Evolution, 55(5), 2001, pp. 943–954

PATTERNS OF ALLOZYME VARIATION IN DIPLOID AND TETRAPLOID *CENTAUREA JACEA* AT DIFFERENT SPATIAL SCALES

OLIVIER J. HARDY[1,2] AND XAVIER VEKEMANS[1]
[1]*Laboratoire de Génétique et Ecologie Végétales, Université Libre de Bruxelles, 1850 Chaussée de Wavre, B-1160 Bruxelles, Belgium*
[2]*E-mail: ohardy@ulb.ac.be*

Abstract.—The extent and spatial patterns of genetic variation at allozyme markers were investigated within and between diploid and autotetraploid knapweeds (*Centaurea jacea* L. sensu lato, Asteraceae) at contrasted geographic scales: (1) among populations sampled from a diploid-tetraploid contact zone in the northeastern part of the Belgian Ardennes, and (2) within mixed populations from that zone where diploids and tetraploids coexist. Our data were also compared with a published dataset by Sommer (1990) describing allozyme variation in separate diploid and tetraploid knapweeds populations collected throughout Europe. Genetic diversity was higher in tetraploids. In the Belgian Ardennes and within the mixed populations, both cytotypes had similar levels of spatial genetic structure, they were genetically differentiated, and their distributions of allele frequencies were not spatially correlated. In contrast, at the European scale, diploids and tetraploids did not show differentiated gene pools and presented a strong correlation between their patterns of spatial genetic variation. Numerical simulations showed that the striking difference in patterns observed at small and large geographic scales could be accounted for by a combination of (1) isolation by distance within cytotypes; and (2) partial reproductive barriers between cytotypes and/or recurrent formation of tetraploids. We suggest that this may explain the difficulty of the taxonomic treatment of knapweeds and of polyploid complexes in general.

Key words.—Autopolyploidy, *Centaurea*, genetic structure, hybridization, isolation by distance, polyploid complex, spatial autocorrelation.

Received March 1, 2000. Accepted January 4, 2001.

Polyploidy has played a major role in the evolution of angiosperms: It is estimated that about half of them are polyploids (Lewis 1980; Masterson 1994), and recent advances in cytogenetics indicate that many diploids are in fact ancient polyploids (reviewed in Leitch and Bennett 1997). The ecological and evolutionary significance of polyploidy can best be investigated on polyploid complexes, that is, groups of closely related taxa with varying ploidal levels (Lumaret 1988a; Thompson and Lumaret 1992; Bretagnolle and Thompson 1995). In recent years, new views have emerged from such studies. Firstly, autopolyploids have appeared to be far more common than previously recognized (Soltis and Soltis 1993), which suggests that the traditional view considering autopolyploidy as maladaptive should be reconsidered. Second, many studies have found evidence for multiple origins of polyploid taxa, both for auto- and allopolyploids, generating complex phyletic relationships between and within ploidal levels (Soltis and Soltis 1993, 1999, 2000; Leitch and Bennett 1997). Consequently, most of the allelic diversity present in diploid parents may be found in the polyploids. Moreover, because populations of autopolyploids experience a lower pressure of genetic drift due to their polysomic inheritance (Moody et al. 1993), they are expected to exhibit higher genetic diversity than diploid populations. Accordingly, increased genetic diversity has generally been observed in autoplolyploids relative to their diploid counterparts (Soltis and Soltis 1993). To perform adequate comparisons between different cytotypes in levels of genetic diversity (Moody et al. 1993) or amount of genetic differentiation (Ronfort et al. 1998; Hardy and Vekemans 1999), statistics have recently been developed to take into account the effect of ploidal level on genetic drift.

Contact and/or hybrid zones between cytotypes of autopolyploid complexes are particularly interesting to study the evolution of reproductive interactions between ploidal levels (Thompson and Lumaret 1992; Petit et al. 1999). Partial reproductive barriers between cytotypes have often been reported (e.g., Van Dijk et al. 1992; Felber-Girard et al. 1996; Petit et al. 1997; Gauthier et al. 1998; Husband and Schemske 1998, 2000). They can result from postzygotic (e.g., triploid block effect, triploid sterility) or prezygotic (e.g., habitat differentiation, phenological differentiation) isolating mechanisms (Petit et al. 1999). Although the consequences of partial reproductive barriers on the origin and persistence of contact zones have been documented (reviews in Thompson and Lumaret 1992; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Petit et al. 1999), their impact on the phyletic relationships occurring within polyploid complexes merits further investigation (Soltis and Soltis 1999, 2000).

Knapweeds (*Centaurea jacea* L. sensu lato) constitute a very polymorphic polyploid complex with two ploidal levels, diploid (2x = 22) and tetraploid (4x = 44; Gardou 1972; Sommer 1990). Allozyme marker studies have demonstrated that tetraploids show a tetrasomic mode of inheritance (Hardy et al. 2000), suggesting an autopolyploid origin and that the diploid and tetraploid cytotypes share a common gene pool in Europe (Sommer 1990). Although different authors have subdivided the complex into a variable number of species (e.g., Dostal 1976; Lambinon et al. 1992), these putative species hybridize freely, at least within a ploidal level (Marsden-Jones and Turrill 1952; Saarisalo-Taubert 1966; Gardou 1972), and cannot be differentiated on the basis of allozyme markers (Sommer 1990). Thus, the whole complex might be regarded as consisting in a single biological species. The major reproductive barrier observed within the complex concerns diploid-tetraploid crosses, which yield low seed set and often unfertile offspring (Gardou 1972; Hardy et al. 2001).

