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High selfing and high inbreeding depression in peripheral populations of *Juncus atratus*

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Abstract

The mating system of a plant is the prime determinant of its population genetic structure. However, mating system effects may be modified by postzygotic mechanisms like inbreeding depression. Furthermore, historical as well as contemporary ecological factors and population characteristics, like the location within the species range can contribute to genetic variability. Using microsatellite markers we assessed the population genetic structure of the wind-pollinated *Juncus atratus* in 16 populations from peripheral and nearly central areas of the distribution range and studied the mating system of the species. In three peripheral populations, outcrossing rates at seeds stage were low (mean $t_m = 5.6\%$), suggesting a highly autogamous mating system. Despite this fact, on adult stage both individual heterozygosity (mean $H_O = 0.48$) and gene diversity (mean $H_E = 0.58$) were high even in small populations. Inbreeding coefficients were consistently low among all populations (mean $F_{IS} = 0.15$). Within the three peripheral populations indirect estimates of lifetime inbreeding depression were surprisingly high ($\delta_{eq} = 0.96$) and inbreeding depression could be shown to act mostly on early seedling establishment. Similar conditions of autogamy combined with high inbreeding depression are typical for plants with a large lifetime genomic mutation rate that cannot avoid selfing by geitonogamy. However, the results presented here are unexpected for small-statured, herbaceous plants. Substantial genetic differentiation among all populations was found (mean $F_{ST} = 0.24$). An isolation-by-distance pattern was apparent on large scale but not on local scale suggesting that the overall pattern was largely influenced by historical factors, e.g. colonization, whereas locally genetic drift was of greater importance than gene flow. Peripheral populations exhibited lower genetic diversity and higher inbreeding coefficients when compared with subcentral populations.

Keywords: genetic differentiation, *Juncus*, mating system, microsatellites, outcrossing rate, wind-pollination

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Introduction

Gene flow among populations and the maintenance of genetic diversity within populations are key factors determining the genetic structure of plant populations. Due to their sessile nature, plants are prone to genetic differentiation as a result of spatial isolation followed by reduced gene flow among individuals, populations, and across the species range. Moreover, genetic variation and population structure reflect both the influence of current ecological and historical processes. It has been suggested that life form and the mating system in particular, which governs the transmission of genes between generations, are the most significant factors affecting the pattern of genetic diversity of plant populations (Loveless & Hamrick 1984; Hamrick & Godt 1996; Glemin *et al.* 2006). Selfing within a population reduces pollen migration among individuals and thus can reduce effective population size. Also, gene flow among populations will be reduced. In inbreeding species individuals are expected to be more homozygous and, compared to outbreeding species, a larger proportion of genetic diversity should be partitioned among the populations.

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The wind-pollinated genus *Juncus* (Juncaceae) comprises more than 300 herbaceous species (Kirschner *et al*. 2002) which have enormous ecological importance mostly in wetland habitats. Nevertheless, mating system, genetic diversity and population structure have not yet been thoroughly analysed in *Juncus* species yet. *Juncus* spp. produce a large number of seeds per capsule which is uncommon for wind-pollinated species and may indicate self-fertilization (Proctor *et al*. 1996). In fact, Buchenau (1890, 1892; p. 378) stated self-fertilization as 'very frequent and successful' in the genus which is anecdotally confirmed for some *Juncus* spp. (e.g. Graebner 1934; Edgar 1964). However, no outcrossing rate estimates are available for species of the genus. For the black rush, *Juncus atratus*, high levels of observed heterozygosity and a low inbreeding coefficient have been reported recently (Michalski *et al*. 2006). Only two facts may account for these results. Either the species is more outcrossed than previously believed for the genus, or despite high selfing, the detrimental effects of inbreeding depression (ID) on selfed progeny maintain observed heterozygosity and a low inbreeding coefficient in the adult generation.

In homozygous individuals, deleterious recessive alleles can be expressed which are expected to cause ID (Charlesworth & Charlesworth 1999; Roff 2002; Carr & Dudash 2003). In frequently outbred species, this will lead to a lowered survival of selfed offspring compared to outcrossed offspring. On the other hand, in species with repeated self-fertilization, purging effects may eventually lead to decreased ID (Crnokrak & Barrett 2002). In a population that is already partially selfing, this mechanism facilitates the spread of an allele that increases selfing. Hence, most theory on the evolution of mating systems in plants predicts that species would be either predominantly outcrossing with strong ID ($\delta > 0.5$) or predominantly selfing with weak ID ($\delta < 0.5$) (Lande & Schemske 1985). However, Charlesworth & Charlesworth (1987) pointed out that if ID is caused by partially recessive, mildly deleterious alleles at many loci, even highly selfed populations will only purge a fraction of their genetic load. Also, a sufficiently high genome-wide mutation rate to recessive lethal alleles can maintain severe ID in populations with substantial levels of selfing (Lande *et al*. 1994; Scofield & Schultz 2006).

Surveys dealing with the distribution of outcrossing rates support the view of bimodality in mating systems (e.g. Schemske & Lande 1985; Barrett & Eckert 1990). However, significant differences in the distributions of outcrossing rates between wind-pollinated and animal-pollinated plants were found which have been a source of controversy for the last 20 years (e.g. Aide 1986; Goodwillie *et al*. 2005). Whereas for wind-pollinated species, as predicted, outcrossing rates show a bimodal distribution, animal-pollinated species more frequently exhibit a mixed mating system (Vogler & Kalisz 2001; but see Igic & Kohn 2006). This difference has been explained by the variability in biotic pollination which may limit the evolutionary response to selection on the mating system. Alternatively, wind-pollinated taxa might lack factors that provide evolutionary stability for mixed mating systems (Goodwillie *et al*. 2005). Also, it has been argued that the difference may be a result of biased sampling, since in the surveys of outcrossing rates wind-pollinated species are represented mainly by either annual, weedy, selfing grasses or by outcrossing conifers. Hence, a wider sampling including more wind-pollinated herbaceous plants may reveal additional abiotic mixed mating systems (Barrett & Eckert 1990).

