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GENECOLOGICAL INVESTIGATIONS ON ZINC PLANTS II INTROGRESSION IN A SMALL POPULATION OF THE ZINC VIOLET VIOLA CALAMINARIA SSP. WESTFALICA (LEJ.) ERNST

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SUMMARY

The two main populations of *Viola calaminaria* ssp. *westfalica* (on a mine and a neighbouring meadow) have been compared. In both, the variability is considerable, but it is greater in the mine population, where hybrids with *V. arvensis* occur much more frequently.

Although the gene flow in the mine population must be considerable, the two populations have not diverged very much and there is no overlap in morphological characters between *V. calaminaria* ssp. *westfalica* and *V. arvensis*.

The latter can be explained by assuming that selection pressure for heavy metal tolerance is very strong. The former phenomenon is more complicated.

The isolation of the two populations is probably of recent origin, so they could not have diverged very much.

If this were the case, an equilibrium between gene flow and selection could explain the remarkable variation in these small populations.

1. INTRODUCTION

In continental Europe there are two subspecies of *Viola calaminaria:* a yellow flowering one in Eastern Belgium and a blue flowering one in Central Germany (See *fig. 1*).

Both subspecies are endemics of abandoned zinc and lead mines and mine tailings.

This paper deals with the blue flowering subspecies, *V. calaminaria* ssp. *westfalica* (Lej.) Ernst. Today there are two populations of this subspecies:

The first is found on an abandoned lead mine near the village of Blankenrode. Here, the dry, partly calcareous soil is very rich in zinc.

The second occurs on a meadow that receives its water from the zinc rich deposits where the first population is growing. A small brook, that originates on the mine, floods the meadow every winter. As a consequence, the soil has a high zinc content (although not as high as on the mine). Ernst (1968) describes the ecological situation in more detail.

Both populations exhibit a striking variation in a number of characters. Of these, flower colour is the most conspicious, but the width of the flowers, the

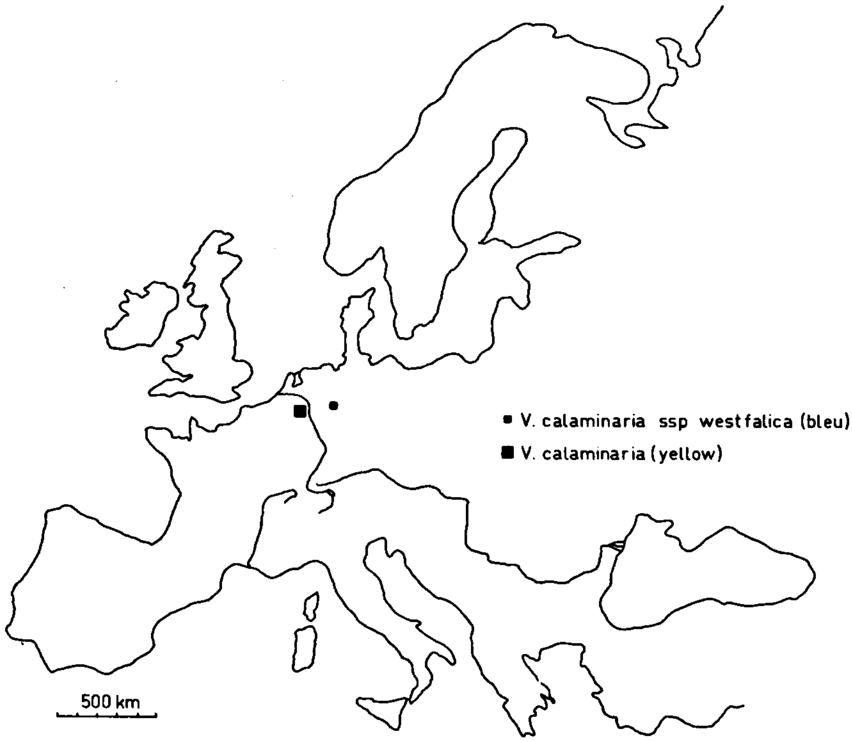

Fig. 1. Map showing the distribution of *V. calaminaria*.

diameter of the stems, the length and form of leaves and stipules are also very variable.

It is very unusual to see such an amount of variation in small isolated populations of very specialized plants.

In 1971 we found on the mine some aberrant plants that were tentatively identified as hybrids between *V. calaminaria* ssp. *westfalica* and *V. arvensis*. The aim of the present investigation was to determine if some of this variation could be contributed to introgression.

2. MATERIAL AND METHODS

Since 1971 all presumed hybrids and a number of normal looking plants have been marked and described.

In view of the small size of the population no transplants were made. All the greenhouse and garden experiments wer made on plants raised from seed. Seed-collections were made in two ways:

A. Seed collections of individual plants: During one or two weeks all the ripe capsules of marked plants were separately harvested.

B. Random seed samples from fifty plants as recommended by CLAUSEN (1960).

Citric acid extractable zinc was measured after MUNK (1956). The zinc determinations were made with a Perkin Elmer atomic absorption Spectrophotometer.

The pollen preparations were made after PETTET (1964). However, we added a treatment with 0.05 mol in 50% ethanol for $\pm$ 1 min. following the eosin staining.

