic differentiation is higher among small populations; and (4) genetic differentiation is higher in Poitou-Charentes than in Burgundy. We used leaf isozymes which generally behave as neutral markers with regard to direct selection pressure and have widely been used in population genetics (Hamrick & Godt 1996). In addition, (5) we aimed to identify seed-mediated gene flow and its role in the genetic differentiation in small versus large populations. For this purpose, we investigated the polymorphism at universal plastid DNA markers (Provan et al. 2001).

MATERIALS AND METHODS

Plant material

In Poitou-Charentes in France, *C. cyanus* is considered rare (0.4% of the fields) and has been recorded mainly in a single "hot spot" area, in which we sampled ten populations separated by a few kilometers (see table 1 & fig. 1). Where the populations were large (i.e. generally comprising more than 100 individuals), thirty plants at the rosette stage were transplanted from the fields to pots in the laboratory garden in February 2006. When the populations consisted of fewer than thirty individuals, we sampled leaves only (table 1). In Burgundy, where *C. cyanus* was more frequent (9% of the fields), populations appear to be randomly located, so we chose five large populations, separated by a few dozen kilometers, in order to maximize the chance of inter-population differentiation (fig. 1). We transplanted thirty plants from each population to the laboratory garden in April 2006. Plants were sampled within the field or in field edges when not present within the field (table 1). The area searched was generally 5 × 20 m, within which the plants were sampled at random. Additionally, we collected leaves of two plants in field edges from five distant regions (see table 1 & fig. 1).

Isozyme analysis

A 2-cm^2 leaf sample (i.e. about 40 mg) was crushed in 300 µL of a buffer solution containing Tris-HCl 0.1 M, 2 µg thioglycolic acid and 1 µL β-mercaptoethanol at pH = 7.5, and then centrifuged at 13.000 g for 20 min. Electrophoresis was carried out with 40 µL of extract in a Disc-PAGE system (Discontinuous Poly-Acrylamide Gel Electrophoresis) with a Tris-Glycine basic migration buffer as described by Gasquez & Compoint (1981). After preliminary trials, five isozyme

Table 1 – *Centaurea cyanus* populations sampled.
Burg.: Burgundy; P-C: Poitou-Charentes; A: Alps; Brit.: Brittany, N: Normandy; B-W: Baden-Württemberg; P: Italian Piedmont. Coordinates follow the World Geodesic System). Sampling: 30 individuals transferred to our garden or 2 leaves sampled on up to 23 individuals. Population size: large >100, small<30. *: material used for chloroplast DNA sequencing (two plants per population; populations B7, 051 and 562 were not assayed for ccmps). Sample: sample size for isozymes (NA: not applicable).

Population	Latitude/Longitude WGS84	Crop	Code	Region	Sampling	Size	Sample
Fleurey/Ouche	47°19′/4°51′	Oilseed rape	B4	Burg.	Individuals	Large	30
Norges la Ville	47°24′/5°04′	Oilseed rape	B5	Burg.	Individuals	Large	30
Le châtelet	47°03′/5°08′	Field edge	B6*	Burg.	Individuals	Large	30
Thury	47°02′/4°32′	Field edge	B7*	Burg.	Individuals	Large	30
Oigny	47°34′/4°43′	Oilseed rape	B8*	Burg.	Individuals	Large	30
La Blottière	46°13′/-0°25′	Oilseed rape	P1	P-C	Individuals	Large	30
Champoly	46°16′/-0°18′	Wheat	P2*	P-C	Individuals	Large	30
Biaouré	46°12′/-0°22′	Fallow	P3*	P-C	Individuals	Large	30
Biaouré	46°12′/-0°22′	Wheat	P4*	P-C	Individuals	Large	30
Le Breuil	46°11′/-0°22′	Fallow	P5*	P-C	Individuals	Large	30
La Blottière	46°13′/-0°26′	Peas	P6	P-C	Leaves	Small	23
Les Vignettes	46°13′/-0°22′	Wheat	P7	P-C	Leaves	Small	23
Croix Careil	46°14′/-0°26′	Oilseed rape	P8	P-C	Leaves	Small	23
Le Sapin	46°14′/-0°26′	Wheat	P9	P-C	Leaves	Small	21
Les Chauvinières	46°14′/-0°23′	Wheat	P10	P-C	Leaves	Small	18
Guillestre	44°39′/6°38′	Wheat	051*	A	Leaves	Small	NA
Le Pont Gasnier	47°57′/-3°43′	Wheat	562*	Brit	Leaves	Small	NA
Rouen	49°26′/1°06′	Field edge	R*	N	Leaves	Small	NA
Ulm	48°24′/9°58′	Field edge	A*	B-W	Leaves	Small	NA
Gran Paradiso	45°32′/7°16′	Field edge	I*	P	Leaves	Small	NA