Together with mating system and inbreeding depression, the genetic structure of populations is also shaped by geographical distance to other populations, habitat suitability or by the historic colonization and migration pattern (Pannell & Dorken 2006). The quality and impact of these factors are likely to be heterogeneous across the species range due to several reasons. In the periphery of a species range, abiotic and biotic environments may differ from those in the centre and it is likely that there are less suitable habitats. Hence, it is generally assumed that population frequency, population size and density are decreasing in the periphery (Brown 1984; Lawton 1993). Smaller and less dense populations are more prone to the consequences of founder effects, inbreeding and genetic drift which are expected to result in a lower genetic diversity (Barrett & Kohn 1991; Ellstrand & Elam 1993; Leimu *et al*. 2006). Increased spatial isolation between peripheral populations will reduce gene flow between populations which in turn causes also higher genetic differentiation among these populations.

In European species, historical effects can also contribute to the lowered genetic diversity at the periphery of a species range. For plants with the ability for long-distance dispersal, postglacial range expansion from glacial refugia may have occurred rapidly. However, the repeated founding events along colonization routes could have lead to decreased heterozygosity in peripheral populations compared to that of populations closer to refugia (Hewitt 1996). Although most of the allelic variation may reside in central populations, peripheral, disjunct populations can contribute to genetic variation at the species level as they often harbour distinct genotypes which make them important for potential adaptation and for conservation (Lesica & Allendorf 1995, e.g. Eckstein *et al*. 2006). Eventually, the spatial pattern of plant population genetic structure may be superimposed by contemporary anthropogenic effects. The human impact may evoke habitat fragmentation and alteration of the natural habitat also in the centre of a species distribution leading to similar effects on genetic diversity as expected in the periphery of a species' range (Young *et al*. 1996).

Table 1 Geographical position and estimated population size of sampled populations and measures of genetic diversity at eight microsatellite loci: N, number of samples analysed; A, mean number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient (**$P < 0.001$, *$P < 0.05$) and number of private alleles

Population ID	Geographical origin	Location coordinates	Estimated population size	N	A	H_O	H_E	F_{IS}	Private alleles
GGUK	Germany, Parey	52°42′N, 12°13′ E	200	24	4.03	0.464	0.562	0.158**	2
GGUS	Germany, Gülpe	52°44′ N, 12°13′ E	7	7	2.50	0.357	0.434	0.178**	0
GHAV	Germany, Havelberg	52°49′ N, 12°05′ E	150	24	4.07	0.484	0.614	0.189*	1
GLEN	Germany, Lennewitz	52°54′ N, 11°57′ E	20	14	3.76	0.441	0.590	0.492**	1
GPRZ	Germany, Pritzerbe	52°30′ N, 12°26′ E	250	24	3.51	0.490	0.554	0.214**	0
GSAN	Germany, Sandau	52°48′ N, 12°02′ E	200	24	3.27	0.250	0.487	0.260**	1
GSCH	Germany, Schartau	52°17′ N, 11°47′ E	350	24	2.83	0.379	0.451	0.186**	1
GVEH	Germany, Vehlgast	52°49′ N, 12°11′ E	300	24	3.54	0.443	0.541	0.163**	1
SMAK	Slovakia, Malacky	48°33′ N, 17°00′ E	2500	24	4.02	0.490	0.634	0.056	2
HBAT	Hungary, Battonya	46°16′ N, 21°06′ E	100	15	5.26	0.647	0.684	0.053*	5
HDAR	Hungary, Darvas	47°07′ N, 21°20′ E	200	11	5.28	0.602	0.694	0.172**	3
HGYO	Hungary, Gyomaendröd	47°02′ N, 20°51′ E	500	13	3.26	0.525	0.521	0.119*	0
HSAT	Hungary, Satoraljaújhely	48°22′ N, 21°37′ E	1500	24	5.30	0.630	0.664	–0.010	9
HSZE	Hungary, Szentes	46°46′ N, 20°26′ E	500	24	3.83	0.435	0.540	0.199**	3
POLG	Poland, Góry	52°22′ N, 22°39′ E	350	24	3.23	0.469	0.523	0.123*	2
UUKR	Ukraine, Uschgorod	48°39′ N, 22°18′ E	25	17	4.97	0.615	0.727	0.105*	4

Thus, the interpretation of the population genetic structure of a species has to take into account several factors: (i) the mating system which determines both gene flow and genetic variability at individual and local scales; (ii) postzygotic mechanisms like inbreeding depression that can counteract the effects of selfing on genetic variability at the individual level; and (iii) contemporary ecological and historical aspects which may vary across the species range.

In this study, we provide a comprehensive analysis of the genetic population structure of the herbaceous, wind-pollinated *Juncus atratus* using nuclear microsatellite markers. The black rush, *J. atratus* (Juncaceae), is a diploid ($2n = 2x = 40$), perennial species distributed from central Asia to subcontinental Europe. It reaches its northwestern range edge in Central Europe, where it grows exclusively in wet meadows and open soils with few populations along the floodplains of the rivers Elbe and Havel (Burkart 1995; river corridor plant, Burkart 2001). Flooding dynamics of river systems of Central Europe have been strongly altered by human impact (Müller 1995). Thus, in Central Europe, suitable habitats for *J. atratus*, i.e. seasonal flooded meadows along river plains, and hence, the species itself is highly endangered (Schnittler & Günther 1999).

We first characterize the mating system of *J. atratus* by analysing the outcrossing rate from maternal seed families. As literature suggests a high selfing rate, ID might account for the high observed heterozygosity and the low inbreeding coefficient previously found for one population of the species. Hence, we secondly assess ID by comparing the change in inbreeding coefficients between different life stages in the field and in the laboratory.

Selfing should lower effective population size and pollen flow among individuals and populations. This in turn, can increase genetic differentiation among populations caused by drift. Thus, we eventually determine genetic variation within and among populations and compare the population genetic structure between peripheral and subcentral populations. We hypothesize lower population size, lower genetic diversity and increased differentiation among peripheral populations.

