Relationship between Empty Seed and Genetic Factors in European Beech (*Fagus sylvatica* L.)

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The relationship between percentage of empty seed (*Pes*) and genetic factors was explored in an isolated stand of European beech (*Fagus sylvatica* L.). Nine allozyme loci (GOT-B, IDH-A, LAP-A, MDH-B, MDH-C, MNR-A, 6-PGDH-A, PGI-B and PGM-A) were used to estimate genetic factors. *Pes* ranged from 4.8% to 40.9% for seed samples of 91 trees within the stand and showed an approximate normal distribution. The average *Pes* was 21.4% and the repeatability of *Pes* was 43.4%. The multilocus estimate for outcrossing rate (t_m) based on seed samples of 30 trees within the stand was 1.015(SE = 0.011) and the mean single locus estimate was slight higher at 1.061(SE = 0.026). No evidence of biparental inbreeding was found. Weak positive correlation between *Pes* and maximum selfing rate as well as and significant negative correlation between *Pes* and multilocus outcrossing rate indicated that self-fertilization may be explained as one of the important causes of empty seeds in beech.

Keywords *Fagus sylvatica* L., allozyme, empty seeds, selfing, outcrossing rate, correlation
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1 Introduction

In many forest tree species controlled self-fertilization results in a higher proportion of empty seeds than cross- or wind-fertilization (e.g. Nielsen and Schaffalitzky De Muckadell 1954, Dieckert 1964, Sorensen 1971, Park and Fowler 1982, Kormutak and Lindgren 1996). It may be inferred that free self-pollination also increases proportion of empty seeds. However, no significant correlation has been found so far between parameters of the mating system and the percentage of empty seed (Sorensen 1971, Schroeder 1989, Kormutak and Lindgren 1996, Freytag 1998, Siregar 2000).

Genetically, empty seeds in forest trees can arise from 1) incompatibility (Nielsen and Schaffalitzky De Muckadell 1954, Yazidani and Lindgren 1991); 2) embryo degeneration, which may be the consequence of inbreeding. Homozygous recessive lethal or sublethal alleles cause the embryonic development failure (Sorensen and Miles 1982, Chiliak et al. 1984, Kosiski 1986, Colangeli and Owens 1990, O'reilly and Farmer 1991, Karkkäinen and Savolainen 1993, Sedgley and Granger 1996, Kuang et al. 1999). Environmental factor such as a limited pollen supply (Colangeli and Owens 1990) and insect damage (O'reilly and Farmer 1991) can also cause empty seeds. In many angiosperm species ovules may not develop into seeds because they are involved in self-incompatibility mechanisms (Waser and Price 1991, Seavey and Carter 1996). Self-incompatibility is a system used by many flowering plant species to prevent self-fertilization and inbreeding, thereby promote outcrossing.

European beech (*Fagus sylvatica* L.) is a widespread, monoecious and wind-pollinated tree species. It belongs to the major forest tree species and is of importance of ecology and economy. Since beech is mainly naturally regenerated, this tree species is very interesting with respect to population genetics, e.g. the genetic differentiation between populations, gene flow and other elements of the reproduction system. It has been reported that European beech is characterized by the possibility of self-fertilizaztion but generally showing high outcrossing rates (Merzeau et al 1994, Müller- Starck 1996, Rossi et al. 1996). Nielsen and Schaffalitzky De Muckadell (1954) reported that self-sterility was the reason of empty seed through artificial pollinatiion. Müller-Starck (1996) suggested that selfing was one of important factors in the formation of empty seeds. Freytag (1998) investigated the relationship between maximum selfing rate and the percentage of empty seeds (*Pes*). Till now, the relationship between *Pes* and self-pollination in European beech is not clear. The objectives of this study are to: 1) describe the variation in percentage of empty seeds and test its distribution, 2) estimate parameters of mating system, 3) explore the relationship between *Pes* and genetic factors.

2 Material and Methods

2.1 Seed Material and Stand Information

An isolated pure beech stand 100C (120 m × 160 m), which has 96 adult trees, was used in this study. This stand is located in Solling, which is in the area of a research project of the Forest Ecology Center, University of Göttingen, Germany. Seeds were collected from the ground under each adult tree in 1998. Seeds were sampled around each adult tree at a radius of 3 m within three circular plots, which are 40 cm in diameter and are located north, southwest and southeast of the stem at angles of 120°. All of the seeds encountered in each circle were sampled. All of the trees within the stand were tagged and the location of each tree was mapped.