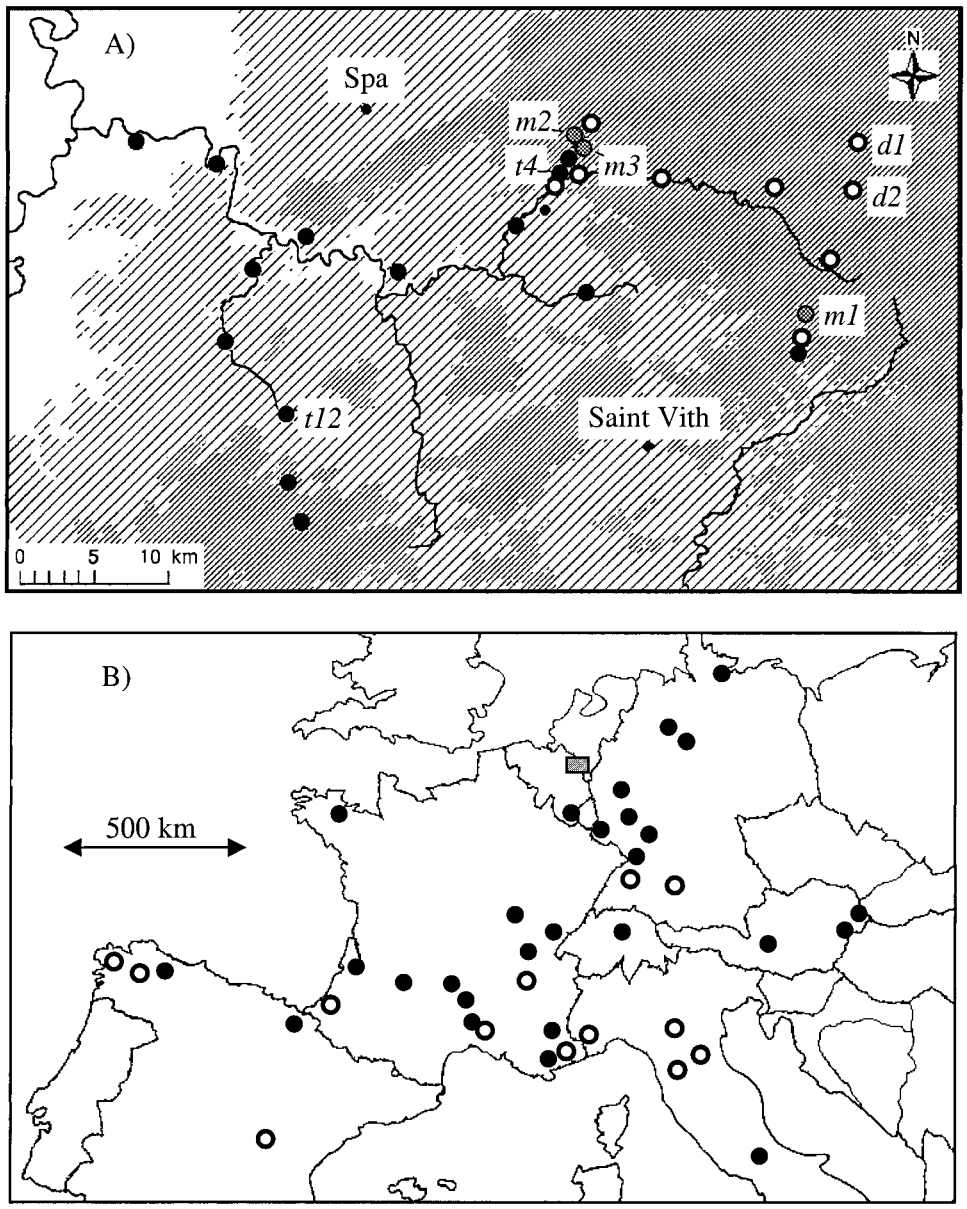

FIG. 1. Distribution of diploid (open circles), tetraploid (closed circles), and mixed (cross-hatched circles) populations of knapweed at two scales: (A) in the northeastern part of the Belgian Ardennes (where hatching indicates altitudes: white: < 300 m; light hatching: 300–500 m; dense hatching: > 500 m); (B) in Western Europe (where the rectangle shows the area covered by panel A). In the Belgian Ardennes, allozyme markers were scored by us in seven populations (the labeled ones). In Western Europe, allozyme markers were scored by Sommer (1990) for all populations shown.

We recently found in the northeastern part of the Belgian Ardennes a contact zone between diploid and tetraploid knapweeds (Fig. 1), with a few populations where both cytotypes grow intermingled (Hardy et al. 2000). In this contact zone, the two cytotypes were well differentiated both morphologically and genetically. No evidence for gene flow between cytotypes was found, that is, no records of naturally occurring triploids, even in the mixed populations, and no evidence for character introgression. Our finding that the two cytotypes were well differentiated at a local scale (Hardy et al. 2000) contrasts with the lack of differentiation between the two cytotypes found at the European scale (Sommer 1990). This suggests that the complexity of the taxonomic treatment of knapweeds might be linked to a geographic scale effect. We hypothesize that this effect might be created by partial reproductive barriers between cytotypes or by infrequent but recurrent formation of tetraploids from local diploids accompanied by a strict reproductive barrier. Indeed, at a small scale, well-differentiated gene pools might occur because the main barrier to gene flow is between cytotypes. But, at a large scale, isolation by distance might be a stronger barrier to gene flow so that cytotypes would not show differentiated gene pools. To test this hypothesis, the level of genetic differentiation should be compared within and between cytotypes at different spatial scales.

In the present study, we use allozyme markers to compare

diploid and tetraploid knapweeds for (1) their levels of within-population genetic variation; and (2) their spatial genetic structure at three different scales: within populations, among populations at a local scale (i.e., the northeastern part of the Belgian Ardennes), and among populations at the European scale (by reanalyzing Sommer's data). To detect gene flow between cytotypes at these different scales, we analyze the spatial correlation of allele frequencies between cytotypes. We also use numerical simulations to investigate how different levels of gene flow between cytotypes can be distinguished based on the pattern of population differentiation in an isolation-by-distance model.

Materials and Methods

Study Organism

Knapweeds are short-lived perennial herbs that are widespread throughout Europe. Diploids have a restricted geographic distribution, being often found in mountainous grassland habitats, whereas tetraploids have apparently a broader ecological amplitude (Gardou 1972). Taxonomic treatments of the complex are controversial. The whole complex may be viewed as belonging to the taxon *Centaurea* subgenus *Jacea* (Mill.) Hayek (Lambinon et al. 1992), and it is sometimes referred to as the *C. jacea–C. nigra* complex. *Centaurea jacea* L. sensu stricto and *C. nigra* L. are indeed taxa often recognized at the species level. In the present study, we instead consider them as morphotypes, given the absence of reproductive barriers, differentiated gene pools, and clear-cut discontinuities between them and the prevalence of intermediate forms in nature (Marsden-Jones and Turrill 1952; Gardou 1972; Sommer 1990; Lambinon et al. 1992). Those intermediate forms are sometimes referred to as *C. thuillieri* (Dostal) J. Duvigneaud and Lambinon (Lambinon et al. 1992). For all three putative species, both diploid and tetraploid cytotypes have been recorded in the literature (Gardou 1972; Sommer 1990). In this paper, unless stated differently, we use the name *C. jacea* in a broad sense to refer to the whole complex.

Centaurea jacea is insect pollinated, and self-pollination experiments have shown that it is self-incompatible, whatever the cytotype or morphotype considered (Gardou 1972; Hardy et al. 2001). In the northeastern part of the Belgian Ardennes, plants flower essentially from mid-June to mid-August. In our experimental garden, transplanted diploids from this area flower approximately 10 days later than transplanted tetraploids (O. J. Hardy and S. Vanderhoeven, pers. obs.), but most of the flowering periods of diploids and tetraploids overlap widely, and this also occurs in situ, within mixed populations (Hardy et al. 2000). In knapweeds, capitula contain approximately 40 central hermaphroditic disc flowers and they may or may not include a ring of sterile ray flowers. According to Lambinon et al. (1992), ray flowers are present in *C. jacea* sensu stricto and absent in *C. nigra,* whereas *C. thuillieri* is polymorphic for this trait. Lack (1982) showed that ray flowers enhance pollinator attractiveness in knapweeds. In the northeastern part of the Belgian Ardennes, all diploids lack ray flowers, whereas all but a few tetraploids possess ray flowers (Hardy et al. 2000). Another important feature distinguishing diploids and tetraploids from that contact zone is the pappus of the achenes, which is more developed in diploids than tetraploids (Hardy et al. 2000). It is worth noting that, in general, these features do not distinguish diploids and tetraploids.

Population Sampling

In the Belgian Ardennes, populations were sampled in a contact zone between diploid and tetraploid knapweeds. Two pure populations of diploids (*d1, d2*), two pure populations of tetraploids (*t4, t12*), and three mixed populations with coexisting diploids and tetraploids (*m1, m2, m3*) were sampled (Fig. 1A). Diploids and tetraploids from this contact zone are well differentiated morphologically, and, according to the nomenclature of Lambinon et al. (1992), tetraploids are intermediate between *C. jacea* and *C. thuillieri,* whereas diploids can be ascribed to *C. nigra.* There is no clear evidence for habitat differentiation between the cytotypes, except that diploids generally grow at higher altitudes (Hardy et al. 2000). Geographic distances between populations range from 0.5 km to 45 km. The site description and the protocol used for cytotype identification (i.e., flow cytometry technique) were given in Hardy et al. (2000).

Within-population genetic structure was studied in two mixed populations: a two-dimensional population (*m2*) extending over an area of about 50 m $\times$ 100 m and a linear population (*m3*) extending over 1 km along a roadside. Diploid and tetraploid cytotypes were approximately equally represented in those populations, and, on a broad scale, they were relatively well intermixed (i.e., cytotypes were not segregated in opposite parts of the population). Overall, 139 (65 diploids, 74 tetraploids) and 200 (108 diploids, 92 tetraploids) individuals were mapped and sampled in the *m2* and *m3* populations, respectively. For the other populations, sample size per cytotype ranged from 19 to 39 individuals.