3. RESULTS

The major differences between *V. calaminaria* ssp. *wesrfalica* and *V. arvensis* are shown in *table 1.*

The results of a biometrical investigation of natural populations of the two species are summarized in *table 2.*

Some floral characters are difficult to measure and were estimated. All measurements and estimates are means of five replicates per plant (ten for stipule length). The characters estimated are:
first: The amount of yellow in the flower. *V. arvensis* is white of yellow, sometimes with a faint blue hue.
V. calaminaria ssp. *westfalica* is violet-blue, always with a yellow spot on the lower petal. Hybrids have various amounts of yellow, starting with the lower petal, then the lateral petals and in extreme cases also the base of the uppermost petals. We estimated the proportion of the flower area that was white or yellow.
second: The labellum. This is a pliable flap of cells that separates the stigma from the pollen reservoir. It is present in the outbreeding *V. calaminaria* ssp. *westfalica,* but absent in *V. arvensis,* which is an inbreeder. *Figs. 2, 3* and *4* show

Table 1. Major differences between *V. calaminaria* ssp. *westfalica* and *V. arvensis.*

V. calaminaria ssp. *westfalica*	*V. arvensis*
Perennial	Annual
With a creeping rhizome, aerial stems not branched	Without a creeping rhizome, aerial stems branched
Flowers $>$ 20 mm, Violet	Flowers $<$ 17 mm, Yellow
Labellum more than 0.1 × the stylar knob	Labellum less than 0.1 × the stylar knob
Outbreeding, insect pollinated	Mainly inbreeding, but sometimes visited by insects
2n=52	2n=34
Majority of pollen four-colpate	Majority of pollen five-colpate

Table 2. Means and 95% confidence limits of some metric characters in natural populations of *V. calaminaria* ssp. *westfalica* and *V. arvensis*. All values in mm.

Population	Width of flower	n	Stem diameter	n	Leaf length	n	Stipule length	n
V. westfalica mine pop. (B)	23.86 ± 4.94	19	1.26 ± 0.38	20	23.76 ± 13.64	20	10.70 ± 8.91	20
V. westfalica meadow pop. (G)	28.18 ± 5.15	21	1.45 ± 0.48	21	29.85 ± 10.13	21	15.18 ± 5.61	21
Significance of difference B/G	$p < 0.001$		$p < 0.001$		$p = 0.001$		$p < 0.001$	
V. arvensis	11.82 ± 5.54	6	2.26 ± 1.58	6	36.58 ± 20.60	4	23.91 ± 10.16	5
Significance of difference B/arv.	$p < 0.001$		$p < 0.001$		$p = 0.10$		$p = 0.005$	

Table 3. Means and 95% confidence limits of some metric characters in *V. calaminaria* ssp. *westfalica*, *V. arvensis* and their hybrids. All values in mm.

Origin of plants	Width of flower	n	Stem diameter	n	Leaf length	n	Stipule length	n
V. westfalica mine pop. (B)	26.49 ± 6.61	24	1.77 ± 0.48	25	33.56 ± 10.66	28	17.60 ± 8.22	27
V. westfalica meadow pop. (G)	30.44 ± 6.14	25	1.92 ± 0.42	25	34.18 ± 9.35	24	19.54 ± 8.47	26
Significance of difference B/G	$p < 0.001$		$p = 0.96$		$p = 0.44$		$p = 0.02$	
V. arvensis	11.96 ± 1.71	25	2.61 ± 0.77	25	42.94 ± 7.66	25	24.85 ± 6.30	25
F_1 west. × arv.	24.58 ± 3.14	24	2.63 ± 0.42	24	n.d.		n.d.	
F_2 west. × arv.	14.92 ± 5.41	23	2.00 ± 0.53	25	33.76 ± 11.08	25	19.43 ± 6.91	25
B_1 west. × arv.	20.00 ± 9.02	27	2.15 ± 0.69	23	36.08 ± 13.03	29	22.58 ± 8.95	24

Fig. 2. *V. calaminaria* ssp. *westfalica*. Lateral view of a flower. One of the lateral petals and the lower petal have been removed to show the stigma and the labellum.

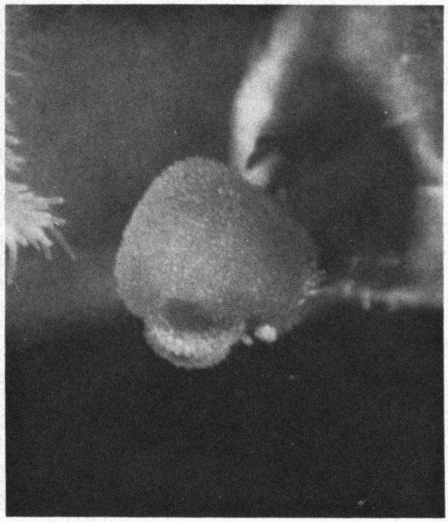

Fig. 3. *V. calaminaria* ssp. *westfalica*. Frontal view of the stigma with the labellum.

Fig. 4. *V. arvensis*. Lateral view of a flower. One of the lateral petals and the lower petal have been removed to show the stigma. Note the absence of the labellum.

flowers with and without labellum. The size of the labellum is estimated with a hand lens as the ratio a/b of *diagram 5*.

Fig. 6 summarizes the results of field observations on *V. calaminaria* ssp. *westfalica* and *V. arvensis*. The figures for the latter are population means, as it was impossible to take more than one measurement on each individual.