Methods

Sampling and genotyping

Juncus atratus individuals were sampled from 16 natural populations (Table 1). Because localities where the species was sampled were coherent and clearly delimited spatially, all plants growing at a particular site were defined as one population. The area of single populations ranged from ~5 m^2 up to 7×10^4 m^2. Populations were situated in nearly central parts of the distribution range in southeastern Europe (subcentral populations) up to the northwestern range edge (peripheral populations; Table 1, Fig. 1). Here, the frequency of known populations is very low. The sampled German populations even represent all known occurrences of *J. atratus* in this country and are the most western populations of the species. In the comparative analysis of central and peripheral populations, the German and Slovakian populations (Fig. 1, nos 1–9) were treated as peripheral, and the Hungarian, Ukrainian and Polish populations (nos 9–16) were treated as subcentral. Leaf

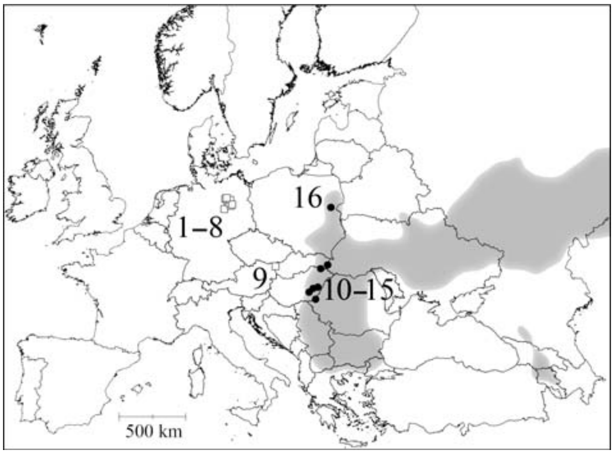

Fig. 1 Map of populations sampled for this study. 1, GGUK; 2, GGUS; 3, GHAV; 4, GLEN; 5, GPRZ; 6, GSAN; 7, GSCH; 8, GVEH; 9, SMAK; 10, HBAT; 11, HDAR; 12, HDAR; 13, HGYO; 14, HSZE; 15, UUKR; 16, POLG. The first letter of the population code refers to the country of origin: G, Germany; S, Slovakia; H, Hungary; U, Ukraine; P, Poland. Circles represent populations treated as subcentral populations. Open squares represent peripheral populations. The grey-shaded area represents the main distribution of *Juncus atratus* in Europe (based on a distribution map by Eric Welk, unpublished). The main distribution of the species reaches as far as Southwest Siberia and Northwest China.

tissue was sampled between 2003 and 2005 from seven to 24 (mean = 19.8) randomly chosen individual plants per population and preserved in silica gel. During sampling, population size was estimated as the approximate number of distinct tufts. Habitat conditions and sizes of habitat patches were found to be very inhomogeneous for both peripheral and subcentral populations.

DNA was extracted with the DNeasy 96 Plant extraction kit (QIAGEN). Samples were genotyped at eight microsatellite loci previously developed for *J. atratus* (Michalski *et al*. 2006). Multiplex polymerase chain reactions (PCR) were performed in a total volume of 15 μL. Conditions for the amplification and detailed primer descriptions follow Michalski *et al*. (2006), except for the multiplexing reaction which comprised the loci Ja29 (Label: FAM), Ja28 (JOE), Ja31 (TAMRA) in one, and loci Ja42 (FAM), Ja07 (FAM), Ja01 (JOE), Ja47 (JOE) and Ja21b (TAMRA) in another PCR amplification. PCR products were separated on an ABI 310 genetic analyser (Applied Biosystems) with internal size standard MapMarker 400 (BioVentures). Individuals were genotyped using GENOTYPER version 2.0 (Applied Biosystems).

Estimation of outcrossing rate and inbreeding depression

Open-pollinated seed families were collected haphazardly from three German populations (GPRZ, GSAN, GVEH, Table 1). Seeds of subcentral populations were not available at the time of sampling. By analysing seeds rather than seedlings, possible biases resulting from early inbreeding depression during germination and seedling establishment can be avoided. However, the primary selfing rate at fertilization might be higher than our estimates due to selective postzygotic seed abortion.

Individual seeds were crushed with a glass rod on plates with shallow slots and dissolved in 6 μL 10× PCR buffer with $(NH_4)_2SO_4$ (Fermentas). After 10 min of denaturation at 95 °C, the mixture was held at 4 °C for 4–7 days to allow the DNA to dissolve. For the microsatellite analysis, 1 μL of the extract was used in each PCR amplification. The maternal genotype did not interfere with the signal from the seeds. Multilocus and single-locus outcrossing rates (t_m, t_s) and parental inbreeding coefficients (F) were estimated using MLTR version 3.1 (Ritland 2002). For populations GPRZ and GSAN, the maternal genotype of seed families was known, whereas for GVEH, it was inferred from the progeny arrays. We used iterations of the Newton-Raphson method with predefined starting values of $t_m = 0.9$ and $F = 0.1$ to obtain maximum-likelihood estimates of the mating parameters.

Inbreeding depression ($\delta = 1$ — relative fitness of selfed progeny) was quantified indirectly using two approaches. First, we used parental *F*-values and outcrossing rates to quantify Ritland's equilibrium estimator of inbreeding depression (Ritland 1990a, equation 3), assuming that populations are at inbreeding equilibrium: $\delta = 1 - [2tF/(1 - t)(1 - F)]$. ID assuming inbreeding equilibrium was calculated for the three populations GPRZ, GSAN and GVEH and is depicted as δ_{eq}. Ninety-five per cent confidence intervals of all mating system parameters were reported based on 1000 bootstrap replicates with the progeny array as the unit of resampling.

Second, ID was quantified by comparing maternal and seedling *F*-values through time. Assuming random outcrossing at rate *t* and no selection against inbred individuals, the offspring inbreeding coefficient F_i is related to the adult F_{i-1} as $F_i = (1 - t)(1 + F_{i-1})/2$ (Ritland 1990a). ID should counterbalance this relationship by decreasing the relative fitness of the selfed offspring. The relative fitness parameter *w* can be estimated according to Ritland (1990a, equation 1): $w = 2tF_i/(1-t)(1 + F_{i-1} - 2F_i)$. By replacing t, F_i and F_{i-1} with their estimates (i.e. t_m, $F_{IS\ seedlings}$ and $F_{IS\ parents}$) an estimate for the ID ($\delta = 1 - w$) up to this point can be derived. We quantified ID in seedlings of *J. atratus* using this approach first in the field and second under controlled conditions without competition in the laboratory. For the field estimate of ID, all putative mother individuals in an area of 15 × 40 m in the population of Sandau (GSAN) were sampled. This population was chosen because seedlings which are only rarely encountered in the field were present in large numbers at this location. Seedlings within the same area were mapped and collected using quadrants of 0.04 m^2. Up to four seedlings were randomly sampled per quadrant.

Plant material was collected in August, at which time seedlings were 3 to 4 months old and between 2 and 18 cm high. Thus, at this time, seedlings were already established under conditions of competition in the natural habitat. DNA extraction of seedlings was performed using the protocol of Edwards *et al*. (1991) modified for small-scale extraction. DNA extraction of adult plants and microsatellite analysis were done as described above. Genotypes were obtained from a total of 131 seedlings and 73 adults. Inbreeding coefficients (F_{IS}) were computed for the seedlings as well as for the adult generation.

