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The segregation ratio in *waxy*-heterozygous barley plants

INGRID FRYKMAN AND BENGT O. BENGTSSON
Department of Genetics, Lund University, Sölvegatan 29, S-223 62 Lund, Sweden
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Summary

In seven experiments the segregation ratio in barley plants heterozygous for the *waxy*-gene was studied. Each experiment entailed the scoring of more than 40000 iodine-stained pollen grains from ten spikes. A significant over-representation of blue pollen grains was found in all experiments, with the segregation distortion (measured as a conversion force) ranging from 0·0063 to 0·0143. The distortion was unaffected by which of two mutations was used and which genetic background the plants had, but was stronger in 1988 than in 1987. Different statistical, methodological and biological explanations of the results are discussed.

1. Introduction

From his analysis of peas, Mendel concluded that heterozygote individuals produce the two types of gametes in equal numbers (Mendel, 1866). This simple 1:1 rule of segregation has become one of the cornerstones of classical genetics.

Today we know that there are exceptions to Mendel's segregation rule. Strong segregation distortion occurs in a number of organisms and situations (Werren *et al.* 1988): the sex chromosomes in the wood lemming, the SD locus in *Drosophila*, the *t* system in the mouse, the B chromosomes in many plant species, and so on. However, these examples can all be regarded as special cases with unique evolutionary histories.

An unresolved question is whether a weak segregation distortion is the rule rather than the exception. Data from careful experiments in fungi have shown that heterozygotes with one normal allele and one allele with a few base pairs deleted often produce more normal alleles than expected from the 1:1 rule. The effect is due to a bias in the conversion process which favours the relatively longer allele. Until recently, the effect was only known from *Ascobolus* and *Sordaria* (see review by Lamb, 1984), but examples of biased conversion in connexion with alleles of different base-pair lengths have recently been found also in yeast (Nag *et al.* 1989).

The effect of biased conversion on the segregation ratio is measured by the conversion force, y, calculated from the ratio between the normal and the mutant alleles, $0{\cdot}5+y:0{\cdot}5-y$. Typical values for the conversion force in *Sordaria* and *Ascobolus* range from greater than 10^{-2} to smaller than 10^{-3}, when the alleles differ by a few base pairs in length (Lamb, 1984). The conversion force associated with base-pair differences is much smaller than the effect associated with deletions and additions. In general, there is no reason to believe that the conversion process functions identically in all organisms. From a theoretical point of view, a bias is expected to evolve in the conversion process so that it acts against the most common type of molecular damage (Bengtsson, 1985; Bengtsson & Uyenoyama, 1990).

Small deviations of the segregation ratio from 1:1 can in many situations be ignored. However, some long-term evolutionary processes are strongly influenced even by small changes of the segregation value (see e.g. Nagylaki, 1983*a*, *b*; Walsh, 1983). This holds, in particular, for the mutation load, which is increased or decreased by many orders of magnitude by a small shift in the conversion force (Bengtsson, 1990).

It is therefore of great importance to investigate the exact segregation ratio for different allele combinations in different organisms. Very few such studies have been performed outside the fungal investigations discussed above, as far as we know. The great experimental and statistical difficulties with studies of the segregation ratio must, however, be realized. If the segregation process has a bias of size y, then y^{-2} randomly drawn gametes must be scored for it to be likely that the 1:1 rule is rejected at the 5% level.

Fungi where the products of meiosis can be recovered and studied together are, of course, the

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ideal material for studies of the segregation process. But it would be unfortunate if no attempts were made to study this important process in any of the other major eukaryote groups, particularly since it is unclear how to extrapolate the information from the fungi to animals and plants with their different chromosome structures, life cycles and gene numbers. It is difficult to see how a sufficient amount of relevant data could be obtained from any animal. Animal gametes can be scored for their genetic composition only with difficulty, and if the scoring is made after fertilization then the segregation ratio will be influenced by many other factors with confounding effects, such as sperm competition, assortative gamete fusion, zygotic viability differences, and so on. This makes pollen studies in plants the only reasonable alternative to the fungal investigations.