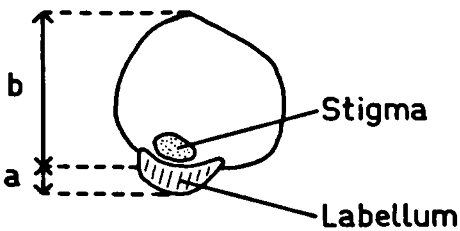

Fig. 5. Diagram showing how the size of the labellum was estimated.

The two violets are clearly separate species, but as we shall demonstrate later there is no strong reproductive barrier between them. This is not surprising since CLAUSEN (1931) showed that violets of the Melandrium section can be easily hybridized, even if they have different chromosome numbers.

In the field, hybrids are recognized by their small, light coloured flowers and branched stems. On closer inspection they often turn out to be intermediate in other characters as well.

Fig. 7 shows the position of the hybrid plants in relation to the parent species. They are always intermediate in some characters, but only rarely in all of them.

The progeny of hybrid plants is extremely variable. Sometimes we find plants that are intermediate in one or more characters, but have a 'normal' progeny.

Only plants of which the progenies were tested and found to be segregating are designated as hybrids in this paper.

If we plot the hybrids with the symbols used in *fig. 9* on a map of the mine (*fig. 8*) we see that their distribution is non random. Most are found along the northern and eastern edges of the mine. On these sides of the mine, cornfields and wasteland occur, where *V. arvensis* is abundant.

VEERMAN & VAN ZON (1965) studied the pollination by insects of pansies. They found that the pollinating bumble-bees fly over short distances. Our own observations show that *V. arvensis*, having small and not very conspicious flowers, is nevertheless visited by bumble-bees, be it less frequently than *V. calaminaria* ssp. *westfalica*. It is quite understandable that the proximity of the male parent plays a major role in the distribution of the hybrids. However, this may not be the only reason.

Zinc determinations show that the areas where hybrids occur have relatively low levels of acid extractable zinc (compare *figs. 8* and *9*).

We also compared plants of the two species grown in the experimental field with the F_1. (See KAKES & EVERARDS (1976) for the crossing procedure.) The results are summarized in *table 3* and *fig. 10*. The F_1 is intermediate in all characters that were measured or estimated, except the diameter of the stem, which is not different from *V. arvensis*. This is not surprising because the hybrids show considerable hybrid vigour.

If we compare *figs. 7* and *10*, we see that there is reasonable agreement between hybrids found on the mine and the experimentally produced F_1. Two differences stand out: Figures for diameter of stem and width of corolla are

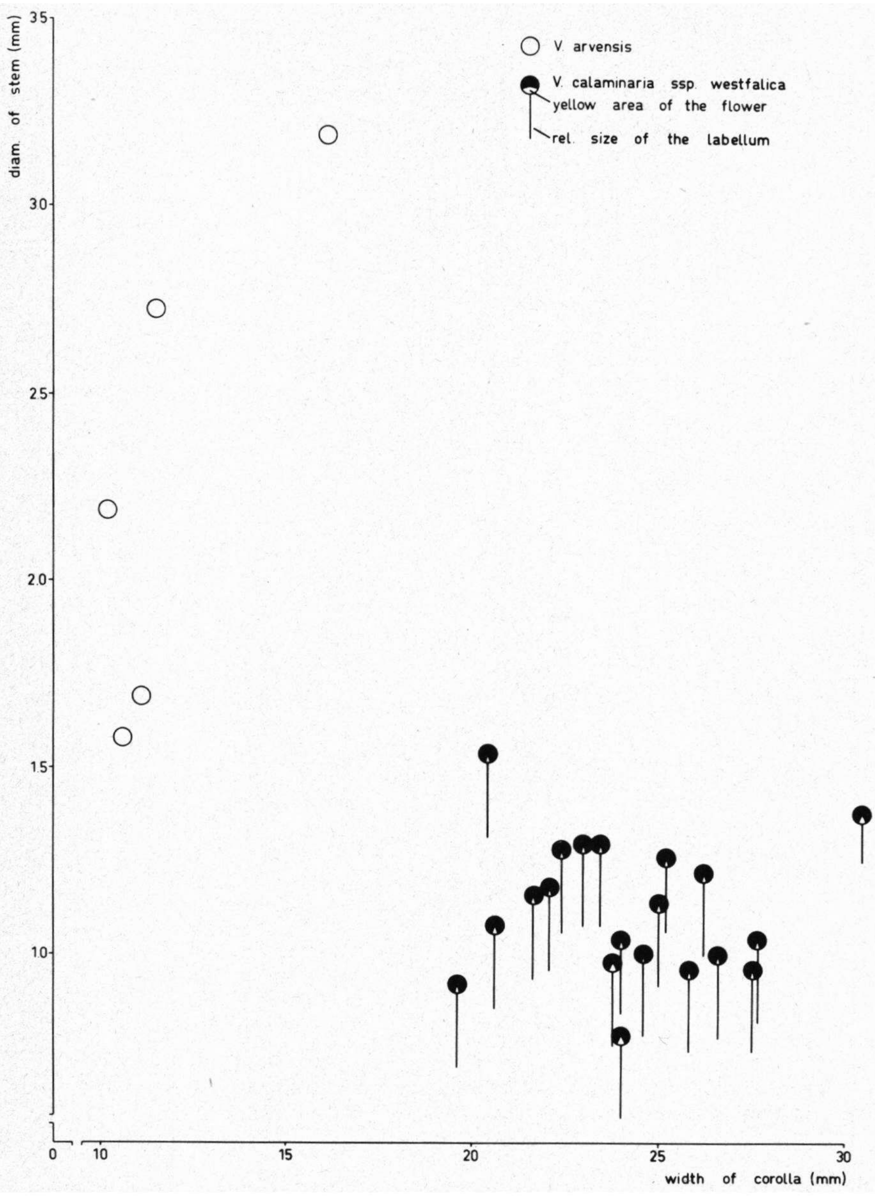

Fig. 6. Pictorialized scatter diagram of field populations of *V. calaminaria* ssp. *westfalica* and *V. arvensis*.