For the laboratory, estimate of ID seedlings were raised in early spring from all 23 seed families used for the initial estimation of the outcrossing rate in the population of Pritzerbe (GPRZ). Twenty-four seeds of each seed family were first germinated individually in PCR plates filled with sand and then kept in a growth chamber. After 3 months, seedlings were repotted into larger pots that were kept at outdoor conditions. Surviving plants were genotyped 6 and 12 month after sowing and inbreeding coefficients were obtained. All F_{IS} values were computed using MSA software version 3.12 (Dieringer & Schlötterer 2003) and averaged over loci. Inbreeding coefficients of adults and offspring were considered to be not significantly different when the F_{IS} value of the adults fell within the 95% confidence interval of the seedlings' inbreeding coefficient obtained from bootstrapping the offspring data 100 times. ID at the seedling stage (δ_{seedl}) was quantified using equation 1 of Ritland (1990a). As t estimates, we used the multilocus outcrossing rates t_m obtained for the respective population. Ninety-five per cent confidence intervals for the ID δ_{seedl} were computed from 100 δ-values obtained by using a constant parental F_{IS}, the F_{IS} values of bootstrapped seedling populations and a randomly assigned t_m bootstrap sample of the corresponding population. Only those loci were included in the analyses which were used for the estimation of the outcrossing rate of the respective population.

Genetic variation and genetic population structure

Genetic diversity at population level was characterized by calculating observed (H_O) heterozygosity and expected heterozygosity (H_E, corrected for sample size) using the software MSA version 3.12 (Dieringer & Schlötterer 2003). Allelic richness (A), calculated by a rarefaction method, inbreeding coefficients (F_{IS}) and their significance levels were all calculated by FSTAT version 2.9.3 (Goudet 2001). Private alleles (A_P) were assessed using GDA version 1.1 (Lewis & Zaykin 2001).

We compared genetic diversity parameters between peripheral and subcentral populations. Significance was evaluated by t-tests and individual P values were evaluated applying a Bonferroni correction to account for the interrelation of the measures. To simultaneously control for possible effects of population size, we also used ANCOVA with population size as covariable using the Akaike information criterion (AIC) for model simplification (Crawley 2007). To test for population subdivision among the populations, pairwise F_{ST} values (Wright 1951), equivalent to Weir and Cockerham's θ (Weir & Cockerham 1984), were calculated using FSTAT version 2.9.3 (Goudet 2001).

The analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) was carried out using ARLEQUIN version 2.000 (Schneider *et al*. 2000). To test for a possible geographical structure of genetic variability over the sampled area, populations were grouped into two regions prior to AMOVA. All German populations formed one group, the Hungarian and the Ukrainian populations a second one. The geographically intermediate Slovakian and Polish populations were not included in this analysis because of their large geographical distance to either group. To test whether genetic differentiation was higher among German populations than in Hungarian and Ukrainian populations differences among mean pairwise F_{ST} values were evaluated using a randomization procedure with 1000 permutations implemented in FSTAT version 2.9.3 (Goudet 2001).

Assuming a stepping-stone model of population structure the null hypothesis that populations are at equilibrium between gene flow and drift can be rejected if (i) pairwise genetic and spatial distances are unrelated and/or (ii) the relationship found fails to be positive and monotonic over all distances (Hutchison & Templeton 1999). Also, under equilibrium conditions, the degree of scatter is expected to increase with geographical distance. Thus, to test for isolation by distance, pairwise F_{ST} values were related to corresponding geographical distances. As a measure for the scatter the absolute values of the residuals obtained from a standard linear regression of genetic distances (F_{ST}'s) on geographical distances were correlated with geographical distances (Hutchison & Templeton 1999). Spearman's rank correlations between pairwise genetic and geographical distances and between the degree of scatter and geographical distances were done for the global scale including all studied populations as well for a more local scale using (i) all German populations and (ii) the Hungarian and Ukrainian populations. The Ukrainian population was situated only 60 km afar from the next Hungarian population sampled, hence Hungarian and Ukrainian populations were treated as one group. Significance of all correlations was evaluated using Mantel tests running 10 000 permutations.

To visualize genetic relationships among the populations, pairwise chord distances (D_c) were calculated following the method of Cavalli-Sforza & Edward (1967). A tree was obtained by the UPGMA method and the statistical reliability of clusters was tested by bootstrapping the genotype frequency data set 1000 times with loci as sampling units.

Table 2 Parental inbreeding coefficient, multilocus outcrossing rate, and inbreeding depression in three populations of *Juncus atratus*. GPRZ, Germany, Pritzerbe; GSAN, Germany, Sandau; GVEH, Germany, Vehlgast. Note that for GPRZ and GVEH inbreeding depression did not differ significantly from unity

Population ID	Sample size *N* families/ total progeny	No. of loci	Parental *F* (95% CI)	Multilocus outcrossing rate t_m (95% CI)	Inbreeding depression δ_{eq} (95% CI)
GPRZ	23/491	2	0.233 (–0.200–0.509)	0.013 (0.001–0.032)	0.992 (0.956–1.001)
GSAN	70/1538	5	0.322 (0.227–0.437)	0.092 (0.054–0.135)	0.904 (0.805–0.955)
GVEH	5/236	8	0.065 (–0.200–0.273)	0.062 (0.039–0.090)	0.991 (0.932–1.021)
Mean			0.207	0.056	0.962

Results

Outcrossing rate and inbreeding depression

Outcrossing rates estimated from seed families collected in the field were low across the three German sites (mean $t_m = 5.6\%$, Table 2). Parental inbreeding coefficients (*F*) ranged between 0.065 and 0.322 and were significantly different from zero in one population (GSAN). Minimum estimates of apparent selfing due to biparental inbreeding ($t_m - t_s$) were found as: 0.003, 0.005 and 0.03 for populations GPRZ, GSAN and GVEH, respectively.

Low estimates for the outcrossing rate combined with low to moderate estimates of *F* indicate that although most of the seeds resulted from self-fertilization, surviving and reproducing plants were almost all outbred. Accordingly, based on the 95% confidence intervals, two of three equilibrium estimates of inbreeding depression did not differ significantly from unity (mean $\delta_{eq} = 0.962$, Table 2).