At the European scale, we reanalyzed data from Sommer (1990), who characterized 59 populations of *C.* section *Jacea* Boissier collected throughout Western Europe using eight enzymatic systems that provided 12 loci. Knapweeds from those populations were identified as belonging to the taxa *C. nigrescens* Willd., *C. pseudophrygia* C. A. Meyer, *C. jacea* s.s. L., *C. nigra* L., or intermediate forms between *C. jacea* and *C. nigra. Centaurea pseudophrygia* and *C. nigrescens* are usually not considered as belonging to the *C. jacea–C. nigra* complex (Sommer 1990). Accordingly, we restricted our analysis to the taxa Sommer referred to as *C. jacea, C. nigra,* and intermediate forms. Thus, by further eliminating populations with small sample size (less than 10), allele frequencies from 13 diploid and 27 tetraploid populations were used in the present study (Fig. 1B). The average sample size per population was 22 individuals. There were no mixed populations in Sommer's dataset, and there was no indication whether populations were collected from diploid-tetraploid contact zones.

Enzyme Electrophoresis

Allozyme markers were used to determine the genotype of each individual collected in the Belgian Ardennes at eight loci, and electrophoreses were run on vertical polyacrylamide gels. Protocols for enzyme electrophoresis and banding pat-

tern interpretations for three enzymatic systems (*Lap, Pgd, Dia*) that each provided one locus were described in Hardy et al. (2000). Three additional monomeric enzymatic systems could be resolved for the present study, providing us with four additional loci: *Pgm* (two loci), *Skd* (one locus), *Est* (one locus). Genotypic interpretation for those additional loci was similar to that of *Lap* and *Dia* (Hardy et al. 2000). The *Est* locus did not lead to satisfactory interpretations in the tetraploids, and thus was only scored in diploids.

Sommer (1990) analyzed the following eight enzymatic systems using horizontal starch gel electrophoreses: *Tpi, Pgi, Pgm, Lap, Got, Skd, Aco, Pgd.* Because the difference in the arrays of enzymatic systems used in our study and that of Sommer is a concern when comparing results from both datasets for some genetic analyses (e.g., levels of polymorphism), most analyses were done twice: first using all loci available and then using the five loci from four enzymatic systems that were common to both studies (two loci for *Pgm* and one for *Lap, Pgd,* and *Skd*).

Analysis of Genetic Variation within Populations

Genetic diversity of diploid and tetraploid populations of the Belgian Ardennes was compared using the average number of alleles per locus, the expected heterozygosity (or gene diversity, $He = 1 - \Sigma_i p_i^2$, where p_i is the frequency of the ith allele), the observed proportion of heterozygotes (or zygotic heterozygosity), *Ho,* and, for the tetraploids, the observed gametic heterozygosity, *Ho′* (Lumaret and Hanotte 1987; Moody et al. 1993). Under tetrasomic inheritance and in the absence of double reduction, the equilibrium *Ho′*-value is equal to the expected heterozygosity (*He*) under panmixia (Moody et al. 1993). Thus, gametic heterozygosity provides a unified approach for comparing genetic variation between diploids and tetraploids. We also computed manually the Shannon-Weaver index averaged over the loci and the equitability (i.e., the ratio of the observed Shannon-Weaver index to its expected value if the alleles had equal frequencies), because those statistics are sometimes used in studies of polyploid complexes (e.g., Lumaret and Hanotte 1987; Lumaret and Barrientos 1990). Mean values for each cytotype are obtained by averaging first over the populations, then over the loci. Differences between cytotypes in statistics of genetic diversity were compared by two-way analyses of variance (ANOVA) with cytotype and locus as main effects, performed with the Statistica software (StatSoft, Inc. 2000).

The total gene diversity observed when diploids and tetraploids are grouped was also computed from population allele frequencies at the European scale and at the local scale (Belgian Ardennes). Total gene diversity was then decomposed into the gene diversities within populations, among populations within a cytotype, and between cytotypes, according to Nei (1987).

Within-Population Analysis of Spatial Genetic Structure

Detailed within-population analysis was performed for the two mixed populations, *m2* and *m3.* The spatial distribution of cytotypes within those populations was investigated using Moran's *I*-statistic with the cytotype of each individual used as the variable of interest.

Genetic structure within populations was assessed by spatial autocorrelation analysis using two statistics derived from population genetics theory (Hardy and Vekemans 1999). The first statistic estimates kinship coefficients, F_{ij}, between pairs of individuals i and j, following Loiselle et al. (1995). The second statistic estimates Wright's coefficient of relationship, ρ_{ij}, between pairs of individuals (Hardy and Vekemans 1999) and corresponds to Moran's *I*-statistic using individual allele frequencies (Dewey and Heywood 1988). To compare the spatial genetic structure of different cytotypes, the coefficient of relationship has the interesting property that it depends only on the pattern of gene flow and is not influenced by the ploidal level nor the selfing rate, contrary to the kinship coefficient (Ronfort et al. 1998; Hardy and Vekemans 1999). These statistics were computed between all pairs of individuals belonging to the same cytotype using multilocus estimates obtained following Loiselle et al. (1995), where the contribution of each allele is weighted by the product $p_{lu}(1 - p_{lu})$, with p_{lu} being the uth allele frequency at locus l. The slopes of the linear regression of those pairwise coefficients on the logarithm of the spatial distance provide estimators of the degree of spatial genetic structure convenient for continuously distributed populations (Hardy and Vekemans 1999). Also, average coefficients $F(d)$ and $\rho(d)$ were computed over given distance intervals d and plotted against d in the form of correlograms.

To detect if the spatial genetic structures of diploids and tetraploids were correlated, spatial correlation coefficients between the allele frequencies of pairs of individuals belonging to different cytotypes were also computed. These coefficients are also called ρ_{ij} because they are equivalent to the coefficients of relationship defined for comparisons within cytotypes. For the comparisons between cytotypes, $\rho_{ij} = \sigma_{ij}/\sigma_d\sigma_t$, where σ_{ij} is the covariance between the allele frequencies of individuals i and j (the population average allele frequencies being specific to each cytotype), and σ_d and σ_t are the standard deviations of the individual allele frequencies for the diploid and tetraploid cytotypes, respectively. Note that for comparisons within a cytotype, $\rho_{ij} = \sigma_{ij}/\sigma^2$, where σ^2 is the variance of the individual allele frequencies for that cytotype. As for within-cytotype comparisons, ρ_{ij} pairwise coefficients for between-cytotype comparisons are regressed against spatial distance and averaged for given distance classes.

Standard errors of the statistics are estimated using a jackknife procedure over the loci (Sokal and Rohlf 1995). Moreover, randomization tests are carried out by comparing observed values of the statistics to their frequency distributions obtained after 10,000 random permutations of the individuals with respect to their spatial positions (only individuals sharing the same cytotype are permuted among one another). This permutation test is suited to test for the occurrence of a spatial genetic structure within each cytotype (Manly 1997). However, for comparisons between cytotypes, care must be taken in the interpretation of significance tests when spatial autocorrelation occurs within each cytotype (Manly 1997). This problem arises because randomization of spatial positions not only uncouples the spatial structures of both cytotypes, as needed, but also suppresses the spatial autocorrelation within each cytotype. Thus, for comparisons between cytotypes, the

tests are likely to be liberal (i.e., the null hypothesis of no spatial correlation between the structures of both cytotypes would be rejected more often than it should).

Computations of the statistics used to describe within-population structure and the randomization procedures were performed using the software Autocorg version 2.0 developed by the authors (available on request).

