Evolution of Viola stagnina and its sisterspecies by hybridisation and polyploidisation

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Chapter

5

Combined analyses of AFLP markers and morphology confirm the taxonomic status of *Viola stagnina* var. *lacteoides*[6]

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Two morphs of *Viola stagnina* have been described in The Netherlands: var. *stagnina* and var. *lacteoides*. The morphological differences between these morphs were controversial which resulted in a debate about the recognition of these infraspecific taxa for *V. stagnina*. This study aims to characterize both morphs using molecular and morphological data and to compare these data with samples collected throughout western Europe in order to provide information on the genetic structure and morphological differences within *V. stagnina*.

Phylogenetic and phenetic analyses of the AFLP data uncovered some genetic differentiation between accessions of both *V. stagnina* morphs. Principal Component Analyses of the morphological data showed that accessions of the morphs belonged to two slightly overlapping clusters and a combined Levene and Student-T test confirmed that 10 out of 13 morphological characters were significantly different between the morphs. A discriminant analysis demonstrated that a combination of four of these characters could correctly identify 92% of both morphs. These results demonstrated that the endemic morph of *V. stagnina* originally described as var. *lacteoides* shows sufficient differentiation to merit recognition as a separate variety.

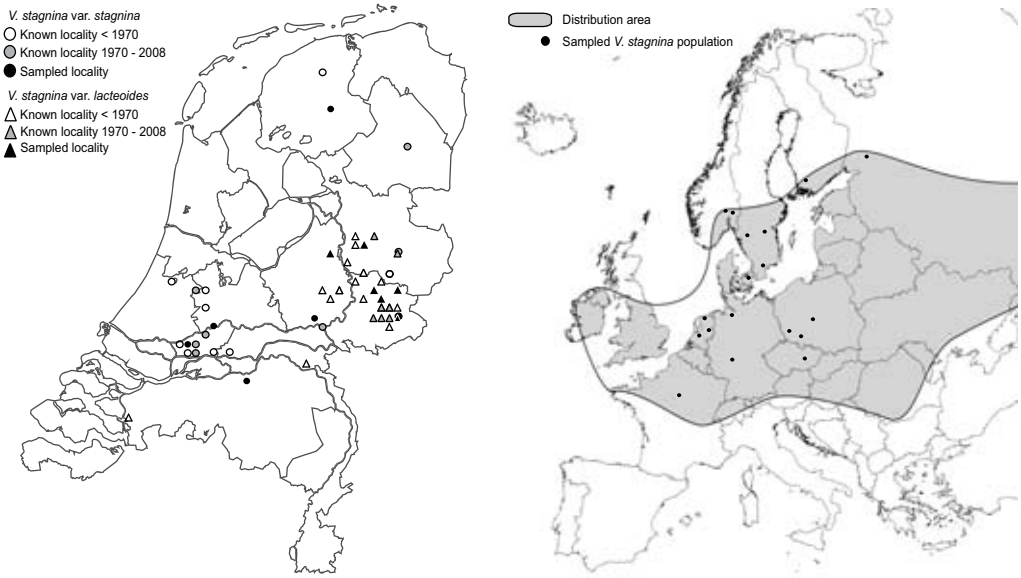
Key words: AFLP, Bayesian analysis, morphometrics, phylogeny, *Viola stagnina*

[6] van den Hof et al., submitted to Conserv. Genet

Introduction

The European Fen Violet, *Viola stagnina* Kit., is a widespread but rare plant species occurring throughout Europe with the exception of the Mediterranean, the southeast and extreme north (Fig. 10). It favors wet and temporarily flooded, sunny habitats such as floodplains, fens and marshes (Valentine et al., 1968; Eckstein et al., 2006*a*; Weeda, 2002). *Viola stagnina* is a member of sect. *Viola* subsect. *Rostratae*, which is rich in species and frequently subdivided into the four series *Arosulatae*, *Mirabiles*, *Repentes*, and *Rosulantes*. *Viola stagnina* is placed in the *Arosulatae* series, whose members are recognised by lacking a basal non-flowering rosette. As a paleotetraploid ($2n = 20$), *V. stagnina* was involved in the alloploid origin of the other arosulate species such as *V. canina* L. and *V. pumila* Chaix (both $2n = 40$; Valentine, 1958; Moore and Harvey, 1961; van den Hof et al., 2008).

Fig. 10. Distribution of *V. stagnina* var. *stagnina* and *V. stagnina* var. *lacteoides* in Europe and The Netherlands.



In many European floras, including the latest edition of the Heukels' Flora of The Netherlands (van der Meijden, 2005), *V. stagnina* is mentioned under the name *V. persicifolia* Schreb. However, in a recent nomenclatural study we (Danihelka et al., in review[5]) have pointed out that this name should be interpreted as referring to *V. elatior* Fries. The name *V. persicifolia* is therefore proposed for rejection (van den Hof et al., in review[5]). For this reason, we chose to 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 & 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. Subsequent authors have spelled *lacteoides* in a number of different ways. In the present publication we use *lacteoides* since we consider this to be the correct spelling. For a more detailed motivation, we refer to van den Hof et al. (submitted[7]).