systems out of the twenty assayed by techniques described by Tanksley & Orton (1983) were retained because they showed polymorphism and displayed a stable and unequivocally identified banding pattern. They were Diaphorases (DIA), Esterases (EST), Glutamate Aspartate Transaminases (GOT), Lactate Dehydrogenases (LDH) and Leucine Amino Peptidases (LAP). However, after leaf storage at 4°C for the transportation from Poitou-Charentes to Dijon and a delay before isozyme extraction, the quality of the leaves collected in small populations was not good enough to perform all the analyses, so that only EST and LAP could be assayed satisfactorily for these plants. Isozyme extraction and migration was replicated for about 10% of the plants in order to ensure homogeneous scoring of successive gels. Bands were identified by the relative mobility (Rf) of their respective protein (electronic appendix 1). Research for null alleles was not carried out systematically, but 12 plants genotyped as showing one band at each locus and presenting combinations of different alleles were crossed: ten seedlings of each plant were analysed as necessary to rule out the presence of two heterozygous parent(s) possessing null alleles, at P = 0.05.

Chloroplast DNA analysis

Total genomic DNA was extracted as described by Doyle & Doyle (1990) from two plants from each of twelve populations (marked with an asterisk in table 1). The twenty-eight primers used in this work are shown in electronic appendix 2. Eighteen primers are derived from Grivet et al. (2001), three from Beltrame (2007), six (corresponding to universal microsatellite sequences: ccmp) from Weising & Gardner (1999), and one was designed from Genbank alignments among the Asteraceae species. Most of these primers are distributed along the Large Single Copy region (Wakasugi et al. 1998). The PCR amplification was carried out in 10 µL buffer (containing 1 M Tris-HCl, pH 8.8, 1 M $MgCl_2$, 1 M β-mercapto-ethanol, 25 mM of each dNTP, 20 $mg.mL^{-1}$ BSA and 1% W1 Polyoxyethylen ether), 8 µL sterile water, 0.8 µL of each primer at 5µM, 0.5 µL of 1/20 diluted *Taq* DNA polymerase and approximately 10 ng template DNA. The PCR program consisted of 37 cycles of 10 s at 95°C, and then 15 s at Tm (melting-point temperature) followed by a final step of Te (elongation time) at 72°C (see Tm and Te values in electronic appendix 1). Amplicons were purified from 0.6% (w/v) agarose gel run and sequenced on both strands using the Sanger et al. (1977) method, by Eurofins MWG Synthesis GmbH (Germany). There were two separate DNA extracts for each plant (i.e. four sequences for each plant).