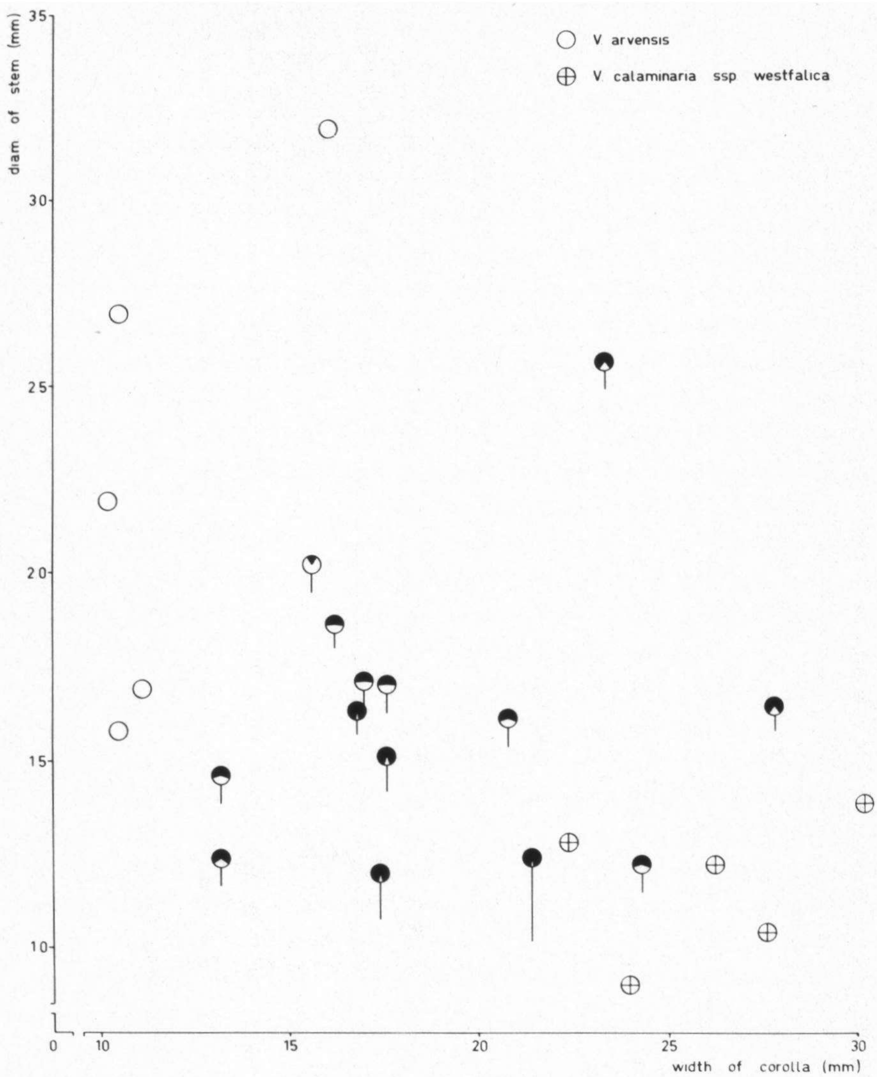

Fig. 7. Pictorialized scatter diagram of hybrids between *V. calaminaria* ssp. *westfalica* and *V. arvensis*. (Sub) population means of the parent species are indicated.

higher for F_1 plants and there is much more variation in hybrids on the mine.

Bearing in mind that the F_1 was raised under uniform and much "better" conditions then those existing on the mine, we may nevertheless assume that not all hybrids pictured in *fig. 7* are F_1's. That hybrid plants can behave quite differently, even if they are closely alike phenotypically, is illustrated by *figs. 11* and *12* showing the progenies of two hybrid plants.

Fig. 12 shows clearly the tendency to *V. calaminaria* ssp. *westfalica* whereas

Fig. 8. Map of the zinc mine showing the position of hybrid plants. Area's where *V. calaminaria* ssp. *westfalica* is dominant in the vegetation are shown in black.

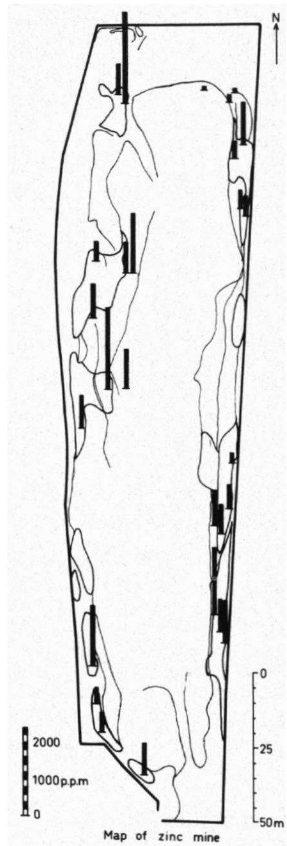

Fig. 9. Map of the zinc mine showing the levels of acid extractable zinc.

fig. 11 is tending to *V. arvensis*.

