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Short Note: Cross-fertilization in a conifer stand inferred from enzyme gene-markers in seeds

By *G.* MÜLLER

(Received February 1978)

Introduction

Cross-fertilization will be described in terms of the probabilities with which the ovules of any given tree in a stand are fertilized by pollen originating from a single specified tree in the same stand as a function of the distance between the respective trees. Information about these probabilities as well as about the probabilities of self-fertilization are required in order to characterize the mating system of trees. It can be assumed that coniferous species such as Norway spruce and Scots pine do not mate at random, since at least two important conditions necessary for this mating system are not fulfilled: firstly, the probabilities of cross-fertilization have been proved to depend on the distance between the mating trees because of limited spatial pollen dispersal, and secondly, the probabilities of self-fertilization of individual trees are on the average higher than the reciprocal value of the population size (KOSKI and MALMIVAARA 1974; MÜLLER 1976 a and 1977). This implies that other mating systems, such as preferential mating between relatives or assortative mating or any combination of both, can be accepted as being more realistic. More precise experimental data are required to characterize the actual mating system of the mentioned tree species and to estimate coefficients of inbreeding and kinship to avoid the well-known detrimental effects of these phenomena on the average expression of important characters in the future breeding populations.

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The probabilities of cross-fertilization cannot be estimated by experiments on the spatial pollen dispersal, because the results would refer only to the probabilities of cross-pollination. It is necessary to identify the pollen contribution of an individual tree ("marker tree") in the viable seeds of other trees in the sarne stand by detecting its genes in the diploid embryo tissue of the respective seeds. This implies that the estimated probabilities of cross-fertilization eomprise the effects of genotypic selection, if this type of celection occurs between formation of the zygote and the embryonic stage.

Identification of pollen can be performed precisely by applying enzyme analysis and using such gene-markers as criteria for identification which guarantee a one-to-one correspondence between enzyme phenotype and genotype. In this paper, an additional field of application of a method is presented which has already been proved to be suitable for the estimation of probabilities of self-fertilization of individual trees in conifer stands (MÜLLER 1976 b and 1977).

Materials and Methods

In the winter of 1976177, cones from each of 105 trees were collected separately from a continuous area of a 120-year-old Scots pine stand *(Pinus sylvestris)* in the forest district of Grebenau (Hessen), compartment 57 b. All trees had been cut down just before this collection. The former position of each tree in the stand before clear-cutting was marked in a map.

The pine stand continues only on the southern side of the experimental area; the other sides adjoin on either young plantations or farm land. In *figure* 1 only those pine-trees are marked with numbers, the seed samples of which

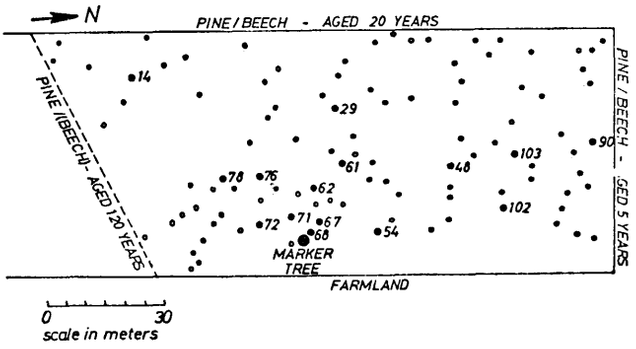

Fig. 1. — Positions of all pine-trees of the experimental area Grebenau/57 b. Numbers refer to analysed sample trees (see *figure 2*).

were used for estimation of cross-fertilization probabilities with respect to the marker tree. The positions of several younger beech trese among the pine trees are not presented.

In order to detect pollen of a marker tree in the seeds of other trees, the system of leucine aminopeptidase (LAP) was analysed in endosperm and embryo tissue of the respective Scots pine seeds. This system has been proved already to be suitable for such type of analyses (for details see MÜLLER 1977):

The LAP-system is controlled genetically by the two polymorphic gene loci LAP-A and LAP-B, each with codominant alleles which are represented phenotypically by distinct isozyme bands (RUDIN 1977; MÜLLER 1978). Such bands are designated as allelic isozymes or "allozymes". The LAP-System is monomeric, so that no hybrid-configurations can be expected in the zymogram of diploid samples. Thus the genotypes at the LAP loci can be identified directly by the respective allozymes. The LAP-System is active in endosperm and embryo tissue, and allozymes, which are controlled by the same allele, are identical in these tissues.