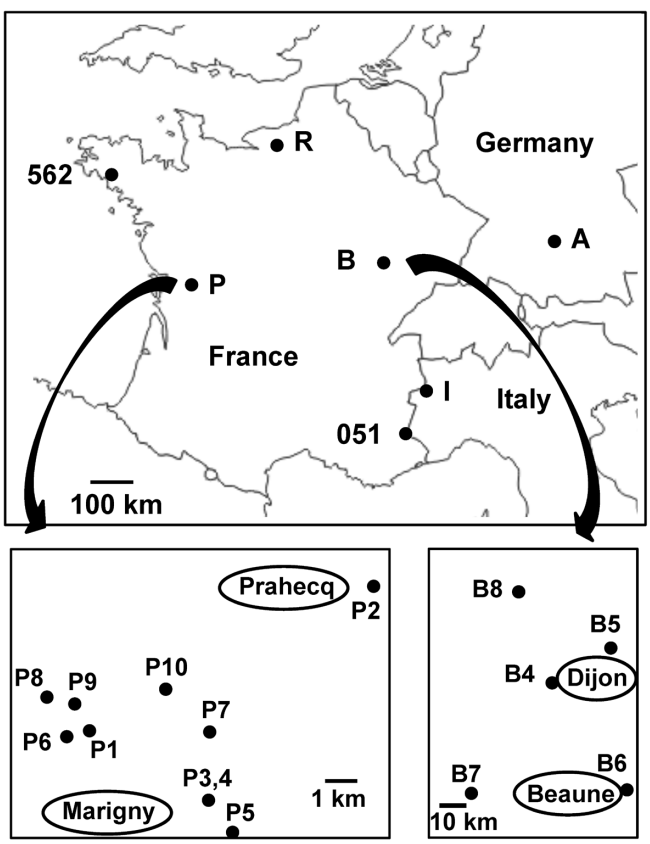

Figure 1 – Maps indicating the locations of the sampled population at three different scales. See codes for populations in table 1.

Data analysis

Estimates of the percentage of polymorphic loci (P, i.e. when the most abundant allele has a frequency lower than 95%), the mean number of alleles per locus (A), observed (Ho) and expected heterozygosity (He, according to Nei 1978) were obtained using POPGENE 3.2 (Yeh et al. 2000). F-statistics of Weir & Cockerham (1984) were calculated with FSTAT (Goudet 2001). First, the intra-population component (Fis) was calculated for each locus and population, and the departure from Hardy-Weinberg equilibrium was tested. Then, the inter-population component (Fst) was calculated for each pair of populations, with a Bonferroni correction, then for each locus by group of populations (Poitou-Charentes versus Burgundy, and small versus large) and, finally, globally for each locus. DNA alignments and sequence editing were performed using BioEdit software (Hall 1999).

RESULTS

Genetic diversity and heterozygosity

The isozyme patterns of the 408 sampled plants allowed for the identification of nine putative loci, seven of which are polymorphic. The monomorphic loci *Got2* and *Lap2* were not included in the analyses. The other loci had two to five clearly distinct alleles, but LAP was difficult to genotype in the Burgundy populations and these data are missing. Most of the alleles were found in all the populations (electronic appendix 3), except *Est1b*, which was present only in one population (P10), and allele *Est2b*, which was present in three populations (P6, P8 and P10). Percentage of polymorphic loci, mean number of alleles, Ho and He values were similar, irrespective of the number of analysed loci, the region and the population size (table 2). A null allele was evident for one locus, *Est3*, because 7% of the plants had a blank at the corresponding Rf while clear bands were noted for the two other *Est* loci, thus indicating that the lack of band was not due to isozyme extraction or reaction. No other case of null

Table 2 – Isozymes data.
Mean values over populations per region of genetic diversity data for large and small populations. (N: number of analyzed plants; P: percent of polymorphic loci; A: mean allele number per locus; Ho: observed heterozygosity; He: expected heterozygosity; SE are indicated in brackets).

Population	N	P	A	Ho	He
Six loci (*Est1*, *Est2*, *Est3*, *Dia*, *Got1*, *Ldh*)					
Burgundy large	150	86	2.6 (1.0)	0.06 (0.11)	0.44 (0.24)
Poitou-Charentes large	150	86	2.6 (1.0)	0.11 (0.15)	0.49 (0.24)
Four loci (*Est1*, *Est2*, *Est3*, *Lap1*)					
Poitou-Charentes small	114	80	2.8 (1.1)	0.27 (0.19)	0.45 (0.29)
Poitou-Charentes large	150	80	2.4 (1.1)	0.24 (0.15)	0.46 (0.29)

Table 3 – Differentiation among populations.
Fst values per locus (7000 permutations) between small and large populations of Poitou-Charentes (P-C), between Burgundy and Poitou-Charentes large populations, and globally among all populations calculated on four or six loci. Bold values indicate significant deviation from 0 at P<0.05.

