Biosystematic Studies in *Disporum* (Liliaceae-Polygonatae)
II. Pollen fertility, meiosis and chiasma frequency
in *D. smilacinum* A. Gray

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The production of abnormal pollen induced by either low or high temperatures is known for *Disporum sessile* (Washiashi 1935, Kayano 1961). The present study which uses natural populations of *D. smilacinum* investigates the production of similar pollen abnormalities. Two approaches are used: a range pollen fertility survey and a detailed meiotic analysis. The eco-evolutionary relationships between meiosis including chiasma frequency, abnormal pollen load, sexual reproduction (seed set) and spring temperature fluctuations are explored and discussed.

Materials and methods

Herbarium records (KYO, KANA, SAPA), spanning the past 90 years, were used for the pollen fertility survey. To complement this range survey, a meiotic analysis of a single population, 40 individuals sampled within a four meter square quadrant in mid-April from near Yatsuo, Toyama Prefecture, was undertaken. The percent normal pollen production for this population, estimated at three different stages (pollen tetrad, anaphase I and metaphase I), is compared with that observed for the range survey. PMCs were fixed in 1:3 acetic ethanol for 30 minutes and transferred to 70% ethanol before squashing in aceto-orcein.

Observations

Survey of pollen fertility: A herbarium survey of the pollen fertility of 45 populations was made by using an aceto-orcein smear technique. The average percent fertility for 15 large populations, based on 2,000 grains each, was 93.8% (S.D.=1.67; n=30,000; range: 90.4-96.7%), while additional 30 populations, each based on a smaller sample of at least 500 grains, had an average fertility of 93.6% (S.D.=2.62; n=17,950; range: 88.2-98.2%). These fertilities are mapped (Fig. 1), with the larger populations depicted by pie-percent diagrams and the smaller ones only by location. No major, significant difference is apparent throughout the range, though there is a slightly lower percent fertility on the Pacific side of Honshu compared with the Japan Sea side.

Overall, this consistently high level of pollen fertility throughout a species' range would cause no great concern. But from each of these scattered popula-

tions, a common type of pollen abnormality was observed. They involved either micronuclei in minute pollen grains or additional micronuclei in regular-sized grains. The origin of these pollen abnormalities and their evolutionary significance were the basis of further investigation.

Fig. 1. Percent pollen fertility for populations of *D. smilacinum*. The percent pie-diagrams are for the 15 large populations based on 2,000 grains each (squares), while the solid circles represent additional 30 populations with a sample size of at least 500 grains.

Pollen tetrad and anaphase I analysis: To ascertain the possible mechanism for this abnormal pollen production, a population of *D. smilacinum* from near Yatsuo, Toyama Prefecture was selected. From 10 individuals, a total of 4,000 pollen tetrads were observed. Of these, 3,138 (78.4%) were normal and 862 (21.6%) were abnormal (Table 1). The percent range of variation for these 10 plants was

Table 1. Analysis summary of tetrad classification
(Yatsuo, 10 plants; 4000 tetrads; 16,000 grains)

Parameter	Tetrads		Pollen grains	
	Normal	Abnormal*	Normal	Abnormal
Number	3,138	862	14,909	1,091
Average %	78.4%	21.6%	93.2%	6.8%
S.D.	4.10	4.10	1.25	1.25
% range	72.4-85.6%	14.4-27.6%	91.1-95.1%	4.9-8.9%

* Containing at least 1 fragment and/or 1 micro-grain, cf. Table 2 for further classification.

Fig. 2. Abnormal pollen in *D. smilacinum*. A, micronuclei in minute grain, 4+ "micro" tetrad configuration. B, 3+1 tetrad. C, 2+2 tetrad. D, 1+3 tetrad. E, tetrad stage with one abnormal dyad containing two fragments. F, pollen grain with two fragments. 575 $\times$.

72.4–85.6% (S.D.=4.10). The grains observed were still surrounded by the original PMC wall. Two sizes of grains with various combinations of chromosomal fragments were observed (Fig. 2; Table 2). In the normal grains, the fragments which became micronuclei were usually separated distally from the major nucleus or the fragments were cytologically eliminated and formed minute pollen grains containing only the fragmental material.