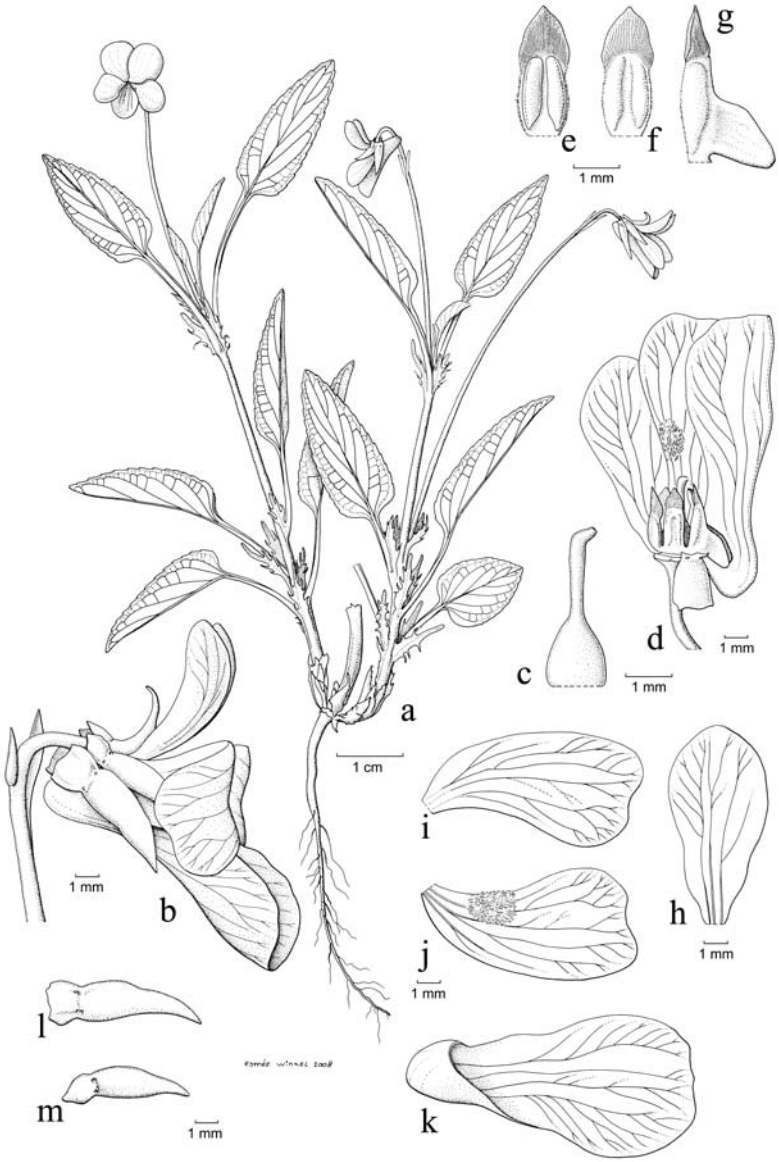


Fig. 9a. *Viola stagnina* var. *stagnina* a. Habit b. Lateral view of the flower c. Lateral view of the flower with male and female reproductive organs d. Gynoecium e. Adaxial view of the upper stamen f. Abaxial view of the upper stamen g. Side view of the spurred lower stamen h. Dorsal petal i. Lateral petal j. Lateral petal with fimbriae k. Ventral petal with spur l. Lower sepal m. Upper sepal.

[7] Chapter 6 of this thesis.