The total amount of seeds sampled under 96 adult tree was 8940. The percentage of empty seed (*Pes*) of each tree was estimated by subtracting the amount of full seeds and insect damaged seeds from the totality of the seeds sampled under each tree. The percentage of empty seeds (*Pes*) of 91 trees, which have more than 40 seeds (from 41 to 176 seeds per tree) were calculated.

2.2 Isoenzyme Electrophoresis

Nine polymorphic enzyme-coding gene loci were utilized for multilocus genotyping (GOT-A, IDH-A, LAP-A, MDH-B, MDH-C, MNR-A, 6-PGDH-A, PGI-B and PGM-A). Genetic control

Table 1. Variance analysis.

Sources of variation	Degrees of freedom	Sum of squares	Mean square square (EMS)	Expected mean	F
Between trees	n – 1	SS_b	V_b	$\sigma_W{}^2 + r\sigma_b{}^2$	V_b/V_w
Within trees	n(r – 1)	SS_W	V_W	V_b/V_w	
Total	nr – 1				

and mode of inheritance of the respective enzyme systems were verified previously (Müller-Starck and Starck 1993). Enzymes were separated from crude homogenate by standard horizontal starch gel electrophoresis. The buds were used for mulitilocus genotyping of 96 adult trees. Seeds sampled under 30 adult trees were analysed with electrophoresis and the total amount of seeds was 1954.

2.3 Statistical Methods

2.3.1 Variation in Percentage of Empty Seeds

The mean, standard deviation and coefficient of variation of *Pes* were calculated. Both χ^2 tests and Kolmogorov-Smirnov tests were used to test hypotheses on the distribution of *Pes*.

When more than one measurement of the character can be made on each individual, the phenotypic variance can be partitioned into the variance within and between individuals (Table 1). The ratio of the between-individual component to the total phenotypic variance is called the intraclass correlation (*Re*). It is the correlation between repeated measurements of the same individual, and is known as the repeatability of the character (Falconer and Mackay 1996).

The repeatability (*Re*) is calculated using following formula.

$$Re = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_W^2} = \frac{V_G + V_{Eg}}{V_P} = \frac{V_b - V_W}{V_b + (r-1)V_W} \qquad (1)$$

where V_G is the genetic variance, V_{Eg} the general environmental variance and V_P the phenotypic variance. V_b is the variance between individuals of *Pes* and V_W is the variance within a tree. r is the number of samples per tree (in this study $r = 3$). *Re* is considered as the upper limit of heritability (Falconer and Mackay 1996).

2.3.2 Estimation of Genetic Factors

Fixation index (*F*) was estimated for seeds sampled under 30 adult trees using the computer program BIOSYS-2 (Swofford and Selander 1997). The fixation index for a single locus is estimated according to following equation.

$$F = 1 - \frac{\sum_{i<j} p_{ij}}{1 - \sum_i p_i^{\,2}} \qquad (2)$$

where F is fixation index for single locus, P_{ij} and P_i are genotype frequency and gene frequency of seed generation (Hattemer et al. 1993).

Within stand, multi-locus genotype exclusion method was used to get seeds, which carry at least one of two alleles at any locus also carried by the adult tree. Then, the adult tree under which the seed was sampled was regarded as seed tree (Wang 2001). And then, the seeds of the seed tree were assumed to be either the result of selfing (*s*) or random outcrossing (*t*), according to the mixed-mating model of Brown and Alland (1970). Population and family multilocus outcrossing rates (t_m) were estimated using the multilocus procedure of Ritland and Jain (1981). The MLTR program of Ritland (1996 version 1.1) was used for the computation multilocus outcrossing rates (t_m). Family t_m was estimated with fixed pollen pool frequencies (p).

The maximum selfing rate ($s_{\max}$) or potential selfing rate, which refers to the proportion of possible self-fertilized seeds when none of all alleles of multilocus genotype in the seed is different from the seed tree, was infered from simple paternity exclusion method (Ellstrand 1984, Hamrick and Schnabel 1985). $s_{\max}$ was estimated with the help of program CERVUS of Marshall (1998 version 1.0).

2.3.3 Correlation and Regression Analysis

Correlation and regression analyses were performed between *Pes* and genetic factors using program of SPSS 8.0.

3 Results

3.1 Dynamics of Percentage of Empty Seeds

According to the 91 observed values of *Pes*, the value of *Pes* ranged from 4.8% to 40.9%. The mean, the standard deviation and the coefficient of variation (cv%) of *Pes* were 21.4%, 8.7% and 40.8%, respectively.

In order to get an overview of raw data it is useful to group the data, meaning that data of about the same size are entered into the same class. When choosing class limits, the following rules should be followed: The class width *b* should be constant: $b = R/(1 + 3.32 \cdot \lg n)$. This is Sturges's rule of thumb, which is usually used to group the raw data (e.g. Köhler et al. 1995). In this formula *n* is the sample size, and *R* is the range of variation.

