Evolution of Viola stagnina and its sisterspecies by hybridisation and polyploidisation

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Chapter

6

Phenotypic plasticity of *Viola stagnina* (Vals melkviooltje)[8]

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At the beginning of the previous century a new variety of *Viola stagnina* Kit. 1824 (syn. *V. persicifolia* auct. non Schreb., Vals melkviooltje) was described, var. *lacteoides* W. Becker & Kloos 1924, endemic to The Netherlands. A recent study demonstrated that this variety is morphologically and genetically distinct from var. *stagnina*, confirming the taxonomic status of a separate variety. In this study, we provide additional evidence for this taxonomic delimitation. Based on a SEM study of fully developed flowers, we conclude that the reported differences in stigma shape are inconsistent. A common garden experiment demonstrated that plant height, leaf color, and stipule size and shape all display large phenotypic plasticity. However, differences in petiole length and lamina size, coinciding with the delimitation of the varieties, have a genetic basis. Furthermore, a crossing experiment and chromosome count provide evidence that the two varieties are not reproductively isolated, yet. Finally, we discuss the nomenclature of the two varieties of *V. stagnina* and formally describe the new combination: *V. stagnina* var. *lacteoides* (W. Becker & Kloos) van den Hof.

Keywords: chromosome count, crossing experiment, nomenclature, phenotypic plasticity, *Viola stagnina* var. *lacteoides*

[8]van den Hof et al., submitted to Plant Ecol. Evol.

Introduction

The European Fen Violet (*Viola stagnina* Kit. syn. *V. persicifolia* auct. non Schreb., Vals melkviooltje) is a widespread but rare plant species, occurring throughout Europe with the exception of the Mediterranean, the southeast and extreme north (Hulten and Fries, 1986; Fig. 10). Populations of *V. stagnina* from Great Britain, Belgium and The Netherlands lie on the western margin of the species' distribution range. In Belgium, the species is considered to be nearly extinct (Zwaenepoel and Vanallemeersch, 2007). In The Netherlands, *V. stagnina* is known from several localities in the Rhine delta and IJssel valley (Fig. 10). Today, only 11 Dutch localities are known where *V. stagnina* still occurs.

Viola stagnina is a pioneer species favoring wet and temporarily flooded, sunny habitats such as floodplains, fens and marshes (Valentine et al., 1968; Eckstein et al., 2006a; Weeda, 2002). In nutrient-rich environments, it is dependent on regular disturbance to successfully compete with other plant species (Eckstein et al., 2006a; Hölzel, 2003). The species can grow on both basic and acidic soil types. The drainage of wetlands and canalization of rivers and brooks have led to a strong decline of *V. stagnina* in many parts of Europe (Weeda, 2002).

Viola stagnina is a member of sect. *Viola* subsect. *Rostratae* (Kupffer) W. Becker, and belongs to a small group of floodplain species characterized by the lack of a basal leaf rosette and frequently referred to as series *Arosulatae*. *Viola canina* L. (Hondsviooltje), *V. elatior* Fries (Hoog viooltje), *V. lactea* Sm. (Echt melkviooltje), and *V. pumila* Chaix (Klein melkviooltje) are the other members of the *Arosulatae* series, which can be found in Belgium. In The Netherlands, the arosulate violets are only represented by *V. canina* and *V. stagnina*. Morphological, cytological and molecular studies have pointed out that *V. stagnina*, as a paleotetraploid (2n = 20), was involved in the polyploid origins of all the other arosulate species, by autopolyploidy in *V. elatior* (2n = 40) (Clausen, 1927; van den Hof et al., 2008) and by allopolyploidy in *V. canina* (2n = 40), *V. lactea* (2n = 40), *V. pumila* (2n = 40) and *V. lactea* (2n = 58) (Valentine, 1958; Moore and Harvey, 1961; van den Hof et al., 2008).

In many European floras, including the latest editions of the Flora of Belgium, the Grand Duchy Luxemburg, north-France and the adjacent areas (Lambinon et al., 2004), and the Heukels' Flora of The Netherlands (van der Meijden, 2005), *V. stagnina* is mentioned under the name *V. persicifolia* Schreb. However, a nomenclatural study (Danihelka et al., in review[5]) has pointed out that this name should be interpreted as referring to *V. elatior* and the name *V. persicifolia* is therefore proposed for rejection (van den Hof et al., in review[4]). We use the unambiguous name *V. stagnina* in the present publication.