Because in Scots pine populations there are usually rare alleles at the LAP-B gene locus, only this locus was analysed. LAP-analyses were performed by homogenizing both endosperm and embryo of dormant seeds and separating the enzymes by means of starch gel zone-electrophoresis in a modified discontinuous buffer system (POULIK 1957; BERGMANN 1973).

The genotypes at the LAP-B locus of all trees of the experimental area were identified by endosperm analysis, using a sample of six seeds per tree. Because all trees carried cones, it was not necessary to identify genotypes by means of needle analyses. If a tree carries a rare allele at the LAP-B gene locus which cannot be detected in the respective tissues of any of its neighboring trees, this tree then is chosen as marker tree. Half of its pollen is marked and thus detectable if there is one rare allele at this gene locus, all pollen if there are two ones.

Since the position of each individual tree is known, within different distances from the marker tree some of its neighboring trees ("sample trees") are chosen randomly for analyses of larger seed samples. Seeds with the contribution of marked pollen can be identified simply by analysis of their embryo tissue, since the respectively female contribution of each sample tree has already been determined by means of the endosperm analyses and can be distinguished definitely from the male contribution. Seeds from 15 such sample trees were analysed using a constant number of seeds per tree.

Results and Discussion

(1) *Endosperm analyses*

Studying endosperm tissues of a total of 105 trees, three alleles were identified at the LAP-B gene locus which are represented phenotypically by single allozyme bands. In accordance with the nomenclature introduced by BERGMANN (1973), the slowest migrating band is designated by B_1, the fastest by B_3 and the medium type by B_2 (for schematic presentation of LAP-B phenotypes see MÜLLER (1976 b)).

Table 1. — Observed genetic structure at the LAP-B gene locus.

	Genotypic frequencies			Allelic frequencies		
	B_1B_2	B_2B_2	B_2B_3	B_1	B_2	B_3
Absolute values	16	88	1	16	193	1
Relative values	.152	.838	.010	.076	.919	.005

Only one out of the 105 trees carried the rare allele B_3, so that the requirements were fulfilled for using this tree as the marker tree. The genotypic frequencies seem to be unusual because of the extremely high percentage of homozygotes (83.8%) and the fact that the three genotypes B_1B_1, B_1B_3 and B_3B_3 are not present. But both phenomena are mainly caused by the extremely high and low frequencies of the alleles B_2 and B_3, respectively. This is demonstrated in table 2 by contrasting the observed genotypic frequencies with the expected Hardy-Weinberg proportions in the equilibrium state.

Table 2. — Observed genotypic frequencies and expected Hardy-Weinberg proportions.

Relative values	Genotypes at the LAP-B gene locus					
	B_1B_1	B_2B_2	B_3B_3	B_1B_2	B_1B_3	B_2B_3
Observed	—	.838	—	.152	—	.010
Expected	.006	.845	$.25\times10^{-4}$	.140	$.76\times10^{-3}$	.009

Deviations between both distributions are rather small. An ordinary statistical test cannot be performed satisfactorily, since only two out of five classes are sufficiently occupied.

In any case, deviations of the observed genotypic structure from any theoretical one must be small. For the present result, the frequency of the heterozygotes cannot exceed $2(1 - p_m) = 2(1 - 0.919) = 0.162$, where p_m is the frequence of the most common allele, which has to be greater than 0.5 (GREGORIUS 1978, Lemma 2). As can be seen, the detected frequency of heterozygotes is even identical to the highest possible value. This indicates the limited informational value of such standards as the Hardy-Weinberg proportions in case of extreme allelic frequencies.