Pollen grains are complete organisms that express their own genetic composition. In most species they are produced in large numbers and are of homogeneous appearance. Different methods have been developed to analyse pollen grains genetically. One of the systems most used is based on the so called *waxy*-gene, coding for starch synthetase (EC 2.4.1.11) which catalyzes the synthesis of amylose in pollen and endosperm (Eriksson, 1969; Rohde *et al.* 1988). Pollen grains in which the gene is active stain dark-blue when tested with an iodine solution, while grains without a functional copy of the gene stain red with a brown tint.

Barley (*Hordeum vulgare*) has a number of advantages for detailed studies of genetic parameters (Bengtsson, 1992). In this species the waxy system has mainly been used in mutation research, for example to investigate the effect of very low doses of mutagens (Eriksson, 1971; Nilan *et al.* 1981), but it has also been used to screen for unstable genetic elements (Simons & Somerville, 1988; Schreiber *et al.* 1991). The gene is less than 4 kb long and contains 11 small introns (Rohde *et al.* 1988).

With these considerations in mind we have performed an extensive study of the segregation ratio in *waxy*-heterozygous barley plants. Two different sodium azide mutations were chosen for study plus two different genetic backgrounds, to see what effect these parameters have on the segregation ratio. The effect of the environment was investigated by including data from plants grown in two different years. Since this was the first study of its type, the methods used as well as our understanding of the results, developed during the course of the study, are outlined in the discussion section.

2. Material and methods

Seeds homozygous for the *waxy* alleles *glx1c* and *glx1g* were kindly provided by Dr R. A. Nilan, Washington State University, Pullman, USA. The mutants were originally induced in the cultivar 'Steptoe' by sodium azide. In a test for intragenic recombination involving seven alleles, the mutant sites in *glx1c* and *glx1g* mapped farthest away from each other with an estimated recombination frequency of about 0·002 (Rosichan *et al.* 1979).

The mutant lines were fertilized with pollen from the locally adapted cultivars 'Alva' and 'Gull', both from Svalöf. Alva is a modern variety which has about half of its parentage from the much older variety Gull. Despite their close relationship, the lines are known to differ with respect to their recombination frequencies in the short arm of chromosome 1 (Säll *et al.* 1990), i.e. the arm on which the waxy gene is situated (Søgaard & von Wettstein-Knowles, 1987).

The F_1 plants were grown in an experimental field in South Sweden during the summers of 1987 and 1988. Ripe spikes were harvested, put in a fixative solution (70% ethanol), and stored at 4 °C for later analysis in the laboratory. The pollen grains were scored by a modified version of the method described by Rosichan *et al.* (1981).

Pollen preparations were obtained by cutting the spike into small pieces in a test tube followed by homogenization in distilled water using a mixer knife. The suspension was filtered through nylon filters (pore sizes 515 and 60 μm). The pollen grains were then rinsed and spun down twice at a very low speed in a centrifuge.

The pollen grains were stained in an iodine solution (500 mg KI and 150 mg I_2 in 100 ml H_2O, diluted 3:5 in distilled water). After a suitable dilution of the suspension, *ca* 800 pollen grains at a time were sucked onto a gridded Millipore filter with a pore size of 1·2 μm.

The grains were counted individually using a preparation microscope with 40 × magnification and a manual counter. Only ripe, normal pollen grains, recognized by their round shape and shiny appearance under the preparation microscope, were scored. Generally, such pollen grains stain red or blue; occasionally a yellow-looking grain with an almost ripe appearance could be found. This type of yellow grains were scored in experiment 3, but were considered unripe and were excluded from the analysis in the other experiments. Pollen preparations with many unripe, yellow grains were not analysed. From each spike at least 4000 pollen grains were scored. Since all the grains on a filter were analysed, somewhat more than 4000 grains were scored from each spike. As summarized in Table 1, ten spikes were analysed from each set of F_1 plants, making a total of more than 40000 pollen grains scored in each experiment.

The scoring of pollen grains was always performed by the same person, I.F. In general, one pollen suspension was analysed per scoring session.

Experiment 7 was similar to experiment 6, except that in this experiment the pollen suspension was stirred extra vigorously before staining.