In The Netherlands, two morphs of *V. stagnina* have been described, var. *stagnina* and var. *lacteoides* W. Becker & Kloos (1924) (Fig. 9). This second morph was by Dutch botanists long held to belong to the related *V. lactea* Sm. (Kloos, 1924). Kloos (loc. cit.) was the first to identify it with *V. stagnina*, and after having consulted the Swiss *Viola* expert W. Becker, they concluded that these specimens did not belong to *V. lactea* but to a new morph of *V. stagnina*, endemic to The Netherlands, which they named *V. persicifolia* var. *"lacteaeoides"* W. Becker and Kloos (1924). As the editor of the genus *Viola* in the flora of Heimans et al. (Kloos, 1924), Kloos introduced this variety to the Dutch flora.

In 1927, *V. stagnina* var. *lacteoides* was mentioned for the first time in Heukels' Schoolflora voor Nederland. Dutch botanists after Kloos, however, had different opinions about the subdivision of *V. stagnina* into two infraspecific taxa and in the following editions

of this flora, the varieties were not mentioned anymore. In the 1977 edition (van Oostroom, 1977), the varieties are mentioned again, this time as subspecies. Den Held described subsp. *lacteoides* in the addenda, saying that its stigma is straight as compared to hooked in subsp. *stagnina*, and that the spur of subsp. *lacteoides* exceeds the calycine appendices which is normally not the case in subsp. *stagnina*. The next edition of the Heukels' flora (van der Meijden, 1983) noted that the taxonomy of the species was being investigated and that the infraspecific taxa within *V. stagnina* were being treated as varieties again, until further notice. In the next edition of the Heukels' flora (van der Meijden, 1990) the differences between the morphs were again considered too small to warrant even infraspecific recognition. In anticipation of the results of the present study and because of preliminary results of a common garden experiment, van der Meijden reinstated the two varieties again in the last edition of the Heukels' flora (van der Meijden, 2005). Weeda (2001, 2002) devoted two papers to *V. stagnina* in The Netherlands. Strongly disagreeing with van der Meijden (1990), Weeda pleaded for a resurrection of the subdivision of *V. stagnina* into two varieties based on the morphological differences mentioned by Kloos (1924) and den Held (in van Oostroom, 1977), but also because in The Netherlands the two morphs of *V. stagnina* have different geographical distributions with only a small overlap. The *stagnina* morph is found in the Holocene part of The Netherlands where it grows mainly in fen meadows and on the floodplains of river and brook valleys. The main distribution of the *lacteoides* morph, on the other hand, is restricted to the Pleistocene part of The Netherlands, where it is found mainly in the valley of the river IJssel on the lower parts of wet heathlands on loamy and peaty soil (Weeda, 2001).

Van den Hof et al.[9] (submitted) intended to settle the ongoing debate among Dutch botanists about the taxonomic status of the two *V. stagnina* morphs by employing the DNA fingerprinting technique AFLPs and by studying macromorphological characters of *V. stagnina* and its closest relatives. They concluded that there are indeed two different morphs of *V. stagnina* present in The Netherlands which can best be recognized as varieties. In the present paper, we provide additional evidence for the fact that we are dealing with two separate varieties of *V. stagnina* 1) by studying phenotypic plasticity of several additional (micro)morphological characters, 2) by testing infraspecific compatibility by means of an infraspecific cross, and 3) by carrying out chromosome counts. In the publications after Kloos' first description, the epithet of the *lacteoides* morph was spelled in many different ways. We therefore also investigated the nomenclature of its scientific and common names and formally describe its new combination under *V. stagnina*.

Material and Methods

Flower morphology

Fully developed flowers were fixed in FAA (18:1:1 of ethanol (50%), acetic acid formalin and water). Samples were dehydrated through ethanol series and dried with a Balzers CPD 030 critical point drier. Dried samples were mounted, sputter-coated with platinum in a BAL-TEC SCD 005 and observed with a JEOL JSM-5300 Scanning Electronic Microscope (SEM). Spurs and stylar heads of both morphs of *V. stagnina* were digitally photographed.

[9]Chapter 5 of this thesis.