(2) *Embryo analyses*

From each of 15 sample trees, the LAP-genotypes of 384 embryos were identified. Of these trees, 13 were homozygous at the LAP-B locus, so that the female contribution could not vary and the pollen contribution was identifiable in the respective embryo tissue without additional analysis of the corresponding endosperm. Two sample trees carried the LAP-genotype B_1B_2 (No. 62 and 78 — *table 3*). Because of the alternating female contribution, the pollen contribution of the respective embryo could be identified without endosperm analysis only in the cases of the LAP-genotypes B_1B_1, B_2B_2, $B♀_1B♂_3$ and $B♀_2B_3♂$ but not in those of $B♀_1B♂_2$ and $B♀_2B♂_1$. To speed up procedure, endosperm analyses were omitted and the frequency of pollen carrying the allele B_1 or B_2 was estimated by assuming 1 : 1 gamete segregation: The number of detected genotypes with alleles B_1 and B_2 was split, so that one-half of the number of embryos contained the allele B_1 and the other half B_2 as the

hypothetic female contribution. Besides that, pollen carrying the B_3 marker allele always could be identified precisely by pure embryo analysis.

Detected or estimated frequencies of pollen contributions are presented in *table 3*. The sample trees are listed according to the distances from the marker tree (see *figure 2*).

Table 3. — Frequency of pollen contribution at the LAP-B gene locus as detected in the embryo tissue of seeds from different Scots pine sample trees.

Sample trees		Embryos of sample trees			
Tree-No.	LAP-genotype	Number per tree	Frequencies of embryos containing following pollen contribution B_1	B_2	B_3
68	B_2B_2	384	14	350	20
67	B_2B_2	384	18	345	21
71	B_2B_2	384	20	348	16
72	B_2B_2	384	35	337	12
62	B_1B_2	384	34*	342*	8
54	B_2B_2	384	19	351	14
76	B_2B_2	384	24	356	4
61	B_2B_2	384	52	328	4
78	B_1B_2	384	29*	347*	8
29	B_2B_2	384	22	354	8
48	B_2B_2	384	8	370	6
102	B_2B_2	384	16	365	3
103	B_2B_2	384	11	371	2
14	B_2B_2	384	13	367	4
90	B_2B_2	384	21	361	2
Total: Absolute values		5760	336	5292	132
Relative values		1	.058	.919	.023

* Estimated (see article)

Besides the alleles B_1, B_2 and B_3, no other pollen contributions were detected in the embryo tissue of analysed seeds. Comparing the relative frequency of these contributions with the allelic frequencies in the tree population *(table 1)*, an obvious deviation can be observed only with respect to allele B_3. This can be interpreted by assuming non-random sampling (felled trees from the experimental area do not represent the whole stand with resepct to allelic frequency), but it can also be caused by extraordinary pollen emission from the marker tree. For the present, this cannot be considered as an definite proof of assortative mating at this gene locus.

The data in table 3 reflect quite obviously the influence of the distance on the respective fertilization probabilities. For instance, the large amount of B_1-carrying pollen detected in the embryos of tree No. 61 can be explained by the fact that this is the only sample tree whose closest neighboring tree is one of the 16 genotypes B_1B_2. Analogously, none of the immediately neighboring trees of sample tree No. 48 carried the allele B_1. The influence of the distance is pronounced when considering pollen with the marker allele B_3: In *figure 2* its relative frequencies are plotted against the distances of the respective sample trees from the marker tree (see also *figure 1*). The direction in which the sample trees lie with respect to the marker tree, was not taken into account, because its influence on the frequency of the B_3-pollen cannot be derived systematically from the presented data. By means of a least square estimation, an exponential distribution function in the general form of $f(x) = \alpha \cdot e^{-\beta x}$ was fitted to experimental data, which represents the probabilities of cross-fertilization as defined above.

The obtained results prove the hypothesis of preferential mating between neighboring trees to be realistic. Under the

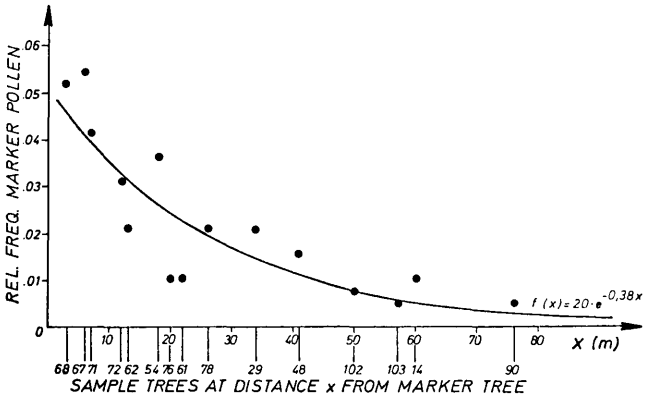

Fig. 2. — Relative frequencies of embryos containing pollen contribution with the marker allele LAP-B_3 as detected in seeds from Scots pine sample trees at distances x from the marker tree. An exponential distribution function is fitted to data.

conditions of the investigated Scots pine stand, the probabilities of cross-fertilization between the marker tree and any other tree at a distance of x meters is represented by the function $f(x) = 20 \cdot e^{-0.38x}$, assuming that pollen from another marker tree did not interfere.