It is noteworthy that the female parent in *fig. 12* has a very small labellum, so the progeny could consist of selfings. The female parent of *fig. 11* has a labellum as big as that in *V. calaminaria* ssp. *westfalica*, so this progeny could mainly consist of backcrosses to the latter species.

To compare the hybrid plants and their progeny we constructed a hybrid index according to ANDERSON (1949). Our index is based on width of the flower, length of the labellum, diameter of the stem and the amount of yellow in the flower. *Table 4* gives the value of this index for the parent species, their F_1 and a number of marked plants. The plants B_1, B9b and B54 are nonhybrids, the rest are hybrids. The correlation hybrid index female parent – hybrid index progeny is significant at the 1 % level. These figures, being a sample of the large

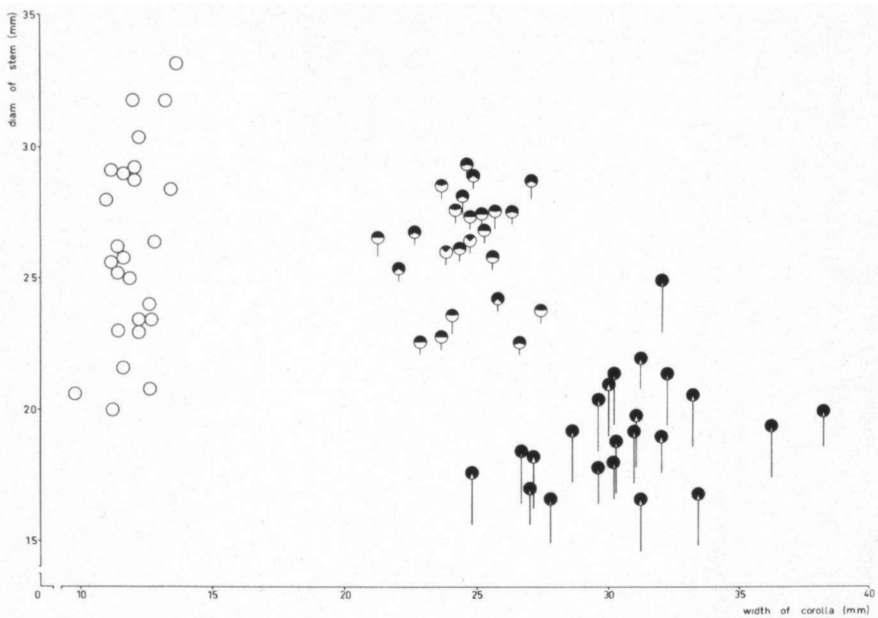

Fig. 10. Pictorialized scatter diagram of *V. calaminaria* ssp. *westfalica*, *V. arvensis* and their F_1 hybrid. Legend as in *fig. 6*.

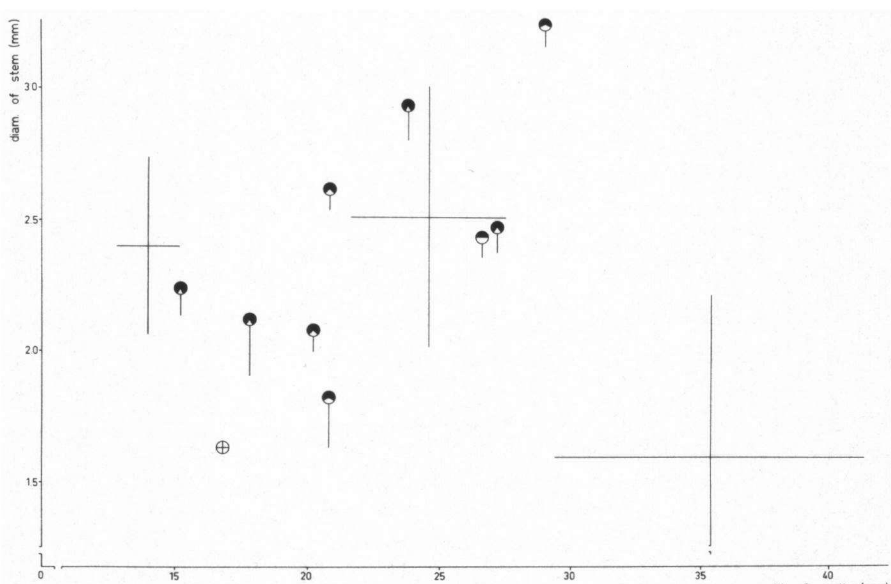

Fig. 11. Pictorialized scatter diagram of the progeny of a hybrid plant. Means and l.s.d. of the parent species and the F_1 are indicated.

Table 4. Mean and standard deviation of the hybrid index of *V. calaminaria* ssp. *westfalica*, *V. arvensis* and their hybrids.