Among-Population Analysis of Spatial Genetic Structure

Two approaches were used to analyze the genetic structure among populations. First, inbreeding coefficients and global differentiation among populations of each cytotype were assessed. For this purpose, standard *F*-statistics and a ρ_{ST}-statistic were computed. The latter can be defined as the intrapopulation correlation coefficient of relationship (similarly, F_{ST} is the intrapopulation correlation coefficient of kinship). It was called ρ by Ronfort et al. (1998), who demonstrated its convenient properties when comparing different ploidal levels, that is, identical expectations under identical gene flow conditions. For diploids, $\rho_{ST} = 2F_{ST}/(1 + F_{IT})$, and *F*-statistics were computed using the program Gen-Survey (Vekemans and Lefebvre 1997). For tetraploids, $\rho_{ST} = 4F_{ST}/(1 + 3F_{IT})$, and *F*-statistics were computed using the program 4X Genetic Structure version 1.0 of J. Ronfort (pers. comm.). Both programs make use of the ANOVA method of Weir and Cockerham (1984). Second, differentiation between pairs of populations as a function of their geographic distance was assessed using the ratios $F_{ST}/(1 - F_{ST})$, distinguishing comparisons within and between cytotypes. F_{ST}-values for population pairs were computed using the formula of Reynolds et al. (1983), which is also based on an ANOVA approach and requires only population allele frequencies and sample sizes. Isolation by distance was inspected through the regression of those ratios on the logarithm of the geographic distance (Rousset 1997; Ronfort et al. 1998). Computations were performed using the software Autocorf developed by the authors (available on request).

Genetic structure was analyzed at two geographic scales: a local one using our own dataset, that is, the populations of the Belgian Ardennes, and the European one, using the dataset of Sommer (1990). Estimation of standard errors was obtained by jackknifing over loci. Statistical randomization tests were made in a similar way as for the within-population analysis, but with populations rather than individuals as permutation objects.

Simulations

Numerical simulations of a stepping-stone model containing diploid and tetraploid populations were performed to investigate the impact of the pattern of gene flow on the statistics used to characterize population structure. In particular, we wished to investigate the effect of combining isolation by distance and reproductive barriers to gene flow between diploid and tetraploid cytotypes. Although reproductive barriers between cytotypes is the rule, gene flow can also occur through different pathways (reviewed in Ramsey and Schemske 1998; Petit et al. 1999). One could distinguish the pathways involving crosses between diploids and tetraploids (which can also involve backcrosses with triploids) and recurrent formation of tetraploids from diploids. The later mechanism causes an unidirectional gene flow from diploids to tetraploids as soon as newly formed tetraploids cross with preexisting tetraploid populations. Thus, it is interesting to investigate bidirectional and unidirectional patterns of gene flow.

The stepping-stone model is a toroidal two-dimensional array of populations where diploid and tetraploid populations alternate like squares of a checkerboard. Gene flow occurs only between adjacent populations. Thus, each population may exchange genes with four populations of the same cytotype and with four populations of the alternative cytotype. The gene flow parameters are the immigration rates of genes coming from populations of the same cytotype (m_w, here fixed to 0.1 for each cytotype) and from a different cytotype (m_b). With bidirectional gene flow, migration rates from diploid to tetraploid and from tetraploid to diploid were equal. With unidirectional gene flow, it occurred only from diploids to tetraploids. The ratio m_b/m_w expresses the intercytotype reproductive barrier (a ratio of zero corresponds to cases where cytotypes do not exchange genes). Simulations were carried out for one diallelic locus with a reversible mutation rate of 10^{-4} in both directions. All populations contained 50 individuals. Four values for the ratio m_b/m_w were used: 0, 10^{-1}, 10^{-2}, 10^{-3}. Simulations of an elongated space of 4×200 populations were run for 10,000 generations (a sufficient time to reach a quasi-equilibrium state), with 100 replicates for each value of the m_b/m_w ratio. Paired $F_{ST}/(1 - F_{ST})$ were computed between all pairs of populations, distinguishing 2x–2x, 4x–4x, and 2x–4x pairs.

RESULTS

Levels of Genetic Variation

In the Belgian Ardennes, four statistics of diversity (i.e., the average number of alleles per locus, the Shannon-Weaver index, the expected heterozygosity [or gene diversity], and the observed zygotic heterozygosity) were significantly higher within tetraploid than diploid populations (Table 1). However, the equitability was similar for the two cytotypes (Table 1), suggesting that the higher diversity of tetraploids was due to the presence of several rare alleles. Indeed, all alleles present in diploids were also found in tetraploids, but the latter possessed nine alleles not found in diploids (Table 2) and that occurred at low frequencies (<5%). The observed zygotic heterozygosity in diploid populations was almost half that in tetraploid populations, but only slightly lower (difference marginally significant) than the observed gametic heterozygosity in the tetraploid populations (Table 1). Expected heterozygosity was usually slightly higher or equal to observed zygotic heterozygosity in diploids and observed gametic heterozygosity in tetraploids (Table 1), showing some level of heterozygote deficiency. As expected for an autopolyploid, fixed heterozygosity was not observed in tetraploid populations at any locus.

Using all available loci, the total gene diversity in the Belgian Ardennes amounted to $H_T = 0.375$, 6.9% of which was distributed between cytotypes and 2.8% among populations within cytotypes. At the European scale, $H_T = 0.317$, 0.8% of which was distributed between cytotypes and 13.0%

TABLE 1. Statistics of genetic variation within diploid and tetraploid populations of knapweeds from the Belgian Ardennes: mean number of alleles per locus (*A*), Shannon's index (*S*), equitability (*E*), expected heterozygosity (*He*), observed zygotic heterozygosity (*Ho*), and observed gametic heterozygosity (*Ho′*, defined only for tetraploids). Locus *Est1* was discarded from the computations because data were missing for one cytotype. Locus *Skd1* was discarded for the equitability parameter because it was monomorph in diploids. Difference between cytotypes tested as the cytotype effects in a two-way ANOVA with cytotype and locus as main effects. The locus effect and locus-cytotype interaction are significant for all parameters.

Cytotype	Population	*N*	*A*	*S*	*E*	*He*	*Ho*	*Ho′*
Diploid	*n1*	19	2.17	0.48	0.77	0.29	0.29	
	n2	37	2.33	0.50	0.77	0.31	0.27	
	m1	33	3.00	0.78	0.75	0.45	0.31	
	m2	65	2.17	0.46	0.60	0.27	0.27	
	m3	108	2.33	0.53	0.69	0.31	0.32	
	mean		2.25	0.50	0.68	0.30	0.29	
Tetraploid	*t4*	25	3.60	0.65	0.65	0.36	0.51	0.33
	t12	39	3.50	0.69	0.65	0.37	0.55	0.35
	m1	30	3.80	0.67	0.68	0.36	0.45	0.27
	m2	71	3.67	0.72	0.73	0.41	0.58	0.36
	m3	92	3.50	0.74	0.70	0.40	0.61	0.37
	mean		3.54	0.69	0.68	0.38	0.54	0.33
$F_{(1,43)}$			99.9***	36.8***	0.0001	13.6***	65.5***	2.8*,[1]

*** $P < 0.001$; * $P < 0.1$.
[1] *Ho′* in tetraploids is tested against *Ho* in diploids.

among populations within cytotypes. Thus, the distribution of the total gene diversity between cytotypes and populations differs markedly according to the geographic scale. The higher total gene diversity observed at the local scale relative to the European scale is an artifact due to the different arrays of enzymatic systems used at these two scales. Indeed, when this analysis was conducted on the five loci common to both datasets, $H_T = 0.350$ in the Belgian Ardennes (3.7% between cytotypes, 2.8% among populations within cytotypes) and $H_T = 0.383$ at the European scale (0.7% between cytotypes, 13.2% among populations within cytotypes), and the average gene diversities within population were very similar (0.327 for our dataset, 0.330 for Sommer's dataset). Most importantly, the difference in distribution of gene diversity between the two scales is confirmed using the five common loci.

Genetic Structure within Populations

Individuals were more likely to share the same cytotype when they were separated by less than about 15 m in the two-dimensional *m2* population and about 50 m in the one-dimensional *m3* population (Fig. 2). Thus, there was some grouping of identical cytotypes on a small spatial scale. At larger distances, Moran's *I*-values were only slightly negative, that is, there was no broad scale spatial segregation of the two cytotypes, in agreement with our direct observation in the field.