In the laboratory, of all seeds sown for the estimation of inbreeding depression from progeny arrays 8.7% (48 of 552) survived until genotyping at an age of 6 months and 2.4% (13 of 552) survived for 12 months until the following spring. In both the field and the laboratory population, seed F_{IS} values were higher than maternal F_{IS} and decreased with seedling age (Fig. 2). Seedlings almost reached maternal F_{IS} after 4 months in the field or equalled the maternal values after 12 months in the laboratory. However, only after 12 months in the laboratory, seedling F_{IS} differed significantly from that of the seeds. Inbreeding depression was estimated as $\delta_{seedl} = 0.821$ (95% CI 0.338–0.914) in the field population after four months, and 0.954 (CI 0.537–1.005) and 0.990 (CI 0.943–1.004) after 6 and 12 months in the laboratory population, respectively.

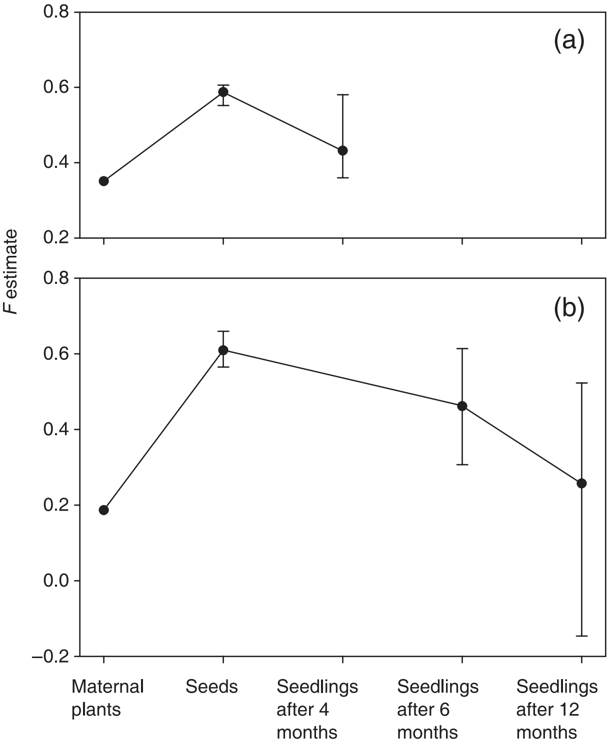

Fig. 2 F_{IS} values of maternal plants, seeds and seedlings under field (a) and laboratory (b) conditions. 95% confidence intervals for F_{IS} values of seeds and seedlings were obtained by bootstrapping the respective populations 100 times.

Genetic diversity at species and population level

In a total of 317 individuals and at eight microsatellite loci analysed, we identified 120 alleles (mean of 15 alleles per locus). The observed heterozygosity (H_O) and the expected heterozygosity (H_E) per locus ranged from 0.233 to 0.631 and from 0.285 to 0.788, respectively. The inbreeding coefficient F_{IS} (Weir & Cockerham 1984) varied from –0.192 to 0.469 per locus. Five of the eight loci had significant positive F_{IS} values (Ja07, Ja21b, Ja28, Ja31 and Ja47), indicating heterozygote deficiency. Genetic parameters at the population level are displayed in Table 1. Genetic diversity was found to be similar among all populations with a mean H_O (SD) of 0.483 (0.106) and a mean H_E of 0.576 (0.087). Across all sampled populations allele number, observed and expected heterozygosities were not significantly related with population size (data not shown). However, the inbreeding coefficient was negatively correlated with

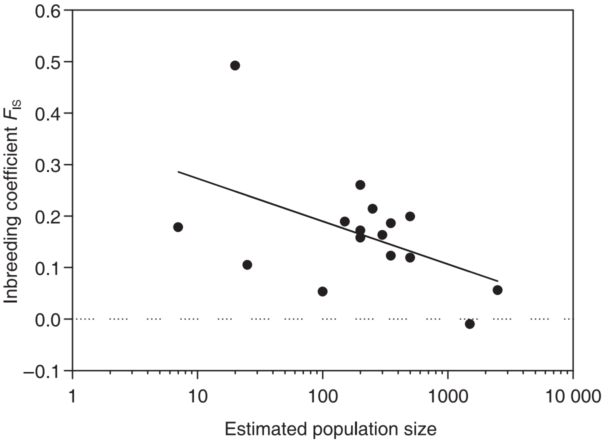

Fig. 3 Inbreeding coefficients F_{IS} plotted against estimated population size. Pearson's correlation $r = -0.489$, $P = 0.05$. The inbreeding coefficients of the two largest populations did not differ significantly from Hardy–Weinberg expectations.

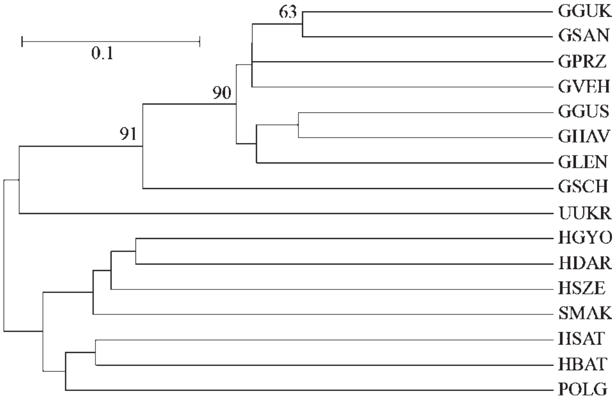

Fig. 4 UPGMA tree of 16 populations of *Juncus atratus* based upon Cavalli-Sforza & Edward's (1967) chord distances. Bootstrap values > 50% are displayed above the branches. The first letter of the population code refers to the country of origin: G, Germany; U, Ukraine; H, Hungary; S, Slovakia; P, Poland.

population size (Pearson correlation $r = -0.489$, $P = 0.05$). The two largest populations (HSAT, SMAK) were even found to be at Hardy–Weinberg equilibrium (Fig. 3).

Peripheral populations had lower parameters of genetic diversity than subcentral populations (Table 3). However, controlling the type I error rate by a Bonferroni correction, only H_O differed significantly, whereas for other parameters, significance was marginal (Table 3). The inbreeding coefficient was found to be higher in the peripheral populations, but also without statistical significance. Population size was not significantly different between the groups. As F_{IS} values were significantly correlated with population size, F_{IS} values were compared between peripheral and central populations by ANCOVA with population size as covariable. In the final model, after removing the interaction, both population size as well as location were retained with statistical significant effects on variability in F_{IS} values (adjusted $R^2 = 0.37$, with $t = -2.374$, $P = 0.03$ and $t = -2.374$, $P = 0.04$ for population size and location, respectively).