Common Garden experiment

To investigate whether vegetative characters such as stipule length, petiole length, lamina size and color are environmentally or genetically controlled in *V. stagnina*, a common garden experiment was carried out. A total of six seedlings from both varieties of *V. stagnina* were collected in the spring of 2008. Individuals of var. *lacteoides* were gathered in Kienveen, a locality near Zutphen. Individuals of var. *stagnina* were gathered from the Bennekomse Hooilanden near Wageningen. Both sites were chosen because individuals could be clearly identified as belonging to either one of the varieties, and because at both localities a relatively large population was present. The seedlings harvested were transplanted to an indoor nursery and grown under moderate light conditions in a substrate containing peat, forest soil and sand. The mean temperature at this nursery was 20 ºC. Measurements on lamina size, petiole length, and stipule length were made after seven months on fresh leaves using calipers. In total, three plants per population were measured.

Seed viability

Manual cross pollinations were carried out between both *V. stagnina* varieties in order to determine whether cross pollinated plants could produce viable seeds. After manual pollination in the field, the plants were bagged to prevent additional pollination by insects. This was done with individuals from both varieties. After six weeks, all resulting seed capsules were harvested. Seeds were stained by macerating them in a 50% lactic acid solution for five days. Viability was assumed when seeds contained an embryo.

Chromosome counts

Freshly harvested root tips of *V. stagnina* var. *lacteoides* plants were fixed in a Carnoy solution (3:1 solution of ethanol and acetic acid, respectively) for at least 24 hours. After fixation, the root tips were transferred to an aceto-carmine solution and shortly boiled. After staining, mitosis of cells in the root tips was observed using Light Microscopy (LM) at 1000x magnification.

Results

Flower morphology

SEM pictures of fully developed flowers of both morphs of *V. stagnina* revealed that stigma shape and spur length as reported by den Held (in van Oostroom, 1977) are variable within each variety. Individuals of both varieties had stigmas that were either hooked or straight (Fig. 17). Spur length varied between 4.5-9.5 mm , 4.0-9.0 mm for var. *stagnina* and var. *lacteoides*, respectively, thus showing an overlap of 90%.

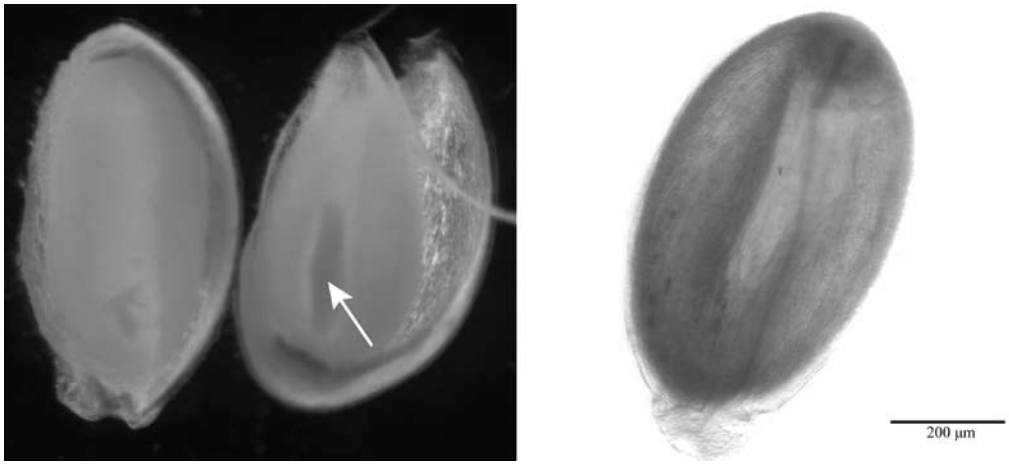

Fig. 17. SEM pictures of the stigma of a mature flower of *V. stagnina* var. *stagnina* (Veenmelkviooltje) at the right, and *V. stagnina* var. *lacteoides* (Heidemelkviooltje) at the left. The white lines indicate the amount of curving.

Common Garden experiment

Differences in lamina color and plant height between both morphs of *V. stagnina* disappeared in the common garden experiment. The leaves of var. *stagnina* became darker, while those of var. *lacteoides* became lighter. Although plants from both varieties grew much bigger than usually observed in the field, the initial differences in petiole length and lamina size remained present. Stipules of both varieties became much more reduced as compared to those of plants in the wild and initial length differences disappeared.

Seed viability

In total, 62 seeds were gathered from the cross-pollinated plants of var. *lacteoides*, of which 59 were considered to be viable (95.2%). The cross-pollinated var. *stagnina* plants yielded 118 seeds, of which 111 were considered to be viable (94.1%) (Fig. 18).