Table 2. Abnormal tetrad summary and pollen classification
(Yatsuo, 10 plants; 862 abnormal tetrads; 3448 grains)

Tetrad configuration Normal cells + Abnormal cells	Number of tetrads	Avg. % of abnormal tetrads	Avg. % of total tetrads	Pollen grains	
				Normal	Abnormal
4+1 micro.	68	7.9%	1.7%	204	68
3+1 (1f)	530	61.5%	13.3%	1590	530
3+1 (2f)	58	6.7%	1.5%	174	58
3+1 (1f)+1 micro.	21	2.4%	0.5%	63	21
2+2 (1f)	116	13.5%	2.9%	232	232
2+1 (1f)+1 micro.	30	3.5%	0.8%	60	60
1+3 (1–2f)	34	3.9%	0.8%	34	102
0+4 (1f)	5	0.6%	0.1%	0	20
Totals	862	100.0%	21.6%	2357	1091

Since light squashing pressure was used, further classification of these abnormal tetrads into abnormal and normal grains was possible (Table 2). When these normal grains were pooled with the normal grains from the normal tetrads, a total of 14,909 (93.2%) normal grains and 1,091 (6.8%) abnormal grains resulted. In this way, the same plants had average fertility percents ranging between 91.1% and 95.1% (S.D.=1.25). Though these values of fertile pollen production are higher than their tetrad estimates (Tables 1, 2), they are quite similar to those obtained from the range-wide pollen fertility survey.

From another 10 individuals, 5,400 anaphase-telophase I PMCs were scored for their normalcy (Table 3). The normal dyad percentage was 91.3% (S.D.=3.87; range: 85.5–97.5%). The observed abnormalities which totaled 470 (8.8%; S.D.=1.56) ranged from bridges with short or long fragment to only fragments. Frequently the fragments had what appeared to be primary constrictions. The

Table 3. Dyad analysis (Anaphase-telophase I)
(Yatsuo, 10 plants; 5400 dyads)

Parameter	Dyads		Bridges plus		Fragment only	
	Normal	Abnormal	Short f.	Long f.	Short	Long
Number	4930	470	156	70	166	78
Average %	91.3%	8.7%	2.9%	1.3%	3.1%	1.4%
S.D.	3.87	1.56	1.03	0.61	1.47	0.96
% range	85.5–97.5%	2.5–14.5%	1.6–4.6%	0.7–2.3%	1.8–3.6%	0.5–2.5%

case of a bridge and no fragment was not observed among the 5,400 dyads. Bridges with either long or short fragments occurred 4.2% of the time, compare with 4.5% for fragments only. On the other hand, dyads with bridges and short fragments (2.9%) corresponded in frequency to dyads with short fragments only (3.1%). Similarly, the class with bridges and long fragments (1.3%) corresponded to the long fragment only class (1.4%).

Assuming that the normal dyads (91.3%) resulted in four normal pollen grains and that the abnormal dyads result in slightly less than the 8.7% abnormal grains, a second and confirming estimate is available for the amount of viable pollen production.

Meiosis and chiasma frequency: Knowledge of a species' somatic karyotype is crucial before a critical analysis of meiosis, especially chiasma frequency, can be undertaken. The diploid complement of this population (Fig. 3; cf. Utech and Kawano 1974) can be expressed as:

$$K(2n)=16=2 J_1+2 V_1+2 J_2+2 V_2+2 j_1+2 j_2+2 j_3+2 v,$$

Fig. 3. Somatic karyotype of *D. smilacinum* from Yatsuo ($2n=16$).

where each somatic element can be distinguished based on size and centromeric position. No clearly defined secondary constriction representing a nucleolar organizing region (NOR) or telomeric satellites were observed for this population. Neither was triploidy, aneuploidy or aneustomaty encountered in their somatic cells. The small number of relatively large, distinguishable chromosomes are well suited for further meiotic analysis. Easy cross identification is possible, due to the relative size correspondence between the somatic and meiotic karyotypes.

