GENETIC STRUCTURE OF AN INSECT POLLINATED FOREST TREE: A STUDY ON *PRUNUS AVIUM* L. USING MICROSATELLITES [1]

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ABSTRACT

We present a genetic analysis in a natural population of wild cherry (*Prunus avium* L.) in northern Germany. Seven microsatellite markers that had originally been characterised in peach were used to describe the genetic variation and the spatial genetic structure of a single population with 75 adult trees. Four to nine alleles and a heterozygosity from 0.423 to 0.916 were observed. An excess of heterozygote genotypes at all loci is indicated by the negative fixation index. A weak spatial genetic structure was found only for locus UDP96-001. The effects of life history traits on the observed genetic structure and the causes for the absence of a reliable spatial genetic structure are discussed.

Keywords: *Prunus avium*, wild cherry, SSR, genetic variation, heterozygote excess, spatial autocorrelation

INTRODUCTION

Wild cherry (*Prunus avium* L.) is widespread throughout temperate mixed forests in Europe, western Asia and North Africa. As a scattered tree species with high potential for colonisation, *P. avium* occurs at wood edges and in younger forests especially. The pioneer character of this species is maintained by animal dispersed seeds and by root suckers (FRASCARIA 1993, DUCCI & SANTI 1997). Insect pollination and strict allogamy determine the reproductive system of wild cherry.

As valuable timber tree and secondary species with benefit for the biological diversity of temperate forests, wild cherry has attracted increasing attention in research and management during the last decade (*e.g.* KLEINSCHMITT & STEPHAN 1997, MARIETTE *et al.* 1997, KOWNATZKI 2001, MOHANTY 2001a and b). Yet, detailed studies of the genetic system, particularly on gene flow within and between populations, are still missing. This may partly be due to the lack of suitable genetic markers. The use of DNA markers, like microsatellites (single sequence repeats, SSRs), has already enabled such studies in other forest tree species (*e.g.* oaks: DOW & ASHLEY 1998, STREIFF *et al.* 1999). As SSRs provide codominant inheritance and a great number of alleles they are qualified markers for studies of actual, realised gene flow mediated by pollen and seeds as well as for studies of the spatial genetic structure (SMOUSE & PEAKALL 1999).

SSR markers that had originally been characterised in peach [*Prunus persica* (L.) Batsch] by TESTOLIN *et al.* (2000) and were adapted to *P. avium* by SCHUELER *et al.* (2003), were used to determine the genetic structure of a wild cherry population. The population consists of 75 adult trees, which were mapped in 2001. The study area of 7 ha is dedicated to intensive monitoring and thus referred to as "intensive study plot" (ISP). Here, we present a first detailed analysis of the genetic structure of this stand, with special regard to its spatial genetic pattern, assessed with autocorrelation analysis. Furthermore, we estimated the power of the microsatellites for ongoing studies on actual gene flow.

MATERIAL AND METHODS

Study site

The intensive study plot (ISP) is located in northern Germany (Fig. 1), at the west bank of the lake