In both the *m2* and *m3* populations, a spatial genetic structure occurred within each cytotype (Fig. 3). In the two-dimensional *m2* population, this structure was essentially apparent in the first distance interval (0–2 m) for which kinship coefficients were equal to 0.079 (SE = 0.038; $P < 0.001$); and 0.181 (SE = 0.060; $P < 0.001$) for tetraploids and diploids, respectively (Fig. 3A; SE is the standard error estimated by jackknifing over loci; *P* is the probability value given by the randomization test). In the one-dimensional *m3* popula-

TABLE 2. Number of alleles per population at seven allozyme loci and total numbers per cytotype. All alleles found in diploids were also present in tetraploids.

	Diploid						Tetraploid					
	n1	*n2*	*m1*	*m2*	*m3*	Total	*t4*	*t12*	*m1*	*m2*	*m3*	Total
Lap1	4	5	5	4	4	6	7	8	9	9	7	10
Pgd1	3	3	2	2	3	3	4	4	4	4	3	4
Dia1	2	2	2	2	2	2	2	2	2	2	3	3
Pgm1	2	2	—	2	2	2	—	2	2	2	2	2
Pgm2	1	1	—	2	2	3	3	3	—	3	4	4
Est1	2	2	—	2	2	2	—	—	—	—	—	—
Skd1	1	1	—	1	1	1	2	2	2	2	2	3

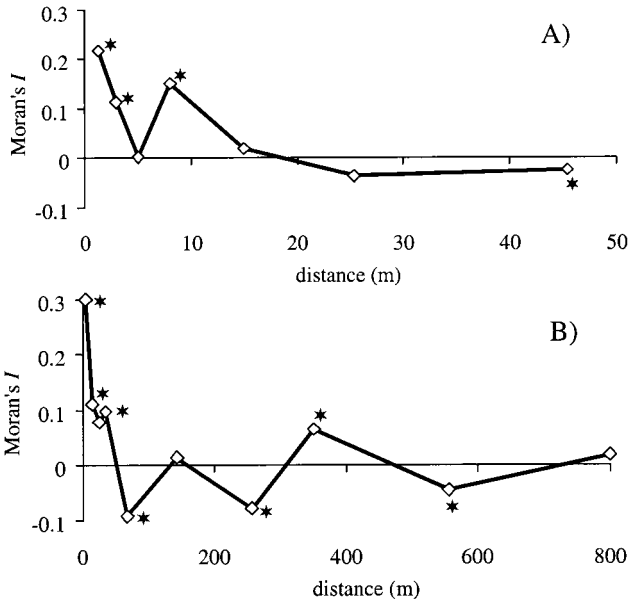

FIG. 2. Autocorrelograms showing the spatial segregation of the cytotypes in the mixed populations: (A) two-dimensional *m2* population; (B) one-dimensional *m3* population. Moran's *I*-values are computed using the individual cytotypes as variable. Star symbols show values that deviate significantly from zero ($P < 0.05$).

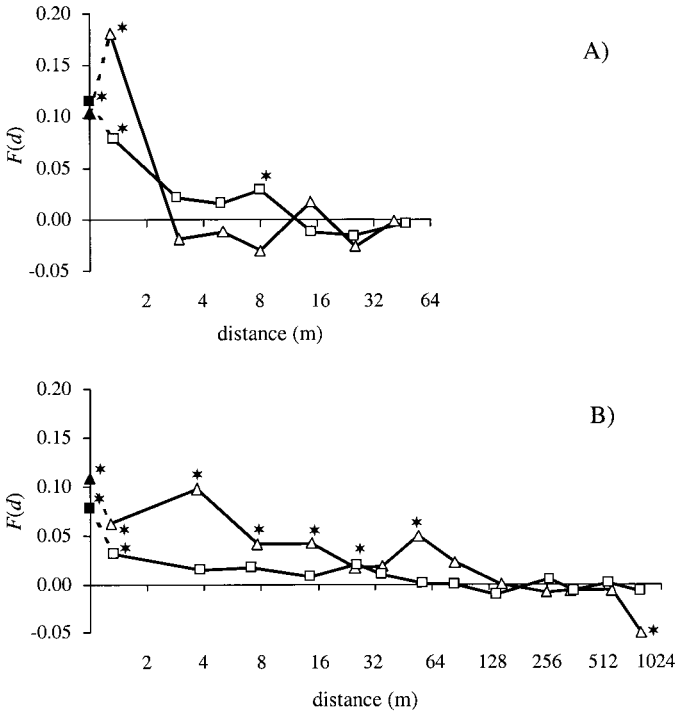

FIG. 3. Coefficients of kinship as a function of the distance (log scale) for each cytotype in the mixed populations: (A) two-dimensional *m2* population; (B) one-dimensional *m3* population. Triangles refer to diploids, squares to tetraploids. The filled symbols situated on the vertical axis show values of the inbreeding coefficients. Star symbols show values that deviate significantly from zero ($P < 0.05$).

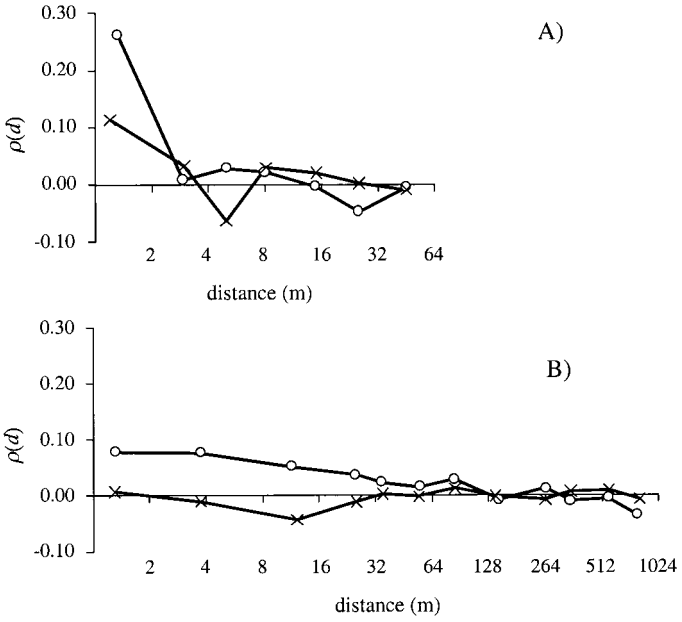

FIG. 4. Coefficients of relationship as a function of the distance (log scale) in the mixed populations: (A) two-dimensional *m2* population; (B) one-dimensional *m3* population. Circles refer to comparisons within cytotypes (values for diploids and tetraploids pooled), crosses refer to comparisons between cytotypes.

tion, the structure was more developed, and the correlograms crossed the horizontal axis at a distance between 100 m and 200 m (Fig. 3B).

Inbreeding coefficients for diploids and tetraploids were equal to, respectively, 0.10 (SE = 0.06; $P = 0.08$) and 0.11 (SE = 0.04; $P < 0.001$) in the *m2* population, and 0.11 (SE = 0.10; $P = 0.004$) and 0.08 (SE = 0.02; $P < 0.001$) in the *m3* population. Those values were similar to the average kinship coefficients between individuals separated by the first distance interval (Fig. 3). Because knapweeds are self-incompatible, positive inbreeding coefficients should reflect biparental inbreeding.

To compare the spatial genetic structure of diploids and tetraploids, the coefficients of relationship (ρ_{ij}) were more useful because they are not affected by the lower drift inherent to tetraploids relative to diploids. The regression slopes of these coefficients on the logarithm of the distance for diploids and tetraploids were equal to, respectively, −0.021 (SE = 0.016; $P = 0.098$) and −0.036 (SE = 0.009; $P = 0.003$) in the *m2* population and −0.032 (SE = 0.013; $P < 0.001$) and −0.015 (SE = 0.005; $P = 0.001$) in the *m3* population. Thus, diploids and tetraploids have similar levels of spatial genetic structure.