Conclusion

The method of analysing enzymes in endosperm and embryo tissue has been used so far solely for the purpose of estimating probabilities of self-fertilization.

In this paper, the application of this method is extended to the probabilities of cross-fertilization, so that conditions are fulfilled which allow estimation of mating probabilities in general. The present study can be applied analogously to other conifer species.

The selected Scots pine stand offers optimal experimental conditions in the sense that the probability of interference by external pollen carrying the marker allele can be considered to be very close to zero (see *figure 1*). If such external pollen can be expected to be more frequent (*e.g.* experimental area in the midst of a large pine area), there is an additional contribution to each of the values of the respective distribution function. This contribution cannot be determined exactly, but an appropriate estimation can be obtained by using the relative frequency of the marker allele as parameter. If there is another marker tree among the experimental trees, the overlapping range of both distribution functions must be determined additionally.

In a previous study (Müller 1977), the rate of self-fertilization of the marker tree No. 69 was estimated to be on average 0.0465. This was also done by applying the enzyme method of seed analysis, using the same LAP-B marker allele. Therefore, under experimental conditions as described above, it can be stated for the present that the mating system of Scots pine is characterized by preferential mating between neighboring trees, including self fertilization.

Acknowledgements

I wish to thank S. Krakuhn and U. Rodeck for technical assistance and Prof. H. Hattemer, as well as Dr. H.-R. Gregorius for critical reading of the manuscript. Cone collection was faciliated by friendly help from the Forstamt Grebenau (Hessen). This study was financially supported by a grant from the Deutsche Forschungsgemeinschaft, Bad Godesberg.

Summary

By analysing allozymes in viable seeds, cross-fertilization can be studied in conifer stands. As an example, the probabilities are estimated with which the pollen contribution

of an individual marker tree in a Scots pine stand can be expected in the seeds of any other tree as a function of the distance between the respective trees:

The genotype at the LAP-B locus of each of 105 neighboring trees is identified by endosperm analysis. The observed genetic structure is presented and compared with the corresponding Hardy Weinberg proportions. The marker tree is the only one carrying the LAP-B_3 allele. To detect its frequency in seed samples of neighboring trees at different distances, embryo analyses are performed. The allelic frequencies at the LAP-B locus of all detected pollen contributions are compared with those of the 105 parental trees. There is no definite indication of genotypic assortative mating at this locus. The frequency of the B_3 marker allele is definitely reduced with increasing distance from the marker tree. By means of the experimental data, the function $f(x) = 20 \cdot e^{-0.38x}$ is derived which represents the estimated probabilities of cross fertilization with respect to the marker tree.

In a previous study of the author, seeds from the same marker tree were identified as originating from self-fertilization. Therefore the mating system of Scots pine can be defined for the present as a system of preferential mating between neighboring trees, including self-fertilization.

Key words: Cross-fertilization, conifer, enzyme, endosperm, embryo.

Zusammenfassung

Durch Analyse von Allozymen in lebensfähigen Samen läßt sich Fremdbefruchtung in Nadelholzbeständen untersuchen. Als Beispiel werden die Wahrscheinlichkeiten eingeschätzt, mit denen der Pollenbeitrag eines einzelnen Versuchsbaumes in einem Kiefernbestand in den Samen beliebiger anderer Bäume in Abhängigkeit von der Entfernung der jeweiligen Bäume erwartet werden kann:

Mit Hilfe von Endospermanalysen werden die Genotypen am LAP-B Genlocus von 105 benachbarten Bäumen identifiziert. Die gefundene genetische Struktur wird präsentiert und mit den korrespondierenden Hardy-Weinberg-Proportionen verglichen. Der Versuchsbaum weist als einziger das LAP-B_3 Allel auf. Um dessen Häufigkeit in Samenproben von unterschiedlich entfernt gelegenen Nachbarbäumen nachzuweisen, werden Embryonanlaysen durchgeführt. Die Allelhäufigkeiten am LAP-B Genlocus aller dabei identifizierten Pollenbeiträge werden mit denen der 105 Bäume aus der Elterngeneration verglichen. Es gibt keinen definitiven Hinweis auf genotypisch assortative Paarung an diesem Genlocus. Die Häufigkeit des Marker-Allels B_3 nimmt eindeutig mit zunehmender Entfernung vom Versuchsbaum ab. Mit Hilfe der experimentell gefundenen Werte wird die Funktion $f(x) = 20 \cdot e^{-0.38x}$ hergeleitet, welche die Schätzwerte für die Fremdbefruchtungswahrscheinlichkeiten bezogen auf den Versuchsbaum darstellt.

In einer früheren Untersuchung des Autors wurden unter den Samen desselben Versuchsbaumes Nachkommen aus Selbstbefruchtung identifiziert. Das Paarungssystem der Kiefer kann deshalb vorläufig als ein System der bevorzugten Paarung benachbarter Bäume unter Einschluß von Selbstbefruchtung bezeichnet werden.

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Buchbesprechungen

Vom II. Internationalen Lärchenprovenienzversuch. Schriftenreihe d. Forstl. Fak. der Universität Göttingen und Mittlgn. der Nieders. Forstl. Versuchsanstalt, Band 49. Von R. Schober. J. D. Sauerländer's Verlag, Frankfurt am Main. 1977. Mit 68 Abb., 35 Tab., 360 S., DM 49,—.

Vom Verfasser wurde vor 20 Jahren der II. Internationale Lärchenherkunftsversuch mit Alpen-, Sudeten-, Tatra-, Südkarpaten- und Polenlärche sowie Absaaten der Schlitzer und Harpker Lärche, außerdem einer Japan-Lärche und der Dunkeld-Hybride initiiert. Das vorliegende Werk gibt neben einem Überblick über die Vorbereitungen, Herkünfte, Anlage und Zielsetzung des Versuchs die ersten Beobachtungsergebnisse auf 31 in Norddeutschland liegenden Versuchsorten wieder. Insgesamt wurden 68 Provenienzen geprüft, deren Anzahl mit den Versuchsorten von einer bis 33 variieren.

Die Ergebnisse dieses (und anderer internationaler Versuche) zeigen im Höhenwuchs die Provenienzen der Sudeten, Tatra und Polen als eindeutig überlegen. Auch die Wienerwald-Lärche weist einen guten Höhenwuchs auf. — Gute bis sehr gute Schaftform bestätigen in besonderem Maße auch in diesem Versuch Alpenherkünfte wie Wienerwald und aus der nördlichen Alpenzwischenzone sowie Lärche der Hohen Tatra. — Lärchenkrebs ist offenbar standortsabhängig; außerdem waren Unterschiede zwischen Arten und Herkünften eindeutig. So waren Japan-Lärche und F_2-Hybriden frei und die Alpenherkünfte wiesen etwa 4 bis 5mal stärkeren Stammkrebs auf als Sudeten-, Tatra- und Polenlärchen. Innerhalb der Alpenlärchen waren Herkünfte der Salzburger und Berchtesgadener Kalk-Alpen sowie aus dem Salzkammergut am wenigsten befallen. Entsprechende Standortwahl und waldbauliche Behandlung lassen jedoch Alpenherkünfte hervorragender Schaftform bevorzugt anbauwürdig erscheinen.

Die wirtschaftliche Bedeutung der Lärche wird insbesondere im Übergang vom reinen Buchen- zum Buchen-Lärchenmischwald bei richtiger Wahl geeigneter Lärchenherkünfte mit hohem Wertholzertrag gesehen.

Die Fülle des dargebotenen Materials kann hier auch nicht im entferntesten diskutiert werden; das Werk stellt eine Fundgrube für jeden der mit der Europäerlärche arbeitenden Wissenschaftler, den akademischen Lehrer, den interessierten Studenten und nicht zuletzt den mit der Lärchenfrage konfrontierten Praktiker dar, zumal die Ergebnisse ausführlich im Lichte neuer Erkenntnisse aus den internationalen Parallelversuchen besprochen werden.

Melchior