Locus	*Est1*	*Est2*	*Est3*	*Dia*	*Got1*	*Ldh*	*Lap1*	All loci
Four loci (P-C populations)								
Small vs large	0.018	**0.031**	-0.004				0.021	**0.015**
Fst global	0.017	**0.097**	**0.037**				**0.078**	**0.059**
Six loci (large populations)								
Burgundy vs P-C	0.006	**0.068**	0.011	**0.155**	-0.005	0.002		**0.041**
Fst global	**0.028**	**0.055**	**0.023**	**0.228**	**0.023**	**0.027**		**0.068**

allele was detected in the progeny of the 12 crossed plants (i.e. all 10-seedlings families were either homozygous one-banded or heterozygous two-banded, except five families for *Est3*). In turn, the likelihood of the absence of null alleles for the other loci in a small sample of 12 plants is comprised between 0 and 14 % at P = 0.05; hence, our experiment is not a proof of absence in the populations.

All except one and three of the 30 Fis values calculated for each of the six loci (*Est1*, *Est2*, *Est3*, *Dia*, *Got1*, *Ldh*) in the five large populations of Burgundy and Poitou-Charentes, respectively, were significantly positive (ranging from 0.36 to 1, Fis = 0.86 and 0.76 on average for Burgundy and Poitou-Charentes, respectively, 0.81 overall). This indicates a general excess of homozygotes when compared to expectations from the panmixia hypothesis. Specifically, most individuals were homozygous for *Dia*, *Got1* and *Ldh*. In the small populations in Poitou-Charentes, which have not been analysed for those three loci, only nine in 20 Fis values were positive (ranging from 0.33 to 0.78, Fis = 0.34 on average), and population P9 was at Hardy-Weinberg equilibrium for the four loci. By comparison, the large populations of Poitou-Charentes analysed without those three loci had 14 in 20 Fis values significantly positive (Fis = 0.47 on average), which indicates that small population size does not increase the excess of homozygotes.

Comparison between regions (large populations)

Significant differentiation was found among large populations (overall Fst = 0.068, P < 0.01). Globally, the two regions were differentiated (Fst = 0.041, P < 0.05, table 3), the differences being mainly due to the loci *Est2* and *Dia*. The differentiation was more apparent between Poitou-Charentes populations (Fst = 0.069, P < 0.01) while that of Burgundy populations bordered significance (Fst = 0.027, P = 0.05, although both Fst values were not statistically different). The calculation of Fst, by pairwise comparison of the large populations, confirmed the homogeneity of the Burgundy populations (table 4). The large populations of Poitou-Charentes were more heterogeneous, e.g. P1 and P5 were differentiated from P3 and P4 and they were differentiated from all the Burgundy populations. Interestingly, P4 was differentiated from P3 although they were in adjacent fields, but one was growing with wheat while the other was situated in a fallow. P4 showed a genetic constitution similar to that of the populations of Burgundy and was not significantly differentiated from them.

Comparison between small and large populations

The large populations of Poitou-Charentes were not differentiated when using the four loci *Est 1*, *Est2*, *Est3* and *Lap1* (electronic appendix 4). The large population P1 was differentiated from the small population P9. P9 was differentiated from the small, nearly adjacent populations P6 and P8. Small-sized populations were differentiated from the large ones for *Est2* and when the four loci were considered together (Fst = 0.015, significantly different from 0, P < 0.05, table 4). The Fst value for all the Poitou-Charentes populations and the four loci was 0.059, significantly different from 0 (P < 0.05).

Table 4 – **Pairwise differentiation**.
Fst values (4500 permutations) between large populations from Burgundy and Poitou-Charentes. Bold values indicate significant deviation from 0 at P<0.05 after Bonferroni correction.