In a UPGMA phenogram of pairwise genetic distances (D_c) depicting the genetic relationships (Fig. 4), the peripheral German populations formed one distinct cluster supported by high bootstrap values. All other populations were tied by relatively long branches without clear groupings.

Population differentiation

An analysis of molecular variance (AMOVA, Table 4) revealed that the majority of genetic variation (71%) resided within the populations. The remaining variation was partitioned to nearly equal amounts among the two groups of peripheral (German) and subcentral (Hungarian/Ukrainian) populations (15%) and among the populations within the groups (14%). Similar results were obtained in an analysis in which all non-German populations formed the second group. Genetic differentiation among the 16 populations measured by pairwise F_{ST} values was found to be moderately pronounced [mean $F_{ST} = 0.243$ (SD = 0.094)] and significant in all but one comparison of two German populations (GGUS vs. GHAV, $F_{ST} = 0.043$, $P = 0.07$). Among peripheral German populations, the same genetic differentiation as among subcentral Hungarian and Ukrainian populations was found (mean $F_{ST} = 0.14$ (SD = 0.08) and

Table 3 Comparison (*t*-test) of genetic diversity of Juncus atratus at eight microsatellite loci between northwestern range edge (German, Slovakian, $n = 9$) populations and subcentral populations (Hungarian, Ukrainian, Polish, $n = 7$). Populations size was not significantly different between the groups (*t*-test $P = 0.97$). *Significant after Bonferroni correction

	Range edge populations	Subcentral populations	
	Mean (SD)	Mean (SD)	*t*-test *P*
Observed heterozygosity, H_O	0.42 (0.08)	0.56 (0.08)	0.005*
Expected heterozygosity, H_E	0.54 (0.07)	0.62 (0.09)	0.062
No. of alleles, *A*	3.50 (0.55)	4.45 (0.97)	0.027
No. of private alleles, A_P	1.00 (0.71)	3.71 (2.81)	0.014
Inbreeding coefficient, F_{IS}	0.21 (0.12)	0.11 (0.07)	0.065

Table 4 Analysis of molecular variance (AMOVA) displaying the genetic variation between the two groups of German and the Hungarian and Ukrainian populations, populations within the groups, and individuals (***$P < 0.001$)

Source of variation	d.f.	Sum of squares	Percentage of variation
Among groups	1	136.9	14.50***
Among populations within groups	12	231.4	14.25***
Within populations	524	1176.1	71.25***
Total	537	1544.4	

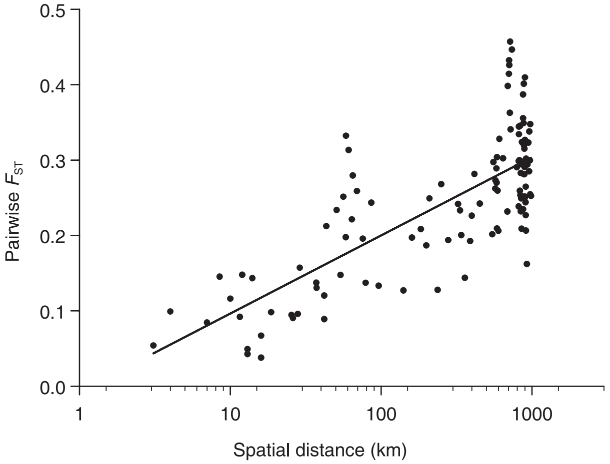

Fig. 5 Scatterplot of pairwise F_{ST} vs. spatial distances of all sampled populations of *J. atratus*. Spearman's rank correlation $r = 0.664$, Mantel $P < 0.001$.

mean $F_{ST} = 0.19$ (0.05), respectively; $P = 0.44$). Isolation by distance was tested by the association between pairwise genetic differentiation (F_{ST}) and pairwise geographical distances (Fig. 5). Correlations were analysed over all populations [$n = 16$, mean pairwise spatial distance 516.1 km (SD = 367.0 km)] and on more local scales for only the peripheral German populations [$n = 8$, 30.5 km (21.6 km)], or all Hungarian and Ukrainian populations [$n = 6$, 142.4 km (80.5 km)]. Across all populations, there was a significant positive correlation (Spearman's rank correlation $r = 0.664$, Mantel $P < 0.001$). For the peripheral German populations, a statistically supported positive correlation was also found ($r = 0.667$, $P < 0.05$), whereas no correlation was found in the Hungarian/Ukrainian populations ($r = 0.12$). The degree of scatter of pairwise F_{ST} values was not significantly related to geographical distance on both scales ($P > 0.19$).

Discussion

The main result of our study is the paradox of high selfing rates found in three German populations of *Juncus atratus* together with high levels of individual heterozygosity and gene diversity at population level across all populations. Selfing species are expected to exhibit much lower levels of individual heterozygosity and gene diversity than species with intermediate or high outcrossing rates. However, in all populations studied, the within-population genetic diversity (H_O and H_E) was in a similar range as for species with a mixed mating system (see Nybom 2004). Although generalizations for *J. atratus* as a whole cannot be derived by our study, the results for the three German populations could be explained by a very strong selection against inbred progeny. This fact is quite unexpected for an herbaceous species as we discuss in the following. We also assess the possible effects of the mating system on the population genetic structure of peripheral and subcentral populations of *J. atratus*.

Mating system

Juncus atratus develops elaborate inflorescences, and as in other *Juncus* species, its flowers are apparently adapted to wind-pollination. Although it is undisputed that wind is the pollen vector accomplishing the rare outcrossing events, self-fertilization was predominant in the three populations studied. Like other *Juncus* species, *J. atratus* exhibits no self-incompatibility mechanism and very low pollen/ovule ratios (P/O) which seem a common feature throughout the genus (Michalski, unpublished data). Low P/O ratios are very unusual for wind-pollinated species and are often associated with a high degree of autogamy (Cruden 1977). Hence, these morphological features substantiate the low outcrossing rate determined in *J. atratus* which is also in line with earlier observations and assumptions referring to the whole genus that self-fertilization might be prevalent (e.g. Buchenau 1892; Proctor *et al.* 1996).