However, several types of abnormalities are reportable. These include:
i) precocious segregation (disjunction) of the smaller metacentric (no. 8 of Fig. 3)

and of the smaller subtelocentric members (no. 5–7 of Fig. 3), ii) interlocking bivalents, that do not involve an interchange (Fig. 4), and iii) a spontaneous interchange between pairs 4 and 5 (Fig. 4) which results in a quadrivalent (1 IV+6 II).

Fig. 4. Meiosis in *D. smilacinum*. A, $n=8\text{II}$, normal configuration. B, $n=6\text{II}+1\text{IV}$. C, $n=8\text{II}$, with three large bivalents interlocking. D, enlargement of C, showing the same three bivalents. Scale indicated.

The precocious segregation (disjunction) which occurred among the smaller bivalents could easily cause, without care, a miscount of $n=9\text{ II}$ or $n=10\text{ II}$, where $n=7\text{ II}+2\text{ I}$ (i.e. either 2 j or 2 v) or $n=6\text{ II}+4\text{ I}$ (i.e. $2\text{ j}+2\text{ v}$) more correctly expresses the situation. This type of disjunction occurred in 128 cells (3.2%) with the formation of a single pair being the commonest (74.3%). It is noteworthy here that the chiasma frequency of these smaller bivalents (Table 4) is the lowest among the genome. Due to the linear relationship between length and chiasma frequency, the smaller bivalents would merely terminalize sooner than the others. They shouldn't be construed as true transmissional univalents or asynaptic bivalents. Their size is similar to the fragments observed at anaphase I and may indicate in part a possible origin for these fragments, due to their partially impeded centromeric behavior.

The interlocking and interchange bivalents were scored separately, though some of the latter may have been the former. The interlocking bivalents, which usually involved two or three of the large pairs, occurred in nine of the 20 plants (48 cells; 1.2%), while figures classified as interchanges occurred in 14 plants and totaled 284 (7.1%) overall. Theoretically, the segregation of the latter, should yield bridges and fragments depending on the chiasma frequency within the interchanged segment. Since short and long fragments were observed with anaphase I bridges, it could be inferred that chiasmata occurred at different combination points, thus yielding these different fragments. It should be noted that fragments from an interchange without an observable anaphase I bridge may arise due to sister

chromatid fusion, though anaphase II bridges would result and were observed. Interlocking bivalents may also form bridges (side-arm) and fragments spontaneously due to breakage and cross chromatid fusion.

The chiasma frequency for each bivalent and their respective arms was analyzed for 50 genomes from five plants (Table 4). Due to the size similarity of the subtelocentric pairs 5-7, some error has been introduced into their scoring. However, the overall average genome and bivalent chiasma frequencies were 22.00 and 2.75 respectively. There was a higher frequency exhibited among the first four, larger bivalents (3.54) than for the four smaller ones (1.96). An essentially linear relationship (positive chiasma interference) exists between both the total bivalent and arms lengths and their respective chiasma frequencies.

Table 4. Chiasma frequency for Yatsuo population of *D. smilacinum*

Bivalent no. and arms	Total number	Chiasma frequency			Percent within bivalent	Percent of total
		Range	Average	S.D.		
1 Long	163	2-5	3.26	0.40	69.4%	14.8%
(J) Short	72	1-3	1.44	0.13	30.6%	6.5%
Total	235	3-7	4.70	0.53	—	21.3%
2 Long	144	2-4	2.88	0.41	68.5%	13.0%
(V) Short	66	1-4	1.32	0.21	31.5%	6.0%
Total	210	3-7	4.20	0.62	—	19.0%
3 Long	73	1-3	1.46	0.35	54.5%	6.6%
(J) Short	61	1-3	1.22	0.29	45.5%	5.5%
Total	134	2-5	2.68	0.64	—	12.1%
4 Long	72	1-3	1.44	0.22	55.8%	6.5%
(V) Short	57	0-2	1.14	0.20	44.2%	5.2%
Total	129	2-5	2.58	0.42	—	11.7%
5 Long	71	0-3	1.42	0.25	70.3%	6.4%
(j) Short	30	0-2	0.60	0.10	29.7%	2.7%
Total	101	1-3	2.02	0.35	—	9.1%
6 Long	81	0-3	1.62	0.29	71.7%	7.3%
(j) Short	32	0-2	0.64	0.13	28.3%	2.9%
Total	113	1-3	2.26	0.42	—	10.2%
7 Long	70	0-2	1.40	0.21	72.7%	6.4%
(j) Short	27	0-2	0.54	0.10	27.8%	2.4%
Total	97	1-3	1.94	0.31	—	8.8%
8 Long	43	0-2	0.86	0.22	53.1%	3.9%
(v) Short	38	0-1	0.76	0.18	46.9%	3.4%
Total	81	1-2	1.62	0.40	—	7.3%