Pop.	Poitou-Charentes					Burgundy				
	P1	P2	P3	P4	P5	B4	B5	B6	B7	B8
P1	0.000	0.020	**0.073**	**0.102**	0.041	**0.106**	**0.082**	**0.060**	**0.100**	**0.164**
P2		0.000	**0.300**	0.033	0.047	0.049	0.029	0.018	0.044	**0.076**
P3			0.000	**0.071**	**0.116**	0.075	**0.068**	0.035	0.058	**0.111**
P4				0.000	**0.170**	0.009	-0.011	0.014	0.012	-0.004
P5					0.000	**0.205**	**0.163**	**0.107**	**0.158**	**0.240**
B4						0.000	0.003	0.027	0.055	0.014
B5							0.000	0.014	0.017	0.007
B6								0.000	0.005	0.068
B7									0.000	0.057
B8										0.000

Chloroplast DNA variability

Eighteen primer pairs provided appropriate amplification, which allowed reading a total of 371 sequences and more than 12 kb, i.e. 8–10% of the whole chloroplast genome. NCBI Genbank accession numbers (KJ499192, KJ499193 and KJ652201 to KJ652219) are indicated in electronic appendix 2. No variability was found among populations, apart from one plant from Italy that showed a point mutation for the ccmp10 fragment.

DISCUSSION

The analysis of leaf isozymes revealed that all populations, except one, had a deficit of heterozygous individuals. The level of variability was high, and there was low but significant differentiation across populations and across regions. No polymorphism was found among the 371 chloroplast DNA sequences analysed. Considering our initial expectations: (1) genetic diversity was not lower in small than in large populations. Given that *C. cyanus* is pollinated by a wide range of insects and is very attractive for bees (Svensson & Wigren 1984, Roscoe & Irvin 2010), gene flow from nearby large populations could have compensated for the reduced local number of individuals through pollen immigration. (2) Although geographical isolation (430 km), different regional climates and farming systems may account for differences between the two regions, there was no reduced genetic diversity in Poitou-Charentes in comparison to Burgundy, at least for the markers used, thus demonstrating that the abundance of populations in the countryside appeared not to be a critical determinant of genetic diversity in *C. cyanus.* (3) In spite of a loss of precision in the analysis with the reduced number of four loci compared to analyses with six loci, genetic differentiation was significant between small populations while no differentiation was apparent among large populations in Poitou-Charentes. (4) Poitou-Charentes populations showed more among-population heterogeneity than the Burgundy populations, but the Fst values were not significantly different indicating no actual differentiation between regions. Perhaps, the regional impoverishment in *C. cyanus* that could tend to increase genetic differentiation among populations was counterbalanced by lower distance between populations in Poitou-Charentes compared to the sampled populations in Burgundy. (5) Finally, seed migration could not be investigated because of the lack of polymorphism of the chloroplast markers.

In general terms, the genetic variability observed for *C. cyanus* across the two regions (P = 80%, He = 0.48, Fst = 0.068) was very high compared to values published for other Asteraceae (P = 45%, He = 0.127, Gst = 0.204) and for other allogamous annual species (P = 59%, He = 0.186, Gst = 0.191) with regard to similar genetic markers – isozymes – (Hamrick & Godt 1996). We acknowledge that we only selected polymorphic isozyme systems while withdrawing monomorphic loci from the analysis, therefore overestimating the actual genetic diversity. In addition, the indicated average values for Asteraceae and allogamous species encompassed a large diversity of life forms, breeding systems and geographical ranges, so that some care should be taken when comparing them to those obtained for any given species. As expected, the genetic structure contrasted with that of autogamous weed species such as *Chenopodium album* L. (Gasquez & Compoint 1981) and *Amaranthus retroflexus* L. (Mandák et al. 2011) that showed low genetic variability, strong inbreeding within populations and high differentiation between populations. It was more similar to that of allogamous, self-incompatible weed species such as *Alopecurus myosuroides* Huds. (Chauvel & Gasquez 1994) and *Raphanus raphanistrum* L. (Kercher & Conner 1996) with high within-population diversity and low genetic differentiation among populations. However, these species are very abundant and successful weeds in intensive agricultural systems, so it is not clear to what extent their genetic structures are of the same nature as that of *C. cyanus*. In one of the few studies dealing with vulnerable, declining weed species, *Consolida regalis* Gray was shown to have similar high within population diversity, but low among population differentiation (Brütting et al. 2012). A recent study with ten microsatellite markers indicated a similar low, but significant heterogeneity (Fst = 0.061) among populations of *C. cyanus* in another French region (Le Corre et al. 2014). Studies using molecular markers generally confirm the genetic struc-

ture initially described with isozymes, e.g. in *C. corymbosa*, *A. myosuroides* and *C. album* (Fréville et al 2001, Menchari et al 2007, Aper et al. 2012).