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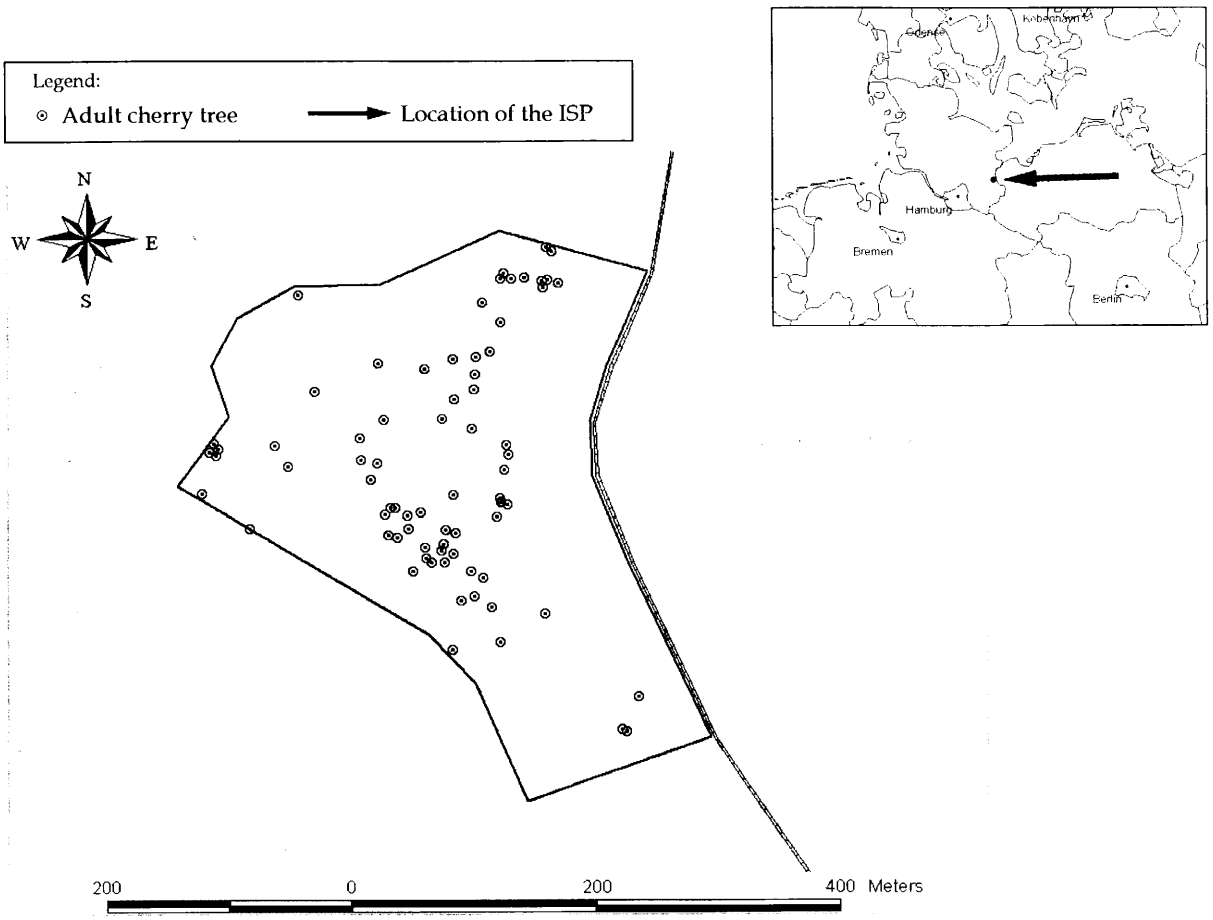

Figure 1. Location and map of the intensive study plot (ISP) with adult wild cherry trees in Northern Germany.

"*Ratzeburger See*" (10° 44' 41" E, 53° 42' 40" N). The ISP is part of a forest band, longitudinal to the lake and belongs to the municipal forest *Ratzeburg*. On its southern and northern border the ISP is neighboring other forest stands, without wild cherry trees 500 m to the southern direction, and with approximately 80 more cherry trees in the northern direction, around 100 m away. A housing estate, founded in the sixties of the last century, is located on the western border of the ISP, including approximately 70 cultural cherry trees (sweet and sour cherry).

Throughout the ISP, which is dominated by European beech we mapped and sampled all 75 adult wild cherries with estimated ages between 10 to 100 year. Diameter at breast height varied from 4 to 70 cm, with a mean of 40 cm, the height ranging from 4 to 34 m.

The wild cherry trees were scattered both as single trees and groups. The fine scale spatial distribution of *P. avium* at the ISP was analysed by Clark and Evans's index (R) (RIPLEY 1981):

$$R = \frac{\bar{r}_{obs}}{\bar{r}_{exp}}; \quad \bar{r}_{obs} = \frac{\sum_{i=r}^{N} r_i}{N}; \quad \bar{r}_{exp} = \frac{1}{2\sqrt{\frac{N}{F}}}$$

where r_i is the distance of the i-th tree to the closest neighbour, N is the total number of trees and F is the size of the plot (m^2). Index values (R) < 1 indicate an aggregated distribution, index values of (R) = 1 a random distribution and (R) > 1 a regular distribution. We calculated a value of R = 0.89, which indicates a trend to spatial aggregation.