Coefficients of relationship between individuals belonging to different cytotypes could only be computed for alleles that were present but unfixed in both cytotypes, reducing the number of informative loci to five (*Lap, Pgd, Dia, Pgm1, Pgm2*). Figure 4 shows the average correlograms for different types of comparisons. The slopes for comparisons within and between cytotypes were equal to, respectively, −0.026 (SE = 0.006; $P = 0.01$) and −0.015 (SE = 0.006; $P = 0.12$) in the *m2* population and −0.016 (SE = 0.005; $P < 0.001$) and 0.004 (SE = 0.015; $P = 0.22$) in the *m3* population. Thus, there was no significant correlation between the spatial genetic structures of diploids and tetraploids.

Genetic Structure of Populations from the Belgian Ardennes

Values of *F*-statistics are given in Table 3. Diploids and tetraploids showed relatively low but significantly positive inbreeding coefficients (mean F_{IS} equal to 0.09 and 0.10,

TABLE 3. *F*-statistics and ρ_{ST}-statistic for the diploid and tetraploid populations of the Belgian Ardennes. The standard errors of the mean estimates (SE) are obtained by jackknifing over loci.

Cytotype	Locus	F_{IT}	F_{IS}	F_{ST}	ρ_{ST}
Diploids	*Lap1*	0.093	0.055	0.040	0.074
	Pgd1	0.141	0.137	0.004	0.008
	Dia1	0.059	0.006	0.053	0.100
	Pgm1	0.060	0.064	−0.004	−0.007
	Pgm2	−0.042	−0.212	0.140	0.293
	Est1	0.299	0.301	−0.004	−0.006
	Skd1	0.036	−0.064	0.094	0.181
	Mean	0.115	0.088	0.030	0.054
	SE	0.047	0.058	0.015	0.026
Tetraploids	*Lap1*	0.128	0.116	0.014	0.039
	Pgd1	0.040	0.020	0.021	0.074
	Dia1	0.054	0.034	0.021	0.071
	Pgm1	0.281	0.250	0.041	0.089
	Pgm2	0.157	0.139	0.022	0.059
	Skd1	−0.016	−0.022	0.006	0.024
	Mean	0.124	0.105	0.021	0.062
	SE	0.036	0.034	0.004	0.011

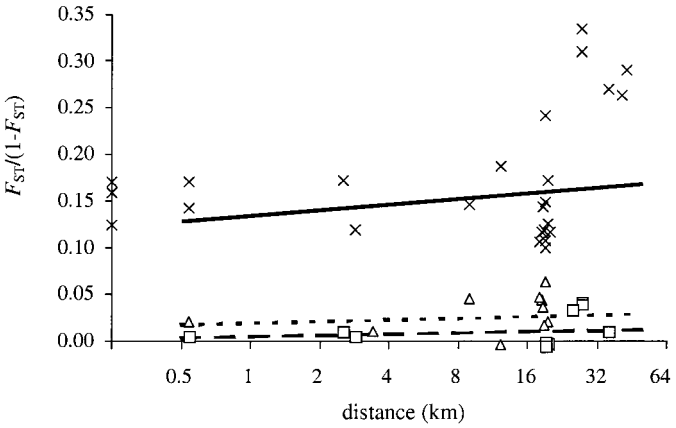

FIG. 5. Ratios $F_{ST}/(1 - F_{ST})$ computed for pairs of populations from the Belgian Ardennes as a function of the distance (log scale). Triangles refer to diploid-diploid population pairs, squares to tetraploid-tetraploid population pairs, crosses to diploid-tetraploid population pairs. Regression lines are also shown. Crosses situated on the vertical axis represent $F_{ST}/(1 - F_{ST})$ values between cytotypes for each mixed population.

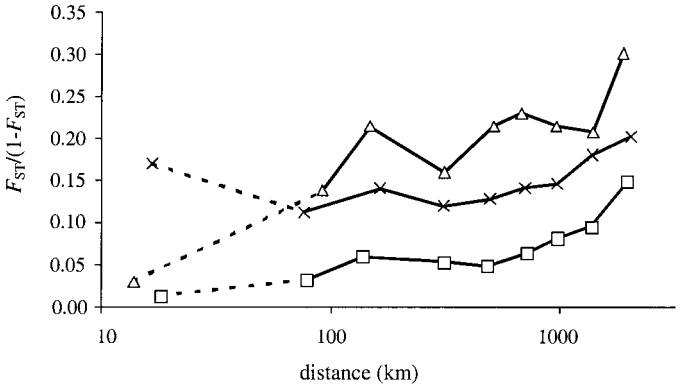

FIG. 6. Average $F_{ST}/(1 - F_{ST})$ ratios per distance classes (log scale) computed for pairs of populations from Europe. Triangles refer to diploid-diploid population pairs, squares to tetraploid-tetraploid population pairs, crosses to diploid-tetraploid population pairs. For the shortest distance class, data from the Belgian Ardennes were used.

respectively). F_{ST}-values were lower in tetraploids (F_{ST} = 0.021) than in diploids (F_{ST} = 0.030), but ρ_{ST}-values were very similar for both cytotypes (Table 3).

Ratios $F_{ST}/(1 - F_{ST})$ for population pairs were much larger for comparisons between cytotypes than within cytotypes (Fig. 5): mean ratios equal 0.030 (SE = 0.013) for diploid pairs, 0.013 (SE = 0.006) for tetraploid pairs, and 0.170 (SE = 0.062) for diploid-tetraploid pairs. Thus, genetic differentiation between cytotypes is an order of magnitude larger than differentiation within a cytotype. In Figure 5, the cross symbols on the vertical axis represent $F_{ST}/(1 - F_{ST})$-values for comparisons between sympatric cytotypes in the three mixed populations. It is noteworthy that those values were not lower than the ones for allopatric cytotypes. As for the within-population investigation, there is again no evidence of gene flow between cytotypes in the contact zone of the Belgian Ardennes.

For the three types of pairwise $F_{ST}/(1 - F_{ST})$ comparisons, positive but statistically nonsignificant slopes were obtained with the logarithm of the distance.

Genetic Structure of Populations from Europe

At the European scale, global F_{ST}-values were equal to 0.153 (SE = 0.024) for the 13 diploid populations and 0.044 (SE = 0.008) for the 27 tetraploid populations, and global ρ_{ST}-values, computed assuming no inbreeding (i.e., F_{IS} = 0), were equal to 0.265 (SE = 0.042) for diploids and 0.154 (SE = 0.029) for tetraploids. Thus, both F_{ST}- and ρ_{ST}-values were higher for the diploid populations. This was also true when ρ_{ST}-values were computed assuming F_{IS} = 0.1, as observed within populations from the Belgian Ardennes.

For clarity, ratios $F_{ST}/(1 - F_{ST})$ computed over population pairs were averaged for a set of distance intervals (Fig. 6). Those ratios were significantly correlated with the logarithm of the distance for all types of comparisons. Slopes were equal to 0.043 (SE = 0.015; P = 0.043) for diploid-diploid comparisons, 0.035 (SE = 0.011; $P < 0.001$) for tetraploid-tetraploid comparisons, and 0.033 (SE = 0.013; P = 0.001) for diploid-tetraploid comparisons. As explained above, the randomization procedure provides a liberal test for the slope of diploid-tetraploid comparisons. However, this slope is nearly three times the value of its standard error obtained by jackknifing over loci, so that it is very likely that it is truly statistically significant. Thus, contrary to the pattern observed at a local scale, at the European scale diploid and tetraploid populations were more closely related to each other when they were geographically closer. It is also interesting to note that the $F_{ST}/(1 - F_{ST})$-values for within-cytotype comparisons at the regional scale in the Belgian Ardennes (populations on average 15 km apart) were much lower than those for the shortest distance class at the European scale (populations on average 80 km apart), whereas, for comparisons between cytotypes, values at the regional scale were similar to those observed for distances up to 1000 km (Fig. 6). This pattern also occurs when analyses are restricted to the five loci common to the local and European scales (results not shown).