Type of plant	Hybrid index	N. of plants	Hybrid index of female parent
V. calaminaria ssp. westfalica	34,5 ± 2,3	20	
V. arvensis	7,5 ± 1,0	20	
F_1 west × arv.	16,0 ± 2,0	24	
Progeny of a marked plant B_1	31,9 ± 2,0	10	30
Progeny of a marked plant B9b	35,7 ± 2,0	10	33
Progeny of a marked plant B54	13,5 ± 4,9	8	13
Progeny of a marked plant B46	26,8 ± 3,4	4	15
Progeny of a marked plant B47	20,9 ± 7,9	10	15
Progeny of a marked plant B49b	30,5 ± 3,3	11	24
Progeny of a marked plant B52i	19,3 ± 3,4	9	18
Progeny of a marked plant B54	31,6 ± 2,6	8	31

body of quantitative and qualitative data we have accumulated since 1972, prove that hybrid plants on the mine are genetically very different and in fact form a hybrid swarm.

The conclusion is strengthened by comparing *fig. 7* (naturally occurring hybrids) with *fig. 13* (F_2west × arv.) and *fig. 14* (B_1west × arv.). There is good agreement between naturally occurring hybrids and the artificial B_1, again

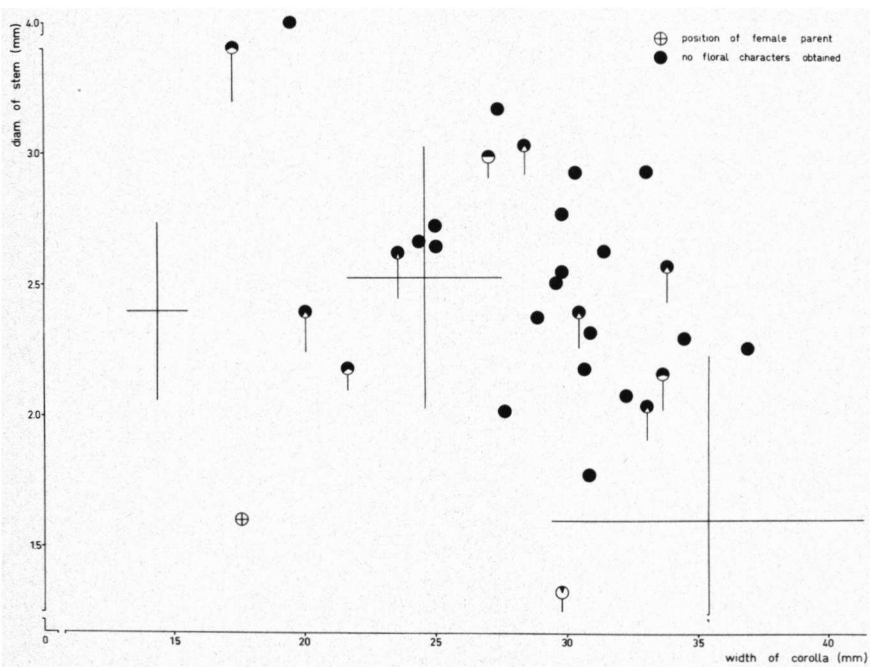

Fig. 12. Pictorialized scatter diagram of the progeny of a hybrid plant. Legend as in *fig. 11*.

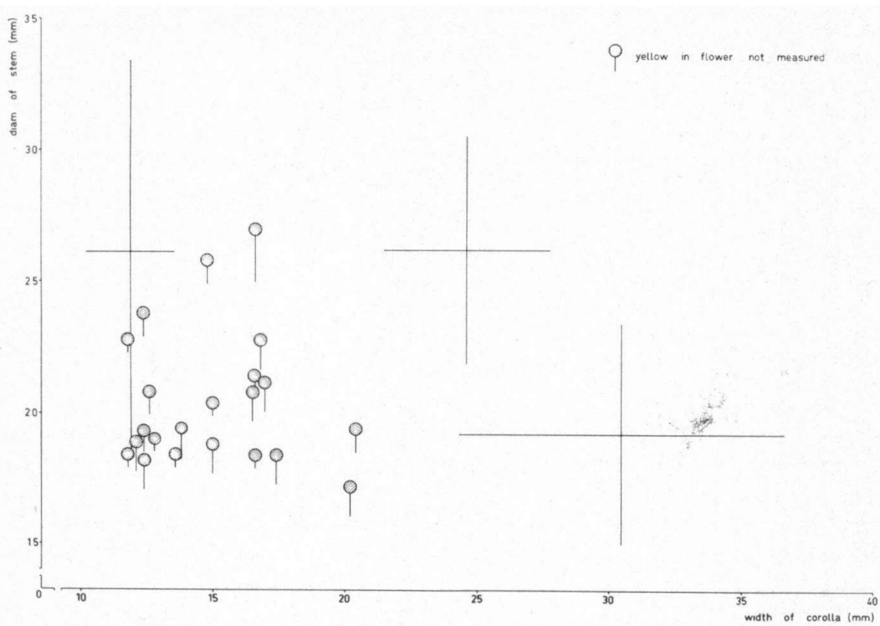

Fig. 13. Pictorialized scatter diagram of the F_2 west. × arv. Means and l.s.d. for the parent species and the F_1 are indicated.