Plant material and DNA extraction

Leaf tissue of all 75 adult wild cherry trees was collected in spring 2001. Each 50–100 mg (fresh weight) leaf material was subjected to DNA

minipreparation. The extraction followed a CTAB-based protocol by DUMOLIN *et al.* (1995) which included an additional and final treatment of the dissolved DNA with 1 µg RNase A at 37 °C for 30 min.

SSR marker analysis

All individuals were genotyped at 7 microsatellite loci (UDP96-001, UDP96-005, UDP97-403, UDP98-021, UDP98-410, UDP98-411, UDP98-412), which had originally been characterised in peach (CIPRIANI *et al.* 1999, TESTOLIN *et al.* 2000) and were adapted to *P. avium* as described by SCHUELER *et al.* (2003). As revealed by the latter study, five markers (UDP96-001, UDP96-005, UDP97-403, UDP98-410, UDP98-411) showed Mendelian inheritance, and the loci UDP98-021 and UDP98-412 were linked to the self-incompatibility locus of cherry. PCR-amplification and SSR detection are described in SCHUELER *et al.* (2003).

Data analysis

Genetic variation: For each locus the number of alleles (A), the effective number of alleles (A_e), the observed and the expected heterozygosity (H_o) and (H_e), and the fixation index (F) were calculated as described by WEIR (1990). An exact test was used to test for significant deviations of the genotypic structure from the Hardy-Weinberg expectations. To test the mean fixation index over all loci for deviation from zero a signed rank test was applied. Statistics and population genetic parameters were calculated with SAS, the SAS-genetics module (SAS Institute Inc. USA), and the SAS-based macro package for population genetics "MacGen" (STAUBER & HERTEL 1997).

Probabilities of exclusion and identity: The probabilities of identity $P_{(ID)}$ and exclusion were calculated with all trees of the ISP. $P_{(ID)}$ characterises the probability that two individuals in the examined population share the same multilocus genotype (unbiased formula, WAITS *et al.* 2001). The measure indicates the power of the cherry markers for the identification of clonal structures, *i.e.* vegetative propagation, in the observed population. The power of the cherry SSR markers for paternity analysis was computed as exclusion probability, following JAMIESON and TAYLOR (1997), when seeds from a known mother were analysed.

Spatial genetic structure: The program SGS (DEGEN *et al.* 2001) was used to analyse and test the spatial genetic structure of all trees showing distinct multilocus genotypes. Moran's index I (SOKAL & WARTENBERG 1983) was computed for both multilocus and single-locus genotypes separately, using twelve different distance classes of 35 m from 0 to 420 m. Running 5,000 permutations, a confidence interval of 95 % was determined.

RESULTS

As *P. avium* may propagate vegetatively by root suckers, we had to ascertain that trees are single individuals. Four pairs of trees with identical multilocus genotypes were detected, each pair being closer than 15 m. Given the extremely low probability of identity ($P_{(ID)} = 1.1 \times 10^{-6}$) in the population,

Table 1. Genetic parameters of the seven SSR loci in the ISP (71 adult wild cherry trees). Number of alleles (A), number of effective alleles (A_e), expected heterozygosity (H_{exp}), observed heterozygosity (H_{obs}), deviation of genotypic structure from Hardy-Weinberg expectations as testen with an exact test (Dev. HW) and fixation index F. The significance of the mean fixation index was tested with a signed rank test.

Locus code*	A	A_e	H_{exp}	H_{obs}	Dev. HW	F
UDP96-001	4	1.6212	0.3832	0.4225	n.s.	–0.1026
UDP96-005	5	3.9093	0.7442	0.9155	n.s.	–0.2302
UDP97-403	8	4.4453	0.7750	0.8169	n.s.	–0.0541
UDP98-021	4	2.4125	0.5855	0.6056	n.s.	–0.0343
UDP98-410	6	3.4838	0.7130	0.7887	n.s.	–0.1062
UDP98-411	9	3.1194	0.6794	0.7042	n.s.	–0.0365
UDP98-412	6	3.7203	0.7312	0.7887	n.s.	–0.0786
Mean	6	3.2445	0.6588	0.7203		–0.0918*

* Codes are the same as in CIPRIANI *et al.* (1999) and TESTOLIN *et al.* (2000); n.s. = not significant; (*) = $p<0.05$.

we considered them to be ramets of the same clone, discarding the younger tree of each pair from further analysis. Moreover, the exclusion probability for paternity analysis on the 7 SSR loci analysed was 0.984.