Simulations

Figure 7 shows how variation in the intercytotype reproductive barrier affects values of $F_{ST}/(1 - F_{ST})$ for pairs of populations of the same or different cytotypes, assuming bidirectional gene flow between cytotypes. When cytotypes exchange genes to some extent, $F_{ST}/(1 - F_{ST})$-values for comparisons between cytotypes were correlated with the distance and they eventually reached the average values obtained for comparisons within cytotypes (Fig. 7). The distance at which between cytotype $F_{ST}/(1 - F_{ST})$-values reached the average within-cytotype values was larger with lower intercytotype gene flow, approximately equal to 100, 20, and four lattice units for an m_b/m_w ratio equal to 10^{-3}, 10^{-2}, and 10^{-1}, respectively (Fig. 7). In the absence of gene flow between cytotypes (results not shown), $F_{ST}/(1 - F_{ST})$-values for comparisons between cytotypes were higher than for comparisons within cytotypes and they were not correlated with the spatial distance. When simulations were run with unidirectional gene

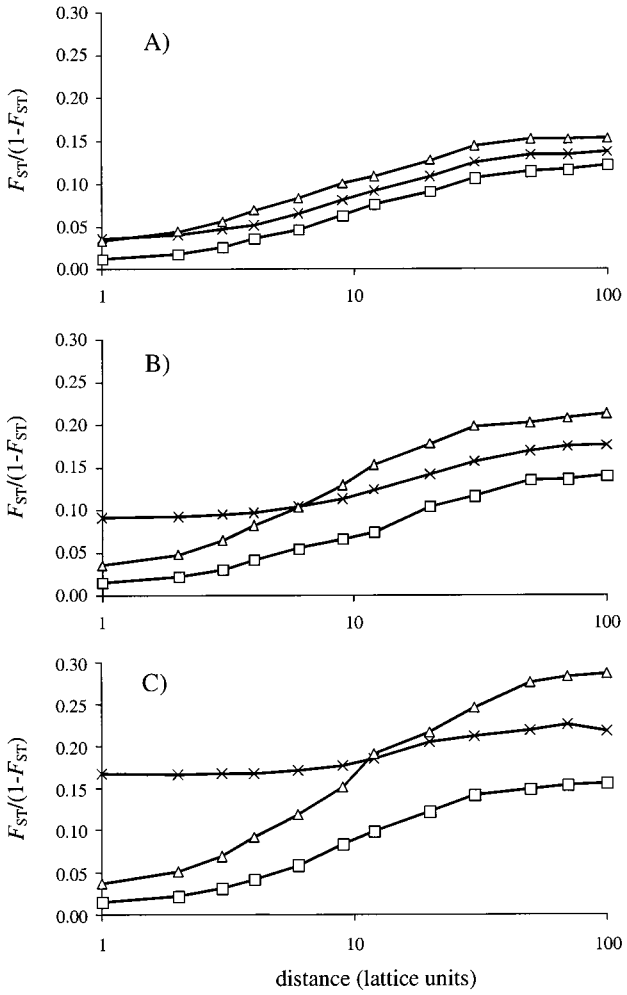

FIG. 7. Average $F_{ST}/(1 - F_{ST})$ ratios per distance classes (log scale) computed for pairs of populations of the simulation model for three m_b/m_w ratios with bidirectional intercytotype gene flow (see text): (A) 10^{-1}; (B) 10^{-2}; (C) 10^{-3}. Triangles refer to diploid-diploid population pairs, squares to tetraploid-tetraploid population pairs, crosses to diploid-tetraploid population pairs.

flow from diploids to tetraploids, as would occur under recurrent formation of tetraploids from diploid populations, a similar pattern was qualitatively observed (results not shown).

For a given distance, average $F_{ST}/(1 - F_{ST})$-values between diploid populations were always higher than values between tetraploid populations (Fig. 7). This pattern is expected because, for equal population sizes, tetraploid populations suffer less genetic drift. However, the difference is less pronounced when intercytotype gene flow is high (Fig. 7).

DISCUSSION

Genetic Diversity of Diploids and Tetraploids

Genetic diversity statistics were higher in tetraploid than in diploid knapweeds. The increased observed zygotic heterozygosity in tetraploids can be accounted for by the increased probability of having at least two different alleles with four homologous genes. But the increased number of alleles and gene diversity per population is not that trivial. Theoretical models based on a mutation-drift balance predict that genetic diversity should be higher in autopolyploids relative to their diploid counterparts because polysomic inheritance increases effective population size (Moody et al. 1993; Ronfort et al. 1998), and this expectation has been observed in several autopolyploid complexes (e.g., Grant et al. 1984; Lumaret and Hanotte 1987; Lumaret 1988b; Ness et al. 1989; Soltis and Soltis 1989; Lumaret and Barrientos 1990; Wolf et al. 1990; Shore 1991; Soltis and Soltis 1993; Hokanson and Hancock 1998; Mahy et al. 2000).

If diploids and tetraploids had similar population sizes, the total gene diversity in tetraploids would be expected to reach $2H_{T(2x)}/(1 + H_{T(2x)})$, where $H_{T(2x)}$ is the total gene diversity in diploids (Moody et al. 1993). Removing the *Est* locus (not scored in tetraploids), the total gene diversity in diploids from the Belgian Ardennes was 0.31, so that the expected value in tetraploids amounts to 0.48, whereas a value of 0.39 was observed. Similarly, using the data at the European scale, $H_{T(2x)} = 0.30$, which results in an expected value for tetraploids of 0.46, but the observed value is 0.32. Within the contact zone of the Belgian Ardennes, no differences in population sizes between diploids and tetraploids were noticed (O. J. Hardy, pers. obs.). However, at the European scale, as tetraploids are more common and widespread than diploids (Gardou 1972; Sommer 1990), total metapopulation size for tetraploids is probably substantially higher than for diploids, so that we would rather expect higher gene diversities in tetraploids under mutation-drift equilibrium. Several causes may explain why gene diversity in tetraploids is not as high as expected, for example, differences in current or past population sizes, a convergence of genetic diversity due to hybridization, or the slow rate at which mutation-drift equilibrium is reached, assuming the tetraploids are fairly recent. However, care must be taken in such interpretation given the limited number of loci and populations investigated.

Spatial Patterns of Genetic Variation within Cytotypes

In the Belgian Ardennes, population differentiation and spatial genetic structure within populations were similar for diploids and tetraploids when using appropriate statistics based on the coefficient on relationship (ρ). Assuming the observed genetic structures are representative of migration-drift equilibrium for both cytotypes, this result suggests that diploids and tetraploids have similar gene dispersal capabilities. As noted previously, two morphological characters that may be involved in gene dispersal capabilities distinguish diploids and tetraploids from the contact zone investigated: ray flowers occur only on tetraploids, and pappus are more developed in diploids (Hardy et al. 2000). Thus, there is no evidence that those differences had a substantial impact on the extent of pollen and seed dispersal. Higher F_{ST}-values for diploids have been observed in other polyploid complexes (e.g., Shore 1991; Petit et al. 1997; but see Mahy et al. 2000), and we show that in knapweeds this pattern could be accounted for by the reduction in pressure of genetic drift due to polysomic inheritance (Moody et al. 1993; Ronfort et al. 1998).

At the European scale, using the ρ_{ST}-statistic, diploid populations were more differentiated than tetraploid ones. Assuming that they have similar dispersal capabilities, this may result from a more scattered distribution of diploid populations, which was indeed observed at the European scale (Gardou 1972; Sommer 1990) but not at the local scale (Fig. 1; Hardy et al. 2000). Care must be taken, however, in such interpretation because, on a large geographic scale, a genetic structure may necessitate a very long time to reach its equilibrium (Whitlock and McCauley 1999), so that historical factors such as the pattern of past colonization are likely to be most determining (Ibrahim et al. 1996).