91 values of *Pes* were divided into eight classes by using *b* ($b = 4.6\%$). The expected frequency for each class was estimated according to the distribution function of a normal distribution. The hypotheses of normal distribution of *Pes* was tested with χ^2. The value of χ^2 is 6.593 $< \chi^2_{0.05}(5) = 11.07$. In addition, Kolmogorov-Smirnov test was also used to test the goodness of fit for 91 observed values of *Pes*. The Z-value was 1.077 and probability (*p*) was 0.197 > 0.05 (with the program SPSS 8.0). Both χ^2 test and Kolmogorov-Smirnov test were not significant and showed that the *Pes* approximates normal distribution.

From the seed lots collected under 91 trees, 59 were chosen to calculate repeatability (*Re*). These lots comprised more than 20 seeds in each of three probes under one tree. The three probes were considered as repeated observations of *Pes* for one tree. The amount of seeds for the 51 trees ranged from 69 to 176 and the total number of seeds was 6580. The average of *Pes* of the 59 trees was 21.3%. The result of variance analysis (ANOVA) is shown in Table 2. The *Pes* between trees showed highly significant differences. According to variance analysis, the repeatability (*Re*) of *Pes* was estimated and the value was 43.4%.

3.2 Estimation of Genetic Factors

The total amount of seeds which were analyzed with electrophoresis and used in estimation of genetic parameters was 1954. These seeds were sampled under 30 adult trees. The multilocus estimate for outcrossing rate in the population was 1.015(SE = 0.011), and the mean single locus estimate was slight higher at 1.061(SE = 0.026). The multilocus estimates were slightly lower than the mean single locus estimates indicating no "biparental inbreeding". Multilocus family estimates of the outcrossing rate (t_m) based on

Table 2. The results of ANOVA for *Pes*.

Sources of variation	Degrees of freedom	Sum of squares	Mean square (MS)	F
Between trees	58	1.2530	0.02160	3.301***
Within trees	118	0.7722	0.00654	
Total	176	2.0252		

*** P<0.001.

Table 3. *Pes* and part of genetic parameters in seeds of 25 seed trees.

Tree no.	No. of seeds	*Pes* (%)	F_{mean}	s_{max} (%)	t_m (SE)
2	44	6.82	–0.10	13.64	1.28 (0.24)
3	72	37.50	–0.08	32.50	1.05 (0.08)
4	83	19.28	0.02	6.12	1.17 (0.07)
5	88	40.91	–0.08	44.00	0.79 (0.09)
6	176	28.41	–0.02	2.33	1.38 (0.29)
7	112	11.61	–0.03	18.99	0.97 (0.05)
8	129	32.56	–0.06	10.29	1.02 (0.03)
10	98	15.31	–0.05	4.55	1.13 (0.23)
11	58	20.69	–0.06	6.10	1.02 (0.29)
12	89	13.48	–0.08	10.40	1.13 (0.22)
14	62	19.35	–0.02	12.60	1.16 (0.19)
15	53	22.64	–0.10	3.00	1.03 (0.07)
16	93	32.26	0.00	18.00	0.98 (0.11)
17	60	28.33	0.05	17.24	1.04 (0.12)
19	87	8.05	0.00	16.39	1.05 (0.04)
20	121	7.44	–0.07	7.32	1.02 (0.03)
21	95	9.47	–0.02	13.33	1.07 (0.03)
22	125	4.80	0.02	14.93	1.17 (0.06)
26	134	14.18	0.00	19.35	1.06 (0.10)
27	132	29.55	0.00	2.22	1.00 (0.49)
29	41	36.59	–0.09	26.32	0.94 (0.15)
30	90	15.56	–0.05	19.67	1.15 (0.23)
31	105	15.24	–0.08	7.14	1.02 (0.14)
44	44	15.91	0.11	11.11	1.44 (0.29)
45	96	29.17	–0.12	4.00	1.02 (0.49)

Table 4. Average mean of parameters for 25 trees.