Genetic variation

Large genetic variation was found at all seven loci (Tab.1). Four to nine alleles were detected (mean A = 6), while the effective number of alleles was ranging from 1.6 to 4.4 (mean A_e = 2.9). The observed heterozygosity varied from 0.42 up to 0.92. Comparisons of observed genotypic structure with expected frequencies revealed no significant departures from Hardy-Weinberg equilibrium. The fixation index F is negative for all loci ranging from –0.0343 to –0.2302. Moreover, a general excess of heterozygotes was found over loci (mean F = –0.0918; p = 0.0156)

Spatial genetic structure

A weak spatial genetic structure at locus UDP96-001 is indicated by the significant departure from random distribution of genotypes within the first (0–35 m) and the fourth (105–104 m) distance class (Fig. 2). Computations with the other single loci and with the multilocus average revealed no departure from random distribution of genotypes.

DISCUSSION

In the present study, nuclear microsatellite loci were used to describe the genetic and the spatial genetic structure of a wild cherry population. These markers are promising tools for estimating gene flow by pollen and seeds and the identification of vegetative reproduction in this insect pollinated tree species. This was confirmed by the low probability of identity, which allowed to detect the existence of four pairs of ramets and by the high exclusion probability for parentage analysis.

Compared to other forest tree species, wild cherry exhibits only a small number of alleles (4 – 9 alleles) and a low mean observed heterozygosity (H_{obs} = 0.759), in contrast oak: 11 – 33 alleles, H_{obs} = 0.836 (DEGEN *et al.* 1999) or ash: 5 – 25 alleles, H_{obs} = 0.827 (at least) (MORAND *et al.* 2001). Questions arise whether the analysed population may represent an exception in *P. avium*. In a previous study based on isozymes carried out in two wild cherry populations of different ages, MARIETTE *et al.* (1997) found a lower genetic diversity within and a lower differentiation between stands in respect both to the analogous estimates obtained for other tree species and to expectations for outcrossing/animal dispersed tree species (as given by HAMRICK *et al.* 1992). A low differentiation between populations was also observed by FRASCARIA *et al.* (1993) with isozymes and by MOHANTY *et al.* (2001a and b) with cpDNA. Such consistent findings may be related to the biology of this species. AUSTERLITZ *et al.* (2000) discussed the length of the plant juvenile period as

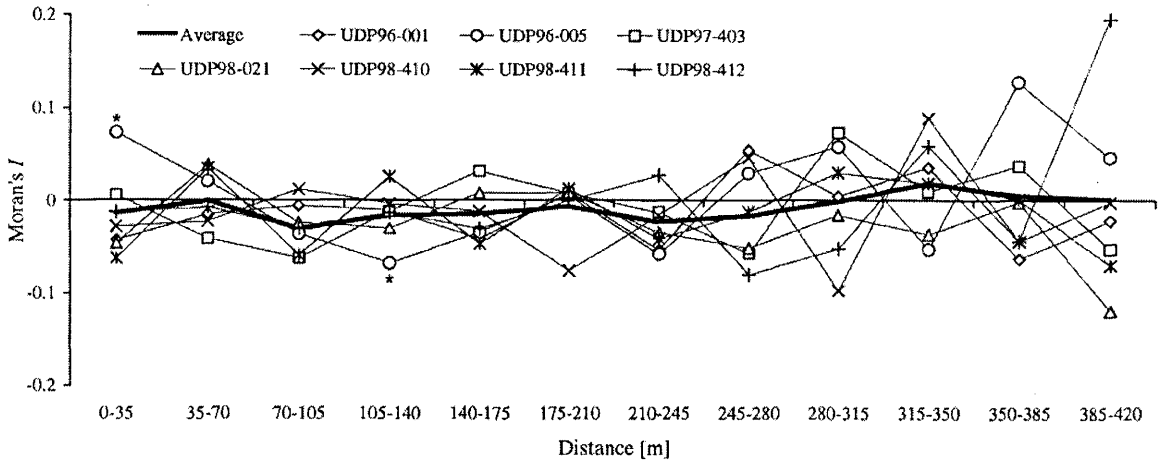

Figure 2. Correlogram with Moran's index I as computed for single loci and the multilocus average in 12 distance classes. A significant spatial genetic structure is given, if the observed value is higher than the expected value in the first distance classes or the observed value is lower than the expected value in the larger distance classes. The significance was tested with 5000 permutations (*) = $p < 0.05$.