Evidence for isolation by distance among populations was not detected at the regional scale but appeared clearly at the European scale. It is interesting to note on Figure 6 that the $F_{ST}/(1 - F_{ST})$-values obtained at the local scale with our dataset are within the range of the ones obtained at the European scale with the dataset of Sommer (1990), which makes sense because isolation-by-distance models predict an approximate linear relationship with the logarithm of the distance (Rousset 1997). This pattern also occurred when analyses were restricted to the five loci common to the two datasets.

Within populations, isolation by distance was observed for both cytotypes and may account for the observed F_{IS}-values through biparental inbreeding. Indeed, kinship coefficients between close individuals (i.e., separated by the first distance intervals) were of the same order of magnitude as the observed inbreeding coefficients (Fig. 3). This is expected for an obligate outbreeder if most pollen grains travel distances included in the first distance intervals.

Reproductive Isolation between Diploids and Tetraploids

The distribution of total gene diversity between cytotypes and among populations and the spatial correlations of allele frequencies between cytotypes gave contrasting results depending on the geographic scale considered. At a local scale, the proportion of gene diversity distributed between cytotypes is substantially larger than that distributed among populations within each cytotype, and allele frequencies were not spatially correlated between cytotypes, suggesting that the latter are reproductively isolated. At the European scale, the reverse pattern occurs, and we checked that differences between the local and European scales in the array of loci investigated was not responsible for this contrast.

Our simulations suggest that the contrasting patterns of population differentiation at different scales could be accounted for by the combination of isolation by distance within cytotypes, on the one hand, and strong but not complete reproductive barriers between cytotypes and/or rare but recurrent formation of tetraploids from diploids on the other hand. Indeed, results shown on Figure 6 for knapweeds are qualitatively similar to those obtained by simulation for a ratio m_b/m_w of 0.01 (Fig. 7B). A sufficient intercytotype reproductive barrier allows cytotypes to differentiate substantially on a local scale, so that cytotypes can behave like separate species. But, on a large scale, isolation by distance is stronger than the intercytotype reproductive barrier, so that cytotypes appear like one species with a common gene pool. In the Belgian Ardennes, diploids and tetraploids are highly differentiated, so that this contact zone is certainly not a primary one (i.e., where tetraploids would have originated from local diploids; Petit et al. 1999), but a secondary contact zone. Actually, most diploid-autopolyploid contact zones described in the literature are thought to be secondary ones (Petit et al. 1999). Thus, although we have genetic data for only one contact zone, it might well be representative of most contact zones between diploid and tetraploid knapweeds. Further studies of other contact zones elsewhere in Europe are necessary to confirm this view.

Other polyploid complexes might show similar scale effect on the patterns of differentiation: On a small geographic scale, Petit et al. (1997) found higher population differentiation between cytotypes than within cytotypes, whereas, on a large geographic scale, Ness et al. (1989) and Soltis and Soltis (1989) found similar levels of population differentiation within and between cytotypes. Thus, partial barriers to intercytotype gene flow may explain the complexity of variation patterns in knapweeds and in polyploid complexes in general.

Intercytotype gene flow may occur through intercytotype crosses (e.g., via the fertilization of a tetraploid by unreduced gametes from a diploid, causing unidirectional gene flow, and/or via backcrosses between a hybrid triploid and one parental cytotype, which can cause bidirectional gene flow; Ramsey and Schemske 1998) or through the recurrent formation of tetraploids (i.e., multiple origin; Soltis and Soltis 1993), which causes unidirectional gene flow only. Under the latter hypothesis, a sufficiently large number of neotetraploids must have been formed to explain that all alleles found in diploid knapweeds were present in tetraploids. Moreover, neotetraploids must have evolved in different places throughout Europe to explain the spatial correlation in allele frequencies between diploid and tetraploid knapweed populations. Simulations suggest that distinction between different modes of intercytotype gene flow is difficult to achieve with current data, but we can at least rule out the alternative hypothesis that tetraploids have evolved in a single or a few instances. This adds to the growing body of evidence that multiple origins of polyploids and/or genetic exchanges among ploidal levels is the rule rather than the exception (Soltis and Soltis 1993, 1995, 1999, 2000).

In the contact zone of the Belgian Ardennes, the two cytotypes do not seem to hybridize, despite showing little habitat differentiation in this area and having widely overlapping flowering periods (Hardy et al. 2000). Moreover, pollinator behavior does not seem to cause assortative mating (Sommer 1990; O. J. Hardy, pers. obs.). Therefore, we suspect that other isolation mechanisms are operating (e.g., triploid block; Petit et al. 1999). This is supported by the significantly lower seed set and germination rates obtained for intercytotype pollination relative to intracytotype pollination in controlled crosses (Gardou 1972; Hardy et al. 2001).

Contrary to what has been observed with *Arrhenatherum elatius* (Petit et al. 1997), there was no increase in apparent selfing rate (F_{IS}-values) in the mixed populations relative to the pure ones. Thus, there has been no breakdown of the self-incompatibility system in response to low hybrid fitness (Petit et al. 1999). Actually, within the mixed populations, a ten-

dency for spatial clustering of identical cytotypes was detected and was probably due to limited seed dispersal (Felsenstein 1975). Because pollen dispersal distances are also likely to be limited, knapweeds being insect pollinated, this clustering should reduce intercytotype pollination. This kind of spatial segregation driven by limited seed dispersal may promote reproductive isolation in diploid-polyploid contact zones and thus facilitate the coexistence of different cytotypes by limiting the impact of the minority cytotype exclusion principle (Levin 1975; Van Dijk and Bijlsma 1994; Husband 2000). The efficiency of such a mechanism was indeed demonstrated in a natural population of *Chamerion angustifolium*, where a deficiency of intercytotype pollinator flights was due largely to the combined effect of limited pollinator flight distances and a tendency for spatial clustering of identical cytotypes (Husband and Schemske 2000).

Contrasting Reproductive Barrier to Isolation by Distance

The approach we used to detect intercytotype gene flow was to contrast the effect of reproductive barriers between cytotypes and isolation by distance within cytotypes on population genetic structure. In this way, simulations have shown that a very low level of intercytotype gene flow could be detected (Fig. 7C), provided that the geographic scale is large enough to ensure that intracytotype gene flow between remote populations is less than intercytotype gene flow.

Such an approach is not restricted to the analysis of polyploid complexes. Any complex with two distinct groups (e.g., morphotypes, ecotypes) that are suspected to exchange genes at a low rate could be analyzed in a similar way (e.g., Vekemans and Lefebvre 1997). An important requirement is that samples of each group show similar geographic distributions; this ensures that for all types of pairwise comparisons (i.e., within and between groups), a full range of geographic distances between samples is present. Actually, Rousset (1999) has modeled analytically genetic differentiation among populations for organisms showing separate ecological types, or source-sink behavior. His conclusions are very consistent with our results. He showed indeed that, under isolation by distance, strong but incomplete reproductive isolation between ecotypes can cause substantially higher population differentiation between rather than within ecotypes for nearby populations, but, for geographically distant populations, differentiation between ecotypes reaches the average within-type differentiation. Rousset (1999) also pointed out that *F*-statistics and gene diversity information provide little insight into the source-sink dynamics and thus into the symmetrical or asymmetrical nature of gene flow.

Acknowledgments

This work was supported by grant 2.4512.97 from the Belgian National Fund for Scientific Research (FNRS), where OH is a research assistant. We are very grateful to M. De Loose and H. Carlier from the Centrum Landbouwkundig Onderzoek in Melle for carrying out flow cytometry analysis and to J. Ochsmann, who sent us a copy of Sommer's thesis. We also thank P. Meerts, J. Lambinon, as well as two anonymous reviewers and the associate editor for their helpful comments on previous drafts of the manuscript.

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Corresponding Editor: M. Dudash