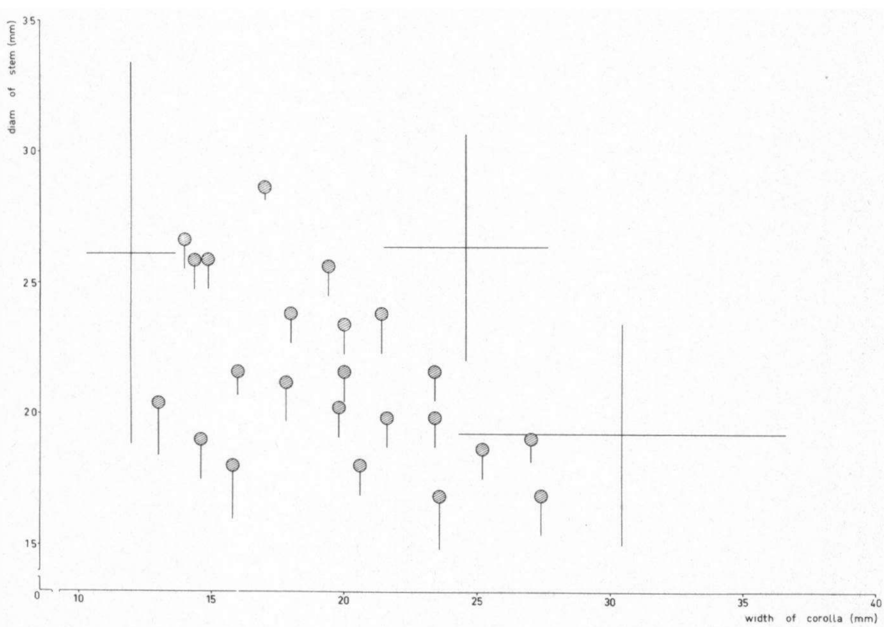

Fig. 14. Pictorialized scatter diagram of the B_1 west. × arv. Means and l.s.d. for the parent species and the F_1 are indicated.

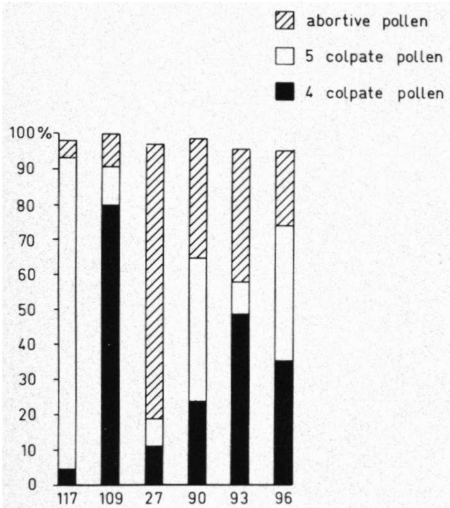

Fig. 15. Pollen assemblages.
117: *V. arvensis*
109: *V. calaminaria* ssp. *westfalica*
27: F_1 *west.* × *arv.*
90, 93, 96: Progenies of hybrid plants.

bearing in mind that measurements have been made under very different conditions.

Pollen morphology was studied in *V. calaminaria* ssp. *westfalica*, *V. arvensis*, their F_1 hybrid and in the progenies of some hybrid plants. The results are summarized in *fig. 15*. *V. calaminaria* ssp. *westfalica* has mainly four colpate pollen and *V. arvensis* mainly five colpate.

The F_1 has about equal percentages of four- and five-colpate pollen, but with a high proportion of abortive cells. Apparently meiosis is very irregular and non-functional gametes are formed. In later stages, the reproductive capacity of the F_1 is also reduced: germination is 32% compared to 59% for *V. calaminaria* ssp. *westfalica* and close to 100% for *V. arvensis*.

The progenies of hybrid plants have very different percentages of four and five colpate pollen; the frequency of abortives is always lower than in the F_1.

From random seed samples of the mine we obtained 16 hybrid plants on a total of 2080 (*table 5*). As the seeds of hybrid plants on the mine were separately

Table 5. Numbers of hybrid plants in random seed samples of *V. calaminaria* ssp. *westfalica*.

Year	'Mine		'Meadow'	
	total	hybrids	total	hybrids
1972	555	6	108	0
1973	453	4	378	0
1974	250	3	153	0
1975	542	3	188	0
1976	280	0	98	0
	2080	16	925	0