an important life history trait imposing a significant genetic diversity. In *P. avium* the juvenile period is relatively short (~ 5 years) compared to other tree species. Furthermore, the relatively short turnover rate (60–120 years), together with their pioneer character may affect genetic diversity. Hence, longer existing populations, as described in MARIETTE *et al.* (1997), may be results of continuous recolonisations/extinctions and high values of interpopulation gene flow by seeds. This may explain that they exhibit a genetic diversity similar to younger populations (see MARIETTE *et al.* 1997). Future investigations on contemporary gene flow, *e.g.* seed endocarp analysis (GODOY & JORDANO 2001, SCHUELER *et al.* 2003), will give insights into processes that shape the genetic structure of the species.

In this study a significant negative fixation index over loci was observed, revealing an excess of heterozygote genotypes. Although a low negative fixation index has been reported for SSR markers also in other tree species (*e.g.* oaks: DEGEN *et al.* 1999), this has never been observed to this extent over all examined loci. A reason for this deficiency of homozygotes over all observed loci may be the strict self-incompatibility of this species, though selection may not be excluded. Isozyme studies have found genotype ratios consistent with Hardy-Weinberg expectations, from a weak excess of heterozygotes in 5 of 9 loci (DUCCI & SANTI 1997) to almost no such excess on 6 loci (FRASCARIA *et al.* 1993).

The spatial genetic structure of the stand was assessed with Moran's *I* by calculating multiallelic single-locus estimators and the multiallelic multilocus average. The latter is commonly used for an unbiased estimation of the spatial genetic structure because typically a high variation can be found among single loci (SLATKIN & ARTER 1991, SMOUSE & PEAKALL 1999). In the present study we found no significant departures from a random distribution of genotypes if we consider the multilocus estimator, but we observed a weak spatial genetic structure at locus UDP96-005. Differences in spatial genetic structure among single loci have been explained by four potential sources (SLATKIN & ARTER 1991): sampling variation, stochastic variation, parametric variation in mutation rates and selection coefficients, and variation in the initial gene frequencies. Except sampling variation, which was minimized by sampling all individuals at the ISP, these sources of variation might apply to our data. Therefore it is difficult to assess whether the weak spatial genetic structure at UDP96-005 is due to restricted gene flow by pollen and seeds, or an effect of one of these sources of variation.

Usually, the spatial genetic structure of plant populations is created by restricted gene flow through pollen and seeds, more generally referred to as isolation by distance (SOKAL & WARTENBERG 1983, BOS & VAN DER HARING 1988, EPPERSON & LI 1990, SMOUSE & PEAKAL 1999, STREIFF *et al.* 1998, DEGEN *et al.* 2001). Also, an aggregated distribution of individuals promotes a spatial genetic structure (DOLIGEZ *et al.* 1998). Wild cherry shows an aggregated spatial distribution and it is expected that gene flow by pollen is limited. A spatial genetic structure can therefore be expected also in wild cherry. GÖMÖRY and PAULE (2001) analysed the spatial genetic structure of a Slovakian wild cherry population by using isozymes. They detected a weak spatial genetic structure, but they could not ascertain whether this pattern is due to restricted gene flow or to vegetative propagation alone, because the enzyme polymorphism was too low to assure the extent of vegetative propagation. The genetic markers in our study allowed the exclusion of vegetative propagules, but the weak spatial genetic structure at only one single locus does allow conclusions on isolation-by-distance processes. The absence of a reliable spatial genetic structure in our study can have several reasons: first, the size of the investigated stand is quite small so that weak spatial genetic patterns cannot be identified; or second, the investigated stand might have been founded artificially so that no spatial genetic pattern is present. Wild cherry has never received much attention in historical documents so that we cannot exclude a human impact. To find out whether wild cherry generates a spatial genetic structure through limited gene flow at our ISP needs therefore an extension of the study area. Also, comparable studies are required on single populations and metapopulations to explore the biology of this fascinating species.

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