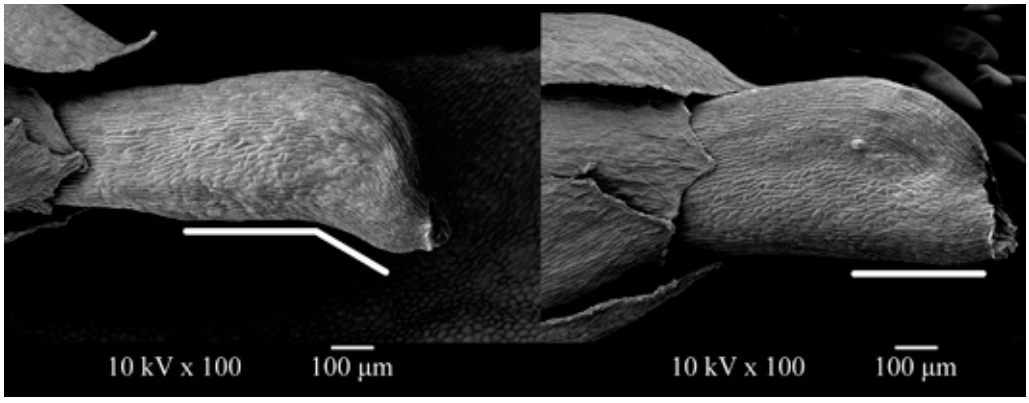

Fig. 18. LM pictures of seeds of crosses between *V. stagnina* var. *lacteoides* (Heidemelkviooltje) and *V. stagnina* var. *stagnina* (Veenmelkviooltje). At the right, a viable seed containing an embryo (indicated with arrow), at the left an aborted seed without embryo.

Chromosome counts

We could not find a single good cell in which all chromosomes were nicely aligned at the equatorial plane in such a way that the chromosomes could be easily counted or even photographed. However, by examining multiple cells in the metaphase stage of mitosis in different root tips, we could determine that *V. stagnina* var. *lacteoides* has 2n=20 chromosomes just like *V. stagnina* var. *stagnina* (Valentine, 1958; Moore and Harvey, 1961).

Discussion

Flower morphology

In contrast to den Held, we consider the described difference in stigma shape between both varieties of *V. stagnina* too variable. Samples of both varieties had straight and hooked shaped stigmas. Therefore, this character is of no use to distinguish between var. *lacteoides* and var. *stagnina*. Presumably, the occasional presence of hooked stigmas in chasmogamous flowers is probably attributed to transitions towards cleistogamous flowers which are self-pollinating and occur later in the season in both varieties. Another floral character that has been used to distinguish between *Viola* species is the indument of the style. This character is, for instance, used to distinguish between *V. laricicola* and *V. riviniana* (Marcussen, 2003), where *V. riviniana* has a densely papillose stigma, while *V. laricicola* has a glabrous stigma. In both *V. stagnina* varieties, however, papillose and glabrous styles were found. Floral characters that are significantly different between both varieties are quantitative. The lateral and ventral petals of var. *lacteoides* are less wide than those of var. *stagnina*. Furthermore, the fully developed sepal appendages are significantly longer in the var. *stagnina* causing the spur to exceed less than is the case in var. *lacteoides*, without actually being shorter. This difference in length of the sepal appendages between the two varieties is probably not caused by a shift in pollinator preference, because the spur length does not significantly differ between var. *lacteoides* and var. *stagnina*. The spurred flower of most *Viola* species is adapted to a wide array of pollinating insect species with medium and long sized tongues (Beattie 1971, 1974). Although pollinators of *V. stagnina* have never been studied, it is unlikely that this species has developed a very specialized pollinator preference, because its flower morphology is highly similar to those of other *Viola* species adapted to a variety of pollinating insects (Beattie 1971, 1974). The differentiation between the two varieties therefore is probably not caused by a shift in pollinator preference but by environmental factors linked to the different habitats.

Common Garden experiment

Leaves are considered very responsive to the light intensities under which they develop (Dengler, 1994) and the light environment is considered an important determinant of leaf form. Differences observed between 'sun' and 'shade' leaves are usually large (Evans et al., 1988) and many studies demonstrate fundamental differences in form and

function of sun and shade leaves. Differences in plant height, leaf color and stipule length between both morphs of *V. stagnina* disappeared in our common garden experiment which indicates that these characters are responsive to environmental conditions and display a large phenotypic plasticity. Differences between the two varieties in petiole length and lamina size, however, remained present over time, indicating that these characters are probably genetically determined.