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Table 1. *Segregation in waxy-heterozygous barley plants*

Expt. no.	Mutant	Pollen parent	F_1 plants cultivated year	Number of Spikes analysed	Number of Pollen counted	Blue pollen n	Blue pollen %	Goodness of fit to 1:1 χ^2	Goodness of fit to 1:1 P	Heterogeneity between spikes χ^2	Heterogeneity between spikes P
1	*glx1c*	Alva	1987	10	41458	21072	50·83	11·35	< 0·001	2·33	> 0·95
2	*glx1g*	Alva	1987	10	41810	21188	50·68	7·66	< 0·01	2·43	> 0·95
3[a]	*glx1c*	Alva	1988	10	42726	21974	51·43	34·95	< 0·001	1·52	> 0·99
4	*glx1g*	Alva	1988	10	41257	21174	51·32	28·85	< 0·001	1·85	> 0·99
5	*glx1c*	Gull	1987	10	41794	21262	50·87	12·75	< 0·001	3·31	> 0·95
6	*glx1g*	Gull	1987	10	40589	20603	50·76	9·38	< 0·01	5·50	n.s.
7[b]	*glx1g*	Gull	1987	10	40631	20683	50·90	21·22	< 0·001	8·14	n.s.

[a] Yellow pollen grains were counted separately in this experiment; see the text for details.
[b] This experiment was identical to experiment 6 except that the pollen suspension was extra vigorously stirred.

3. Results

The results from the experiments with the *waxy*-heterozygous plants are described in Table 1. The first six experiments make up the original investigation, while the seventh was based on a slightly different method and was performed only to investigate a particular statistical effect. This experiment is therefore not included in the analyses of variance of the segregation ratios.

In general, a clear distinction could be seen between the pollen grains that stained blue and those that stained red. In most of the experiments, ten or fewer pollen grains (out of more than 40000) were found to stain almost dark brown. The reason for this we do not know. Their numbers were so small that they do not influence the results reported below. During the course of the study we came to worry about whether red pollen grains were judged unripe or abnormal more often than blue pollen grains. For this purpose almost normal looking yellow staining pollen grains were scored in experiment 3, to see if they could possibly be grains that should have been scored as red.

More blue then red pollen grains were found in all the experiments. Indeed, this holds for every one of the 70 spikes analysed. In all experiments the deviation from the expected 1:1 segregation ratio was strongly statistically significant, as seen from the goodness-of-fit values in Table 1.

Only a marginal effect was obtained by including the yellow pollen grains (altogether 458) among the red ones in experiment 3; the frequency of blue pollen grains fell from 51·43 to 50·88% (still highly significant). The deviant segregation values generally obtained could therefore not be explained by a consistent misjudgment of the borderline cases between unripe yellow pollen grains and ripe red ones. The yellow grains were therefore not included in the further analysis.

In experiments 1–5, the segregation ratios from the ten analysed spike preparations were so similar that their frequencies did not show the expected scatter around their overall mean value; see the small heterogeneity χ^2 values in Table 1. This effect was smaller in experiment 6 and disappeared altogether in experiment 7, when the pollen suspension was more rigorously stirred than in the earlier experiments. As seen from Table 1, this stirring affected only the distribution of the segregation frequencies for the ten investigated spikes, and not their overall mean value.

Two analyses of variance were performed to test for the effect of the mutant involved, the choice of pollen parent, and the environment (year) in which the F_1 plants were grown. In the first analysis the experiments made with plants grown in 1987 were compared (Table 2). The frequency of blue pollen grains per spike was, according to this test, not affected by which mutant or pollen parent was used. In the second analysis, Table 3, the experiments based on crosses with the cultivar Alva were compared. Again, there was no effect of which mutant was involved in

Table 2. *Analysis of variance for the experiments cultivated 1987 (nos 1, 2, 5, 6); the analysis is based on the frequency of blue pollen grains among the ten spikes analysed in each experiment*

Source of variation	D.F.	MS	F
Between mutants	1	0·1677	0·73
Between pollen parents	1	0·0403	0·18
Interaction	1	0·0042	0·02
Error	36	0·2282	—

Table 3. *Analysis of variance for the experiments with Alva as pollen parent (nos 1–4); the analysis is based on the frequency of blue pollen grains among the ten spikes analysed in each experiment*

Source of variation	D.F.	MS	F
Between years	1	3·9376	29·08***
Between mutants	1	0·1703	1·26
Interaction	1	0·0038	0·03
Error	36	0·1354	—

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the cross. However, a strong effect was found of the year in which the F_1 plants were grown. Heterozygous plants involving Alva produced 50·78 % blue pollen in 1987 and 51·38 % in 1988.