A notable result was a general deficit of heterozygous plants, apart from one small population (on average Fis = 0.81, Ho << He). This suggests a different genetic constitution for *C. cyanus* than for other allogamous species that are at panmixia (e.g., *Alopecurus myosuroides*, Chauvel & Gasquez 1994, Menchari et al. 2007). There could be several explanations to that deficit. First, there could be undetected null alleles, thus possibly leading to confusion of heterozygotes and homozygotes, which was certainly the case for *Est3*. The presence of null alleles leads to overestimation of allele frequencies, which has a moderate effect on indices for genetic diversity and differentiation, but directly impacts the counting of heterozygous loci. This situation is common in plant isozymes (Tanksley & Orton 1983). Correction of the data for the presence of null alleles in another study showed that significant departure from Hardy-Weinberg equilibrium caused by excess homozygotes was still observed in some populations of *C. cyanus* (Le Corre et al. 2014). Second, the self-incompatibility system could be less stringent than expected, including pseudo-self-compatible plants. This feature has been documented in several Asteraceae species such as *Crepis sancta* (L.) Babc. (Cheptou et al. 2002). We found considerable variation in the strength of self-incompatibility among individuals in a study of the self-fertilization regime of *C. cyanus*, which appeared not to be really inheritable, but at least one plant produced more seeds under self- fertilization than did any of the plants in the outcrossing regime (Bellanger et al. 2014). Both the presence of pseudo-self-compatible phenotypes and self-compatible plants could contribute to the observed deficit in heterozygotes. However, this effect could be limited by the reduced fitness of the selfed plants that contribute less than the outcrossed plants to the next generation (Bellanger et al. 2015). A Wahlund effect is unlikely because of the very small sampling areas and the homogeneity of every arable field.

The lack of chloroplast variation among the few assayed Western European populations is not a trait specific to *C. cyanus:* the compilation of 131 papers dealing with chloroplast DNA variability showed that a lack of variation was reported for sixteen of 117 studied species, although most were Poales and Pinales (H. Arnal, pers. com.). However, the lack of chloroplast variation in *C. cyanus* was unexpected because of the generalized intraspecific variability observed in other Asteraceae (Vijverberg et al. 1999, Coleman & Abbott 2003, Wills et al. 2005, Hufbauer & Sforza 2008, Gaudeul et al. 2011, Shimono et al. 2013, Wang 2013), including rare, endemic *Centaurea* species (Beltrame 2007, Lopez & Barreiro 2013). Perhaps an immigration bottleneck when arriving with cultivated cereals in Western Europe allowed only one haplotype to spread in arable fields; with the provision that variability does occur in the centre of origin, probably Turkey. Thereafter, since the Middle Ages when cornflower was a common component of cereal fields (Rösch 1998), dispersal of seeds contained in the crop harvest through human mobility probably contributed to genetic homogeneity within and among invaded regions. However, such seed dispersal is no longer possible due to the recent use of rigorous sieving procedures during crop seed certification. In addition, agriculture intensification, land management and anti-dicotyledonous herbicides over the past fifty years have fragmented and caused significant decline in populations. This process of decline, especially in Poitou-Charentes, has not yet substantially decreased genetic diversity, probably thanks to the buffering effect of both the soil seed bank and high gene flow mediated by foraging insects. In conclusion, the pattern of genetic variation described in our study, with low but significant regional and population differentiation, could reflect the first effects of the rarefaction and fragmentation of previously abundant, widely distributed and poorly structured *C. cyanus* populations, on the reproduction regime and the increase of local differentiation among populations.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data) and consist of: (1) isozyme electrophoretic patterns with Rf values; (2) characteristics of twenty eight chloroplast DNA regions; (3) isozyme allele frequencies at all loci; and (4) pairwise Fst values between large and small populations from Poitou-Charentes.

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