Mating system estimates for *J. atratus* were only derived from German populations from the northwestern periphery of the species distribution. Lower values of observed heterozygozity (H_O) and the slightly higher inbreeding coefficients (F_{IS}) in peripheral populations may indicate a higher degree of inbreeding in peripheral than in subcentral populations. Differences in the mating system between populations may arise due to a number of facts. First, the mating system in plants can be strongly influenced by population characteristics which can differ throughout the species distribution. In self-compatible plants, population size and/or density can affect the outcrossing rate (Karron *et al.* 1995; Routley *et al.* 1999). However, these population characteristics were not different between studied peripheral and central populations. Second, pollination in anemophilous species should be favoured by certain environmental factors, such as dry conditions (Regal 1982; Ackerman 2000). However, it remains an open question whether environmental factors differ between peripheral and subcentral populations. Third, it is expected that individuals with higher selfing ability would have a selective advantage for the establishment of new sexually reproducing populations following founder events (Baker 1955). Hence, German populations may have been founded by more self-fertilizing individuals during colonization after postglacial range expansion.

Inbreeding depression

In the three populations in which the mating system was assessed, extremely low outcrossing rates were found ($t_m < 0.1$). These estimates are in sharp contrast to the high levels of individual heterozygosity and the low to moderate inbreeding coefficients in these populations. This discrepancy might indicate a strong selection against selfed offspring. In fact, estimates of inbreeding depression (ID) determined with the two methods resulted in similarly high values of ID close to unity.

First, the equilibrium estimator of Ritland (1990a) revealed high levels of ID due to low t_m and only moderate adult *F*-values. Although population estimates of ID are often charged with large errors (Ritland 1990a; see Eckert & Barrett 1994), we obtained very consistent estimates of ID in the three studied German populations. However, in the evaluation of the results, one has to take into account that the equilibrium estimate of ID is based upon several assumptions. (i) The populations are at inbreeding equilibrium. Thus, a bias would arise if the inbreeding coefficient of the population is currently increasing or decreasing (Eckert & Barrett 1994); (ii) The outcrossing rate (*t*) and the inbreeding coefficient (*F*) do not vary among years; and (iii) the inbreeding coefficient (*F*) is not influenced by the effects of genetic drift. However, the influence of deviations from the assumptions of Ritland's model generally appear to be small (Lynch & Walsh 1998). Studies that have compared experimental estimates of ID and indirect methods using Ritland's estimator often found lower ID in experimental approaches (Eckert & Barrett 1994; Kohn & Biardi 1995). However, glasshouse studies may often underestimate ID because plants are usually not followed to reproductive maturity or do not undergo severe episodes of selection that may occur in the field. Thus, although our sampling is far from being extensive, any existing bias by our approach is probably small. Even if assumptions (i) and (ii) were not met, the estimate of ID would be biased downwards only (Ritland 1990a; Eckert & Barrett 1994). Also, it has been stated that the use of genetic markers to estimate inbreeding depression is reliable only for populations with moderate levels of inbreeding (Ritland 1990a). However, despite of high selfing rates, all studied populations of *J. atratus* exhibited a genetic structure similar to mixed-mating populations. Inbreeding coefficients were in the range of the greatest power to estimate inbreeding depression by this approach (Ritland 1990b).

Second, ID was an effective factor selecting on seedling survival and early establishment as shown by the decrease of inbreeding coefficients between seeds and seedlings (Fig. 4). This timing seems reasonable as seedlings of rushes are very delicate and vulnerable to physical irritation. For seedlings of a number of *Juncus* species, a low competitive ability has been described (e.g. Lazenby 1955; Welch 1967; Eleuterius 1984). It has been recently shown that establishment is the limiting life stage in *J. atratus* which depends on disturbance by flooding events or by the creation of open soils (M. Burkart *et al.* in preparation). Also, at least in the German populations, natural conditions for germination and establishment of the offspring seem critical, since seedlings are only rarely encountered, although adults flower regularly.

In an earlier study of ID in four *Juncus*-species, only little evidence for inbreeding depression was found (Edgar 1964). The study examined morphological characters of adults raised from open-pollinated and selfed progeny. If the results for *J. atratus* are transferable to other *Juncus* species, then the failure to find evidence for ID may be explained by both the breeding system and early inbreeding depression. First, open-pollinated seeds are not necessarily outcrossed but may be highly selfed. Second, even if seed sources differed in the degree of outcrossing, early acting inbreeding depression at the germination and seedling-stage will level out most differences before reproductive maturity.

Strong selection against inbred individuals in combination with the maintenance of high levels of self-fertilization is hard to explain by traditional concepts of mating system evolution. If ID is caused by highly deleterious recessive alleles, purging should lower the genetic load as the selfing rate increases and, eventually, autogamy as an evolutionary stable strategy should be associated with low ID (Lande & Schemske 1985). Despite these predictions, a number of species practicing mixed mating or even high degrees of selfing are known which maintain substantial levels of ID (examples in Husband & Schemske 1996; Routley *et al*. 1999; Goodwillie *et al*. 2005).

How the magnitude of ID will evolve with the level of inbreeding, however, may also depend on the mutation rate, the effects of individual alleles and the degree of their expression in heterozygotes. For example, recessive mutations with only slightly deleterious effects or a low dominance coefficient may be an important component of ID that can be maintained even in highly inbred populations (Charlesworth *et al.* 1990; Charlesworth *et al.* 1991). Lande *et al.* (1994) suggested that both selective interference among loci with recessive deleterious mutations and a sufficiently high genomic lethal mutation rate can result in severe ID. If ID approaches unity, lacking variability in the fitness of selfed progeny may even prevent purging of the genetic load despite of high selfing (Lande *et al*. 1994). Scofield & Schultz (2006) hypothesized that the level of ID is associated with the life time mutation rate. Because plants in general do not produce a separate germ line, very high levels of ID ($\delta \approx 1$) should be more likely in long-lived, large-statured plants like trees. This assumption is consistent with the observation that early, severe ID appears to be more frequent in large-statured rather than in small-statured plants (Husband & Schemske 1996). Hence, severe ID and its expression in early life stages in an herbaceous, small-statured plant such as *J. atratus* are quite surprising. The joint maintenance of high ID and high selfing would rather be expected in trees and shrubs that cannot avoid substantial levels of geitonogamous selfing (example in Ishida 2006; Scofield & Schultz 2006).