4. Discussion

In the experiments with *waxy*-heterozygous barley plants, a consistent deviation from the expected 1:1 segregation ratio was found. In this section we discuss what interpretation and importance should be given to this result. Statistical, experimental and biological factors with an influence on the measured segregation ratios are considered. The methodology and the results are discussed in parallel.

(i) *Statistical factors*

Ten genetically identical spikes were analysed for an almost equal number of pollen grains in each experiment. This procedure was adopted since it made it possible to estimate the magnitude of the experimental variation in the investigation. Within each experiment, the between spike variance should contain all important experimental error factors, such as uneven staining, mistakes made during scoring, and so on.

When the results from the first two experiments had been obtained, we were surprised to find the consistent bias in favour of blue pollen grains, but were shocked by the small variance between the ten segregation ratio measurements in each experiment. The heterogeneity analysis showed that the results from the different spike suspensions were much too similar, compared to what was expected by the standard χ^2 heterogeneity test. Before any conclusions could be drawn concerning the biased segregation ratio, this statistical problem had to be solved.

We continued the experiments in an unchanged way to see if the effect was reproducible, which it turned out to be. The heterogeneity between spikes was significantly smaller than expected in five of the six basic experiments, and in the sixth it was lower than the expected value (equal to the relevant degrees of freedom which in all cases were 8).

No ordinary experimental error, with, for example, the staining procedure or the preparation of the pollen suspension, should give rise to an experimental variance smaller than expected. Neither could the method used for scoring the grains produce any such effect, assuming the well defined rule for when to finish the scoring process. When one of the first spike preparations was scored, it was noticed that the preparation was incompletely stained giving far too few blue pollen grains, so we excluded that particular score. This was, however, a clear case of learning to use the scoring method correctly, and it could not be considered a pruning of the data set only to retain the best results.

Furthermore, the segregation ratios were not calculated until the end of each scoring session, making it very difficult to understand how any conscious or unconscious wish of the scorer could have influenced the results.

Finally, at the end of the set of experiments, we came up with the following suggestion which we believe explains the small statistical variance. The explanation follows from the fact that pollen production is not a strictly binomial process, where each grain's genetical composition is statistically independent of every other grain. Four pollen grains are produced from each tetrad in a normal meiosis, and among these grains two carry the normal allele while two carry the mutated allele. This implies that if the scored pollen grains all come from such complete 'quartets' of pollen grains, then there will be no variation at all between the measurements. In a real situation, however, there are two factors that make the between spike variance greater than zero. On the one hand, the mixing and filtering of the pollen grains in the suspension must cause the break up of some of the quartets, thereby increasing the variance by making the scored grains more statistically independent of each other. On the other hand, the process that biased the segregation ratio must also increase the between spike variance, since it introduces a sampling effect between, for example, quartets with 2 blues+2 reds and quartets with 3 blues and 1 red.

When we finally realized that this effect may be the cause of the reduced between spike variation, we repeated experiment six, for which we had more material, one extra time with an extra vigorous stirring of the pollen suspension. It was then found, see experiment 7, that the frequency of blue pollen grains remained unchanged compared with experiment 6, but that the between spike variance had been increased and was now very close to the expected value.

Thus, we conclude that the surprising statistical effect with a small variance between the ten data points which make up every experiment was due to our gentle methods to produce pollen preparations, which generally caused pollen grains derived from the same meiosis to be scored together. In the future, a strong mixing of the suspension will be used.

(ii) *Experimental factors*

The observed small between spike variance becomes of great interest when the generally distorted segregation ratio is discussed. Obviously, the experimental variance, due to staining or scoring, must be very small if the subtle statistical dependence between the pollen grains could be detected. This implies that the constant bias in favour of the blue pollen grains could hardly be due, for example, to overstaining, since this effect probably would be stronger for some

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spikes and weaker for others. It is, likewise, not easy to see how a tendency for red pollen grains to attach to the glass ware and not be spun down on the filter could remain so constant. The effect on the results of spikes with different degrees of maturity must also be next to negligible.

The effect of the year of growth (1987 or 1988) on the segregation ratio was not due to a systematic change in the experimental procedure, since some of the experiments from 1987 were scored later than the experiments from 1988. There was, in fact, no correlation between the segregation ratios estimated in the experiments and the order in which the experiments were performed (data not shown).