In a previous study by Bergdolt (1932), it is stated that leaf color of several different *Viola* species is probably not influenced by abiotic factors, and that it can be considered as a good character for species recognition. However, in the same study he demonstrates that the abaxial side of *V. canina* leaves can become darker colored under the influence of light. This is a common response in plants and may also be the case for *V. stagnina*, it being one of the progenitors of *V. canina*.

Specimens of *V. stagnina* var. *stagnina* mostly grow on floodplains of rivers and brooks, or lakeshores as is mostly the case in Scandinavia. In these environments, the plants are regularly flooded. Specimens of *V. stagnina* var. *lacteoides*, however, grow in wet heath lands, which are only flooded irregularly by rainfall. Soils in which var. *lacteoides* plants grow are also more sandy as compared to soils in which var. *stagnina* usually grows, causing the soil to dry out sooner. *Viola stagnina* var. *lacteoides* plants are therefore required to respond more often to periods of drought, which may account for their smaller habit size and thicker leaves.

Seed viability

The viability of the seeds resulting from our infraspecific cross indicate that both morphs of *V. stagnina* are not reproductively isolated. Experimental crosses between *V. stagnina* and *V. canina* also resulted in the production of many viable seeds. The seeds of these interspecific hybrids ultimately produced well developed but completely sterile plants (Røren et al., 1994). Hybrids of *V. stagnina* with a number of other species of subsect. *Rostratae* are known, but these are all sterile (Moore and Harvey, 1960). Future research with the F_1 resulting from the cross between var. *lacteoides* and var. *stagnina* should point out whether these infraspecific hybrids are fertile or sterile.

Chromosome counts

The chromosome number of 2n=20 of *V. stagnina* var. *lacteoides* indicates that Kloos was indeed right by ascribing this morph to *V. stagnina* and not to *V. lactea* (2n=58) as did the botanists before Kloos. A closer relationship with the other arosulate violets than *V. stagnina* is also unlikely since *V. canina*, *V. elatior*, and *V. pumila* are all octoploids (2n=40).

Another hypothesis put forward by Weeda (2002) that *V. stagnina* var. *lacteoides* might be the result of introgression between *V. stagnina* and the hybrid *V.* x *ritschliana* can also be considered as improbable. F_1 hybrids may also produce occasional gametes with unreduced chromosomes, in this case n=10 and n=20. Introgressed F_2 individuals might therefore have the normal chromosome number of either 2n=20 or 2n=40, making it impossible to detect these introgressed individuals by examining their chromosome number (Røren et al., 1994). In an investigation of chromosome numbers, morphology and fertility in numerous populations of *V. stagnina* and its hybrid with *V. canina* in southern

Norway, Røren et al. (1994) found no evidence of introgression between the two species. Although introgression can have occurred in the case of *V. stagnina* var. *lacteoides*, it is very unlikely. The AFLPs data from both *V. stagnina* morphs and allies by van den Hof et al. (submitted) show that accessions of the hybrid *V.* x. *ritschliana* are very closely related to *V. canina*, while all var. *lacteoides* accessions are very closely related to the common *V. stagnina* variety. When var. *lacteoides* would have been the result of introgression between *V. stagnina* and *V. canina*, the accessions of var. *lacteoides* are expected to be closer related to *V. canina* than to *V. stagnina*.

Nomenclature

In The Netherlands, the common name for *V. stagnina* is Melkviooltje, In Belgium, however, the species is known as Vals melkviooltje, because two closely related *Viola* species occur there with a similar name: *V. lactea* (Echt melkviooltje) and *V. pumila* (Klein melkviooltje). To avoid confusion, we therefore recommend changing the Dutch common name of *V. stagnina* from Melkviooltje into Vals melkviooltje.

The Dutch variety of the Fen Violet was first published as *V. persicifolia* var. *lacteaeoides* W. Becker and Kloos (Kloos, 1924). For a number of different reasons, this taxonomic name should be changed. First of all, Danihelka et al. (in review) and van den Hof et al. (in review) explained why *V. persicifolia* should be changed into *V. stagnina*. Secondly, the correct merge of the two elements 'lactea' and 'oides' from the orginal epithet is 'lacteoides', because it is a compound formed from lactea and '-oides', denoting resemblance. The genitive case of lactea is lacteae. In compounds these 'ae' endings are removed. The suffix '-oides' should in this case be added without a connecting 'i' because 'lacte' ends with a vowel. The correct declination for the Dutch variety of the Fen Violet is therefore *V. stagnina* var. *lacteoides* and the previously used adjectives 'lacteaeoides' (Kloos, 1924; van der Meijden, 2005), 'lacteoïdes (Heimans et al., 1965) and 'lactaeoides' (van der Meijden, 1990) are incorrect. We describe the following new combination:

Viola stagnina Kit. ex Schult. var. *lacteoides* (W. Becker & Kloos) van den Hof comb. nov.: *Viola persicifolia* var. *lacteaeoides* W. Becker & Kloos. Nederlandsch Kruidkundig Archief 33: 192. 1924.