Reproductive biology: To properly assess the evolutionary role of chiasma frequency and the permittable, chromosomal abnormality load, an overview of this species' reproductive biology is essential. Within a given population, a very high percent of the shoot are sterile, being annually produced by stoloniferous offshoots which are subsequently separated from the mother plant. This vegetative mode of reproduction is reminiscent of selfing annuals, since the same geno-

types are preferentially replaced. The fertile individuals, on the other hand, usually have a single (rarely two), pendulous, campanulate flower. The flower is also weakly protandrous and lacks specific floral pollinator adaptations. Whether some type of compatibility system is operative is unknown. Its floral morphology suggests an essentially open, generalized pollinator presentation, which contrasts strikingly with that of *D. sessile*.

Within a given flower, the tricarpellate gynoecium generally has three embryo sacs, while pollen production can reach 5.9×10^4 grains/flower. The floral ratio of ovules to grains (ca. 1:9800) is significant when chiasma frequency and chromosomal heterozygosity are considered. Because of this gametophytic ratio, if a similar abnormality load were to occur on the female side as the male, it would be statistically insignificant as far as transmissional analysis was concerned.

PMC chiasma frequencies are known (Table 4), whereas EMC frequencies are not. Recombination is involved in all the bivalents and represents a mechanism for heterozygous pollen production. One could therefore infer a certain heterozygosity in seed production, though the actual numbers produced are relatively small. Berries collected from Yatsuo the previous fall, for example, averaged 1.32 seeds/gynoecium ($n=50$; S.D.=0.42).

This forest perennial therefore utilizes two reproductive strategies: a successful vegetative propagation of preadapted genotypes and a limited production of genetically heterozygous seeds. An ecological and phenological appraisal of these two modes is currently in progress.

Discussion and summary

A pollen fertility survey for *D. smilacinum* established a type of pollen abnormality common throughout its range. The plants sampled span a 90 year period and suggest a more temporal aspect for their occurrence. A detailed analysis for a single population confirmed a similar pollen fertility, as estimated from three different meiotic stage, and provided a chromosomal mechanism for this abnormal pollen production. An average abnormal pollen load of less than 10% is implied. This load would appear acceptable, since it is compensated by a highly effective vegetative propagation mechanism and by a chiasma frequency which insures heterozygosity in its sexual reproduction. The latter would be instrumental in contributing to its evolutionary rate.

In *D. sessile* which is also common in Japan, the occurrence of an homologous range of pollen abnormalities is also known (Hasegawa 1933, Washiashi 1935). The production of unreduced pollen (Kayano 1961, Washiashi 1935) and triploidy (Hasegawa 1932, 1933) add further complications and distinctions to this species' karyology. A classical demonstration of the chiasma-type hypothesis involving a heteromorphic, reciprocal translocation was presented by Kayano (1960a, b) for this species. However, the artificial production of abnormal pollen by low temperature (7°C) induction (Washiashi 1935) and of unreduced pollen high in univalents by high temperature (31–36°C) induction (Kayano 1961) is relevant to the current discussion.

In *D. smilacinum*, meiosis occurs slightly below the ground or duff layer before canopy closure. At this stage, the underground shoot lengths are between 2–3 cm and the anthers measure ca. 1.4 mm. It is precisely in this vertical zone that the diurnal spring temperature fluctuations are the most intense. Ranges of 30°C or more would not be uncommon (Geiger 1965). The significance of these vertical micro-environmental temperature fluctuations are usually lost in monthly and annual averages. Subsequently, high and low abnormality induction temperatures would be encountered. Since meiosis is synchronized, the control and regulatory aspects of this temperature variation would be a crucial factor in maintaining variability and stability in pollen production.

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