However, a high number of somatic mutations per sexual generation may also be accumulated by long-lived herbaceous plants with a great extent of vegetative reproduction (e.g. Klekowski 1988). *Juncus* species are known for their rhizomatous growth (Richards & Clapham 1941) and a number of species can form extensive stands by vegetative propagation (Snogerup 1993). *J. atratus* produces a creeping rhizome with short internodes (Kirschner *et al*. 2002). However, extensive clonality should result in a large fraction of identical multilocus genotypes within populations, which could not be detected for *J. atratus*. In only two of three German populations studied for ID did we find two multilocus genotypes occurring twice. Therefore, although vegetative spread may occur very locally, at population level clonality does not play a significant role in *J. atratus*.

The strong ID that could be demonstrated in the German populations of *J. atratus* involves severe selective mortality and hence a loss of parental investment. Although the selective significance of such an adaptation is difficult to explain, our results are no complete exception among mating system studies. A very similar pattern to that of *J. atratus* has been demonstrated for the herbaceous, perennial *Aquilegia canadensis* (Herlihy & Eckert 2002). As in the black rush, most ovules were self-fertilized despite high levels of ID. These results challenge the theory on mating system evolution and should provoke further theoretic and experimental work.

Genetic variation within and among populations

Self-fertilization should lead to both reduced effective population size and reduced effective pollen flow which in turn will result in lower genetic diversity and increased genetic differentiation among populations (Charlesworth 2003). However, in species with high selfing rates strong ID can result in the genetic structure of adult plants to converge on that of an outcrossing population (Goodwillie *et al*. 2005; Scofield & Schultz 2006). Despite of high selfing rates in the German populations, individual heterozygosity was high in all populations. Also, *J. atratus* showed considerable high within-population diversity with high gene diversity (H_E, Table 1). Supposedly, high levels of inbreeding depression, as found in the German populations, maintain allelic richness by favouring heterozygosity. This can contribute to a genetic diversity pattern resembling that of more outcrossed populations.

Limited pollen flow due to high levels of self-fertilization and the patchy distribution of the species may contribute to the genetic differentiation pattern in *J. atratus*. Mean pairwise F_{ST} values were moderately high when compared to other wind-pollinated herbs studied with microsatellite markers. For example, *Ambrosia artemisiifolia* (Genton *et al*. 2005) is only weakly differentiated (mean $F_{ST} < 0.1$) across its natural distribution. The self-compatible *Typha angustifolia*, *T. latifolia* (Tsyusko *et al*. 2005) and *Elymus athericus* (Bockelmann *et al*. 2003), and the endangered *Oryza officinalis* (Gao 2005) exhibited population differentiation (F_{ST} or analogues = 0.15–0.44) similar to *J. atratus*. However, F_{ST} values of *J. atratus* appear low in comparison to strong selfers which typically exhibit values of $F_{ST} > 0.5$, like *Arabidopsis thaliana* (Stenoien *et al*. 2005) or *Alliaria petiolata* (Durka *et al*. 2005).

High levels of gene flow by seeds can maintain genetic diversity within selfing species, and at population level, allelic variation is not necessarily decreased (Allard *et al*. 1968; Chauvet *et al*. 2004). *J. atratus* produces numerous seeds which can be easily dispersed by the frequent flooding events occurring in its habitat. Seed trap and buoyancy experiments have shown that the seeds of *J. atratus* have the potential for long-distance dispersal (Alsleben *et al*. 2004). Thus, seed flow may provide opportunities for gene migration between extant populations that can maintain genetic diversity. However, at least at the periphery of the species range, minimal geographical distances of several kilometres between neighbouring populations make recent gene flow quite unlikely. In the cladogram, long branches for individual populations indicate a high degree of isolation between the populations and hence suggest an independent history due to limited gene flow. This pattern is substantiated by the analysis of molecular variance that found the largest part of the variation resided within the populations. Obviously, seed dispersal cannot counteract the effects of genetic drift at the regional scale.

Perennial plant species with long-lasting seed banks are able to resist the effects of fragmentation and isolation on the genetic structure for a longer period of time (Templeton & Levin 1979; Cabin 1996). *Juncus* species are known to build-up highly persistent seed banks (Thompson *et al*. 1997; Jensen 2004). This fact could contribute to the temporal maintenance of genetic variation.

Juncus atratus populations of the peripheral region showed lower genetic diversity values than subcentral populations independent of the population size. Generally it is suggested that the extant localities of *J. atratus* are at least partly of relict nature (Snogerup 1978). Lowered genetic diversity in peripheral populations would be concordant with the decline of populations and suitable habitats of the species in Central Europe (Burkart 1995) resulting in hindered gene migration between populations. However, the degree of genetic differentiation was not different between peripheral German populations and subcentral populations.

Declining genetic diversity towards the periphery of the species' range is a common phenomenon of European plant species (e.g. Kuittinen *et al*. 1997; King & Ferris 1998; Durka 1999; Persson *et al*. 2004; Eckstein *et al*. 2006). This fact has also been interpreted as an effect of repeated founding events along colonization routes during postglacial range expansion (Hewitt 1996). The reduction of gene diversity and particularly the loss of private alleles in peripheral populations of *J. atratus* may be explained by such historical factors, because after bottleneck or founding events with drastically reduced effective population size, expected heterozygosity should increase faster than allelic richness (Nei *et al*. 1975). Postglacial colonization of *J. atratus* is likely to have started from refugia in southeastern Europe and around the Black Sea, where the *Juncus* species group these days still maintains a centre of diversity with *J. thomasii*, *J. alpigenus* and *J. anatolicus* (Snogerup 1978).

In *J. atratus*, an overall increase of genetic differentiation between populations with increasing distance was found (Fig. 2), which would be expected if the populations were at gene flow–drift equilibrium. However, in a pattern consistent with regional equilibrium, the degree of scatter should also increase with spatial distances (Hutchison & Templeton 1999). This could not be validated. Also, at the local scales, the pattern was inconsistent. Genetic and geographical distances were significantly and positively related among peripheral German populations, but not among the Ukrainian and Hungarian populations. At this scale which is only slightly larger than the German area, genetic drift seems more important than gene flow. Despite the expectation that in the centre of a species' range gene flow between populations should be stronger than in peripheral populations, this pattern indicates a high degree of isolation also among subcentral populations. It is likely that the pattern found at the large scale primarily reflects species history, i.e. a fast postglacial colonization combined with various levels of recent gene flow–drift equilibria, depending on local conditions.

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