N	*Pes* (%)	t_m	$s_{max(\%)}$	F_{GOT-B}	F_{IDH-A}	F_{LAP-A}	F_{MDH-B}	F_{MDH-C}	F_{MNR-A}	$F_{6-PGDH-A}$	F_{PGI-B}	F_{PGM-A}	F_{mean}
25	20.60	1.08	13.66	–0.058	–0.027	–0.052	0.032	–0.079	–0.029	–0.058	0.027	–0.032	–0.037

N refers number of trees.

mixed mating model of these 30 trees ranged from 0.79 to 2.00. Maximum selfing rate ranged from 0 to 44%. Because the multilocus outcrossing rate of 5 adult trees gave $t=2.0$, they are excluded from further analysis. Genetic parameters were calculated for seed samples of 25 trees and part of parameters are shown in Table 3. Genetic parameters include the maximum self rate (s_{max}), the multilocus outcrossing rate (t_m) and the fixation index (*F*) for 9 gene loci and their average (Tables 3 and 4).

3.3 Correlation and Regression Analysis

Pearson's correlation coefficient between *Pes* and genetic parameters was calculated for the 25 families and presented in Table 5.

It is clear from Table 5 that there is a significant negative correlation between *Pes* and t_m. s_{max} shows weak positive correlation with *Pes*. The mean fixation index and that of all loci have no significant correlation with *Pes*.

Simple linear regression equation was fitted to the data on *Pes* and t_m and s_{max}. R^2 is the coefficient of determination. It can be seen from

Table 5. Coefficients of simple correlation between *Pes* and genetic factors.

t_m	$s_{\max}$	$F_{GOT\text{-}B}$	$F_{IDH\text{-}A}$	$F_{LAP\text{-}A}$	$F_{MDH\text{-}B}$	$F_{MDH\text{-}C}$	$F_{MNR\text{-}A}$	$F_{6\text{-}PGDH\text{-}A}$	$F_{PGI\text{-}B}$	$F_{PGM\text{-}A}$	F_{mean}
–0.408*	0.369	–0.141	–0.225	–0.275	–.373	0.068	0.013	0.281	0.164	–0.016	–0.199

* $P<0.05$.

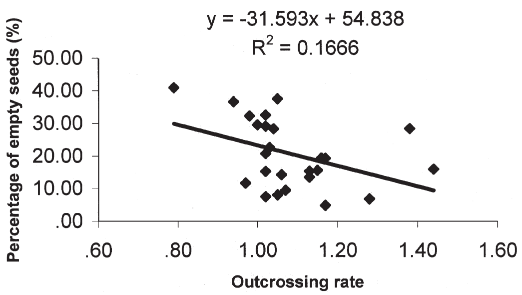

Fig. 1. Regression line of *Pes* on t_m for 25 trees.

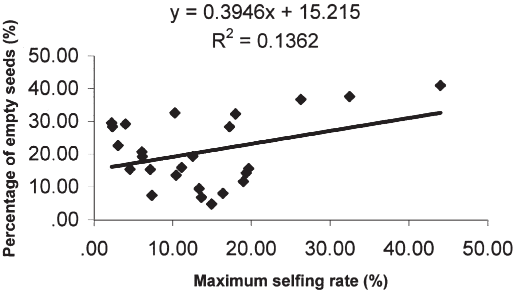

Fig. 2. Regression line of *Pes* on $s_{\max}$ for 25 trees.

Fig. 1 that significant relationship between t_m and *Pes* exists. However, Fig. 2 shows weak positive relationship between $s_{\max}$ and *Pes*.

4 Discussion

4.1 Variation in Percentage of Empty Seeds

Pes of 91 adult trees ranged from 4.8% to 40.9% and showed an approximate normal distribution. The *Pes* within stand showed continuous distribution and implied that this trait shows quantitative differences between trees and is determined not only by genetic factors but also environmental factors. Variance analysis indicated that the differences of *Pes* between trees were highly significant. The estimated repeatability (the upper limit of heritability) of *Pes* is 43.3% and showed that the *Pes*-values of adult trees could be inherited to their progeny at a maximum rate of 43.3%. This is first report on heritability of *Pes* in European beech. Matziris (1998) reported that there were statistically significant differences among clones in the percentage of full seed in Aleppo pine. This author also found that percentage of full seed was moderately inherited (the broad sense heritability was 41%). It has been shown that the *Pes* ranged from 4.1% to 18.2% for seeds sampled under 7 adult trees of European beech (Freytag 1998). Janßen (2000) reported that *Pes* ranged from 19.3% to 69.2% in 25 seed samples and the average was 32.6% within an old stand of European beech.

4.2 Relationship between Percentage of Empty Seeds and Genetic Factors

Primary results of this study showed that there is a significant negative correlation between *Pes* and t_m and a weak positive correlation between *Pes* and $s_{\max}$ on the basis of seed samples of 25 families. These findings indicated that self-fertilization may be explained as one of the important causes of empty seeds in beech. Generally, the t_m and $s_{\max}$ show similar trend to *Pes*. In fact, t_m has significant negative correlation with $s_{\max}$ (correlation coefficient is –0.438*). However, $s_{\max}$ shows the potential value of self-pollination and it is calculated directly from the number of possible self-fertilized seeds. t_m is estimated based on maximum likelihood method and should be closer to the actual selfing rate according to this point.