From these considerations we conclude that our data show that it is possible to obtain an extremely high accuracy in the handling and scoring of pollen grains, making the purely experimental errors very small when the segregation ratio is estimated.

(iii) *Biological factors*

We are convinced that the higher number of blue pollen grains reflects an underlying real biological situation – the *waxy*-heterozygous plants in our experiments *did not* produce ripe pollen grains in the expected ratio of 1:1.

There are many possible causes of the deviation. Let us first exclude some biological effects that may, in principle, change the segregation ratio, but which cannot be the decisive factor in the present case.

A normal allele will occasionally mutate to a non-functional state and a mutant allele will sometimes, though less often, revert to a more or less normal state. In the present case Rosichan *et al.* (1979) have estimated that the *glx1c* and *glx1g* alleles revert to the normal state with frequencies around 2×10^{-5}. Thus, mutation cannot account for the observed bias in the segregation ratio since this effect is far too small.

One type of non-disjunction can also be excluded as a cause of the observed bias. If two copies of chromosome 1 occasionally go to the same pollen grain, at the same time as another viable and naturally maturing pollen grain is formed with no chromosome 1, then this will not lead to a predominance of blue pollen grains in the sample.

However, the situation is different if the microspore with no chromosome 1 fails to be viable. The frequency of red and blue viable grains coming out of the disturbed meiosis will then depend on whether the disjunction occurred during the first or the second meiotic division. If it occurred during the second division, then – on average – there will be no disturbance of the segregation ratio. If it occurred during the first division, then the result will depend on the recombination fraction between the centromere and the *waxy* locus. At most, this type of non-disjunction will lead to the formation of two blue pollen grains and no red ones (which occurs for close linkage with the centromere). This effect is similar to what happens if the first meiotic division fails and two *diploid* blue-staining pollen grains are formed instead of four haploid grains.

It is easy to see that the frequency of blue pollen grains in a sample where a frequency x of the meioses leads to two blue grains while the rest of the meioses lead to two blue and two red grains, is $1/(2-x)$. Thus, the frequency of diploid pollen grain formation and non-disjunction of chromosome 1 in the first meiotic division must be about 3% in 1987 and above 5% in 1988, for these effects alone to produce the segregation distortions observed. We have no direct information on the frequency of meiotic irregularities in our crosses, but from what we know about the regular appearance of meiosis in barley, we regard these values as too high for meiotic disturbances to be the likely cause of the deviations observed. However, the possibility cannot be fully excluded.

Another explanation is that the chromosome segment with the mutated *waxy* allele also carries some genetic material which, due to the mutagen treatment or to a general difference in the genetic background, gives the microspores carrying the *waxy*-allele a slightly smaller chance of maturing into ripe pollen grains than the other microspores. This suggestion is very hard to exclude, but if it were true, then one would have expected to find an effect due to which *waxy*-allele was used in the cross or from which line the normal chromosome was derived. No such effects were seen (Tables 2, 3), so we have no empirical indication that this suggestion holds.

The final possibility is that the bias in the formation of pollen grains is due to some direct process involving the gene segments, probably an allele-influenced bias in conversion. Again, we have no direct information on the existence of any such factor in our crosses.

To conclude, we do not at the present know what factor underlies the deviation from the expected 1:1 segregation. Obviously, it is important to determine the exact reason of the observed segregation distortion, since only segment-specific processes, like biased gene conversion, will have the important evolutionary effects mentioned in the introduction. A methodologically produced bias, and biological processes leading to gametes with reduced viability, will never be of any evolutionary relevance.

(iv) *Future studies*

To improve our knowledge of the experimental system, we would like to perform more experiments of the described type, but now with *waxy* alleles induced with other agents than sodium azide. The interpretation of the conversion results in the fungal studies was greatly improved when it was seen that the direction of the conversion bias depended on whether the mutated allele carried a deletion or an addition of base pairs. Similarly, we wish to see if the segregation

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distortion reported on here can be shown to depend, more or less clearly, on the type of mutagen used for inducing the waxy mutation.

In the future, we hope that it will also be possible to use PCR methods on isolated, stained pollen grains to study the behaviour of flanking RFLP markers in these crosses. Such direct analysis will finally elucidate what happens during meiosis and segregation of heterozygous genes in barley.

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