This variety of *V. stagnina* differs from the more common variety of *Viola stagnina* in its shorter petioles, and smaller lamina. Furthermore, the dorsal and ventral petals are more narrow than those of the common variety and the calycine appendages are shorter so that the spur exceeds the calycine appendages. The variety occurs in wet heathlands on loamy and sandy soil as opposed to fen meadows in river floodplains and brook valleys on loamy and peaty soil where the more common variety occurs.

Key to the arosulate *Viola* species in The Netherlands and Belgium:

1. - Stipules of the upper leaves as long as the petiole or exceeding the petiole. → 2
   - Stipules usually shorter than 2/3 of the petiole, sometimes as long as of the petiole of the upper leaves, but never exceeding the petiole. → 4
2. - Plants puberulent from slightly downwards-pointing hairs; lamina of the middle and

upper stem leaves lanceolate, at the base truncate or rarely subcordate; tall, robust, erect plants (20 - 50 cm). → ***V. elatior* Fries.**
- Plants glabrous or very sparcely pilose. → 3

3. – Spur of the ventral petal clearly exceeding the calycine appendages; flowers very pale blue to white with distinct dark reddish or purplish venation; lamina of the middle and upper stem leaves narrowly ovate to ovate, at the base cuneate, rarely rounded. plants 7 – 25 cm tall. → ***V. lactea* Sm.**
- Spur of the ventral petal only slightly exceeding the calycine appendages; flowers pale blue with dark lilac venation; lamina of the middle and upper stem leaves lanceolate or narrowly oblong, at the base usually attenuate or narrowly cuneate, rarely subcordate or truncate; plants 5 – 30 cm tall. → ***V. pumila* Chaix.**

4. - Flowers blue-violet; lamina of the middle and upper stem leaves broadly ovate to ovate, leaf base cordate or deeply cordate, rarely truncate. → ***V. canina* L.**
- Flowers white or very pale blue; leaves of the middle and upper stem lanceolate or narrowly triangulate, leafbase truncate or subcordate, rarely cordate. → 5

5. - Spur of the ventral petal not or only slightly exceeding the calycine appendages. Lamina lanceolate or narrowly triangulate (2.5 – 5.0 cm long and 0.8 – 2.0 cm wide); petiole 1.2 to 3.2 cm long, plants usually pale green, relatively tall (7-25 cm). → ***V. stagnina* var. *stagnina***
- Spur of the ventral petal clearly exceeding the calycine appendages. Lamina of the middle and upper stem leaves lanceolate or narrowly triangulate but smaller (1.2 – 2.2 cm long and 0.6 – 1.1 cm wide); petiole 0.6 – 1.4 cm long; plants usually dark green, remaining quite small (2.4 – 8.0 cm). ***V. stagnina* var. *lacteoides***

Conclusions

Our morphological studies showed that stigma shape was variable within each variety and that spur length was not found to differ significantly between both varieties of *V. stagnina*. Sepal appendage length, on the other hand, was significantly smaller in var. *lacteoides* and also the ventral and dorsal petals were not as broad as those of var. *stagnina*.

Our common garden experiment demonstrated that plasticity in plant height, leaf color and stipule length and shape in *V. stagnina* are caused by differences in abiotic factors such as soil type, humidity and light intensity. The observed differences in lamina size and petiole length, however, are fixed genetically between the two varieties. A common garden experiment with flowering plants should point out which characters in the flowers are influenced by environmental factors and which characters are determined genetically.

Crossings showed that both morphs are probably not reproductively isolated and chromosome counts showed that they have identical chromosome numbers.

The correct epithets of the common and scientific names of the Dutch endemic morph should be Vals melkviooltje for *Viola stagnina* in general and Heidemelkviooltje and Veenmelkviooltje for var. *lacteoides* and var. *stagnina*, respectively.

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