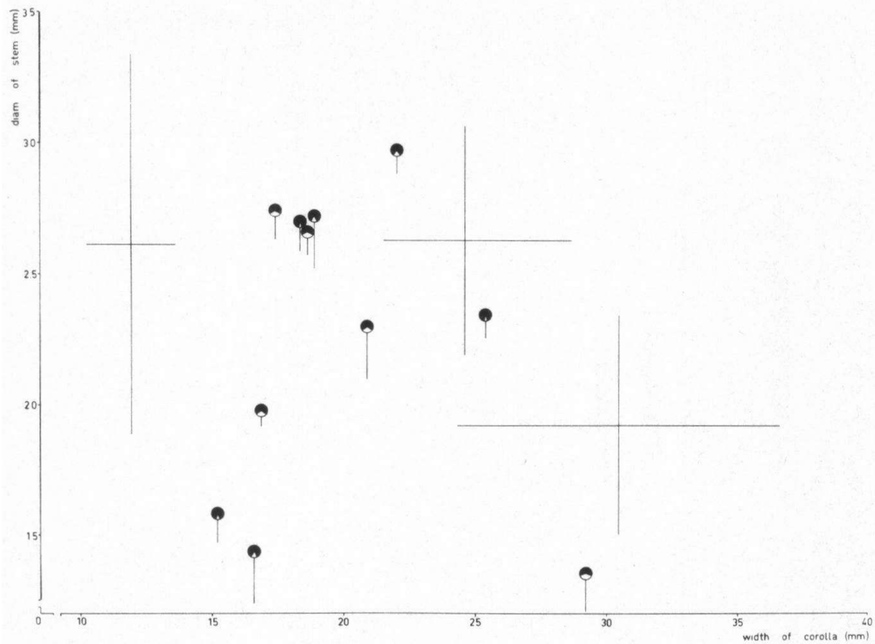

Fig. 16. Pictorialized scatter diagram of hybrids found in random seed samples of *V. calaminaria* ssp. *westfalica*. Legend as in *fig. 11*.

harvested, the former must be the result of pollen flow. Presumably they represent B_1- and later backcross generations. Their position is given in *fig. 16*.

We will now consider the second population of *V. calaminaria* ssp. *westfalica*, viz. that on the zinc meadow.

The ecological situation here is completely different from that on the mine. The soil is a heavy loam and can be very wet in winter and early spring. The amount of acid extractable zinc is fairly high but not as variable as on the mine. In short, we have here a much more uniform and sheltered environment.

From *table 2* we learn that the meadow population has higher values for all characters measured. However, these differences are not significant in plants grown from random seed samples, except the width of the flower (see *table 3*). The latter character must have a genetic basis, the other differences between the two populations can be modifications, due to the different environments.

Two hybrid plants have been found on the meadow since the start of the investigation, but these plants have not maintained themselves longer than one year.

Among 925 plants grown from random seed samples obtained on the meadow, no hybrids were found.

4. DISCUSSION

We conclude from the data given that hybrids between *V. calaminaria* ssp. *westfalica* and *V. arvensis* are formed and can maintain themselves on the mine, especially in border situations with relatively low levels of soil zinc.

The fertility of the hybrids is distinctly lower than that of *V. calaminaria* ssp. *westfalica*, but this is, at least partly, compensated by the hybrid vigour shown by the F_1. This hybrid vigour is particularly strong under culture conditions, but also visible in the field if the plants grow in soil with very low zinc levels. The hybrid plants have, moreover, the advantage of being perennial like *V. calaminaria* ssp. *westfalica*, so once a hybrid is established, it can influence the population for a number of years.

Therefore, we expect a hybrid swarm between the parent species. That this is indeed the case, can be seen by comparing *fig. 7* and *16* with *fig. 13* and *14*.

The hybrids fill the gap between the parent species and their extremely variable progenies show very different combinations of parental characters.

However, the recognition of a rather strong introgression in *V. calaminaria* ssp. *westfalica* raises the question how a very small population can maintain its individually in spite of this constant dilution of its gene pool. As we have seen that hybrids predominate in areas with relatively low zinc content, it is reasonable to assume that hybrids have a low tolerance to zinc and thus are strongly selected against in the greater part of the mine.

Preliminary tests have shown that the F_1 as well as natural hybrids have an intermediate zinc tolerance. The tolerance of the progeny hybrid plants seems to be very variable. However, we feel that more evidence is needed here.

As we saw, the only genetic difference found between the mine- and meadow populations is the size of the flower, the mine population having significantly smaller ones. This happens to be the most conspicious difference between *V. calaminaria* ssp. *westfalica* and *V. arvensis*.

Therefore, although there is evidence of a general influence of *V. arvensis* in the mine population, this influence is no longer apparent in the meadow population.

Although the distance between the two populations is small (1300m), they must be fairly well isolated at present. It has been shown (VEERMAN & VAN ZON (1965)) that even smaller distances prevent pollen transport by insects in *Viola*.

In 1972 a highway was constructed just between the meadow and the mine. Although the populations themselves have not been touched, the situation between them has drastically changed. ERNST (1968) supposes that the zinc vegetation originated on the slopes between the mine and the meadow, where the ore deposits struck the surface before they were exploited.

Even now, *Cardaminopsis halleri*, one of the few zinc tolerant plants that can grow in the shadow, occurs all along the brook that runs from the mine to the meadow, and a small population of *V. calaminaria* ssp. *westfalica* is present on a clearing in the woods about 500 m from the meadow.

Therefore it seems very likely that the isolation of mine- and meadow-violets is a quite recent phenomenom and that they once were connected by numerous small populations.

If we assume that *V. arvensis* has been present around the mine for a fairly long time, we arrive at the following picture: Introgression has been going on for a considerable time and is continuously adding to the variability of *V. calaminaria* ssp. *westfalica*. The two species have remained distinct however, partly because they only meet in a part of the mine, and partly because of selection against the *arvensis* genome in the zinc rich habitat. Since the two populations are now genetically isolated it could well be that the mine population continues to diverge from the stabilized meadow population. Perhaps the difference in flower length is a first indication of this.

An alternative view is that an equilibrium has been established between gene flow and selection. Hybrid plants can only maintain themselves in microniches with low zinc levels. Differences between the mine and meadow populations are caused not only by absence of introgression in the latter, but also by its much more homogeneous environment, where occasional hybrids do not survive.

Only a prolonged observation of the two populations can provide the evidence necessary to reject one of the hypotheses.

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