In many angiosperm species ovules may not develop into seeds because they are involved in self-incompatibility mechanisms (Waser and Price 1991, Seavey and Carter 1996). In European beech, Nielsen and Schaffalitzky De Muckadell (1954) reported that self-sterility was the reason of empty seed through artificial pollination. Müller-Starck (1996) found that the maximum selfing rate was 6.7%, 5.6% and 2.6% for three trees and suggested that selfing was one of the important causes of empty seeds. Freytag (1998) tried to examine the correlation between maximum selfing rate and percentage of empty seed using seed samples of 7 trees. Her result showed that two trees which had the highest maximum selfing rate had also high *Pes* and one tree with the lowest $s_{\max}$ had the smallest *Pes*. However, the other four trees had large variation.

Inbreeding is the likely cause of positive F-values only if similar positive values are observed at most loci. The heterogeneity of fixation indices (Table 4) implied no evidence for a strong influence of inbreeding on the genotypic structures of this isolated beech stand. This result is consistent with the reports that no evidence for an inbreeding structure was revealed by the F-values in European beech (Merzeau et al. 1994, Müller-Starck 1996, Ziehe et al. 1998). Furthermore, the result $t_m < t_s$ could not be interpreted by inbreeding. When there is inbreeding in addition to selfing, the multilocus estimates of t_m are expected to be higher than the mean of the single-locus estimates t_s (Shaw 1981). In addition, the fixation index has no significant correlation with *Pes* in this study. The proportion of filled seed production in *Picea abies* appears to increase until $F = 0.125$ and to decrease at higher level of inbreeding (Andersson et al. 1974). A strong inverse linear relation was found between filled seed per cone and inbreeding coefficient (F) with values less than or equal to 0.5 in Douglas-fir (Woods and Heaman 1989).

It has been reported that the portion of empty seeds increased with increasing level of selfing for several conifer species (Dieckert 1964, Franklin 1971, Griffin and Lindgren 1985). However, no significant correlation was found between the characteristics outcrossing rate and percentage of empty seeds or percentage of full seeds (Sorensen 1971, Schroeder 1989, Kormutak and Lindgren 1996, Freytag 1998, Siregar 2000). It has been suggested that the relation would probably be clearer, when genetic load is the main factor affecting the mating system (Omi and Adams 1986).

4.3 Limitation and Concluding Remarks

This is the first systematic study of relationship between percentage of empty seeds (*Pes*) and genetic factors in European beech. The primary results indicated that self-fertilization may be explained as one of the important causes of empty seeds in European beech.

The mixed mating model is based on certain assumptions (Brown et al. 1985), namely: 1) maternal genotypes outcross at the same rate to a homogeneous pollen pool; 2) for each maternal parent, progeny genotypic classes are independent, identically distributed, multinomial random variables; 3) alleles at different loci segregate independently; and 4) genetic markers are not affected by selection or mutation between the time of mating and progeny evaluation. The assumptions of the mixed mating model are often violated in natural populations and some critiques of estimating mating system based on mixed mating model have been found (Brown et al. 1985, Hamirick and Schnabel 1985, Hamrick 1989, Gregorius 1991, Mitton 1998). In this study, more than one locus in this stand is significant from Hardy-Weiberg structure. This implies deviations from random mating. The method used for identification of each offspring as a result of self or cross-fertilization are not error-free (Gregorius 1991). Ritland and El-Kassaby (1985) reported that 15–25% of family outcrossing estimates did not converge (t was greater than 2) and explained the possible reasons. In this study, the multilocus outcrossing rate of 5 adult trees gave $t = 2.0$ and the reason could be highly heterozyous seed trees.

The percecntage of empty seeds is commonly considered as an indicator of the resulting excess of self-fertilization. In fact, self-fertilization may be explained as one of the important causes of empty seeds in beech. In this study, this point was confirmed from significant negative correlation between *Pes* and multilocus outcrossing rates (t_m) as well as weak positive correlation between *Pes* and maximum selfing rate ($s_{\max}$). However, the genetic factors were estimated based on only 30 families and the seeds were produced through open-fertilization. The genetic factors involved in the proportion of empty seeds must be further elucidated. Korbecka et al. (2002) suggested that genotypic approach has more advantages than phenotype approach to study selective embrtion abortion. It can be seen that molecular techniques and artifical pollination will be used to further study the mechanism of seed abortion.

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