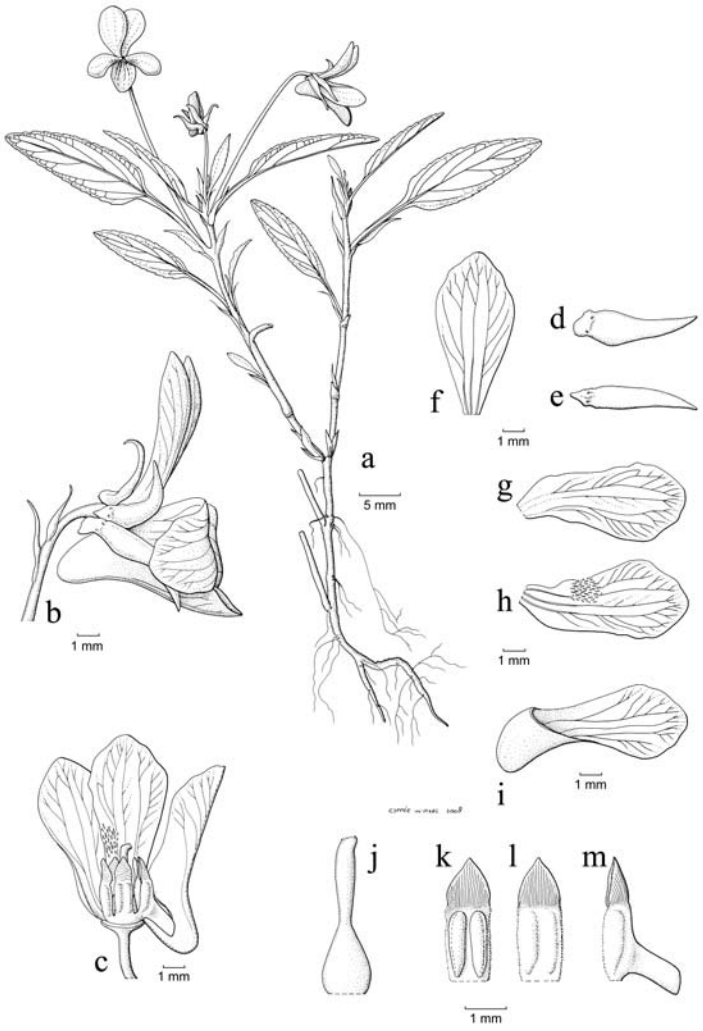


Fig. 9b. *V. stagnina* var. *lacteoides* a. Habit b. Lateral view of the flower c. Lateral view of the flower with male and female reproductive organs d. Lower sepal e. Upper sepal f. Dorsal petal g. Lateral petal h. Lateral petal with fimbriae i. Ventral petal with spur j. Gynoecium k. Adaxial view of the upper stamen l. Abaxial view of the upper stamen m. Side view of the spurred lower stamen.

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 (den Held,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 the ventral petal 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 (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).

With the development of DNA fingerprinting techniques, such as AFLPs (Vos et al., 1995), new possibilities are now at hand to investigate whether the *lacteoides* morph is genetically distinct from the *stagnina* morph. *Viola stagnina* in The Netherlands is very vulnerable and mentioned on the Dutch red list as a rapidly declining and rare species. As a consequence of inbreeding, caused by the small population sizes and cleistogamy, *V. stagnina* does not harbor much genetic variation. Because of this low amount of genetic variation and because AFLPs have the advantage of being highly variable between closely related taxa compared to nuclear DNA sequences we chose to use AFLPs as a phylogenetic and phenetic marker (e.g. Pelser et al., 2003; Eckstein et al., 2006*b*; Kadereit and Kadereit, 2007; Schenk et al., 2008). Other advantages of AFLPs are that these markers are generated relatively cheap compared to DNA sequence markers. Furthermore, AFLPs are sampled across the entire genome and not from specific locations such as nuclear DNA sequences, which normally represent only a single gene (Koopman, 2005). In the past it was often thought that a major drawback of AFLPs is the possible lack of homology between AFLP fragments, since homology is only inferred from fragment size, while source and sequence identity remain unknown (Althoff et al., 2007; Koopman, 2005). This is especially true for more distantly related taxa. A comparison between AFLP variation and nrITS sequence divergence by Koopman (2005), showed that for plant species AFLP markers are still reliable when their nrITS sequences differ less than around 30 nucleotides. A search on NCBI GenBank showed us there was a difference of less than 25 nucleotides between nrITS sequences of *V. elatior* and *V. riviniana* Rchb. We therefore expected that AFLP markers are reliable for recovering the phylogenetic relationships among the taxa included in this study.

We applied AFLPs and morphometrics to Dutch and European accessions of *V. stagnina* to answer the following questions: (1) Is the Dutch endemic *lacteoides* morph genetically distinct from the far more widespread *stagnina* morph? (2) Are there morphological traits separating the two morphs from each other? Assessing whether infraspecific taxa can be recognized within *Viola stagnina* is not only interesting from a scientific point of view. The results of this study are also important for Dutch nature conservation management because the Bern convention of 1981 demands upgrading of the protection of areas when these contain endemics.

Materials and Methods

Taxon selection

Together with the accessions of the two *V. stagnina* morphs, different accessions of *V. canina, V. pumila*, *V. elatior* and the hybrid *V. canina* × *stagnina*, also known as *V.* × *ritschliana*, were used in our analyses, because these species were found to be most closely related to *V. stagnina* based on DNA sequence analysis (van den Hof et al., 2008). Accessions of *V. riviniana* were used as outgroup (Appendix 1). Unfortunately, no material of *V. lactea* could be included for AFLP analysis due to an inferior quality of the DNA isolates from the specimens available. We do not consider omitting *V. lactea* from our analyses a serious drawback to this study. The chromosome number of $2n = 58$, combined with habitat ecology and distribution suggests it is not closely related to *V. stagnina*.

AFLP

Total genomic DNA was extracted using the Dneasy Plant Mini Kit (Qiagen) and the CTAB method of Doyle and Doyle (1987) with some modifications. For a detailed description of this extraction protocol see van den Hof et al. (2008). *Eco*RI and *Mse*I restriction enzymes were used to digest between 200 - 500 ng of DNA for each sample. The digestion of the DNA was done overnight at a temperature of 37°C. Subsequently, adaptors of a known sequence were ligated to the fragmented DNA, after which preselective amplification of the DNA took place with *Eco*RI+A and *Mse*I+A primers. Selective amplification was conducted with two different primer pairs, *Eco*RI+ACT and *Mse*I+ACT, and *Eco*RI+ATC and *Mse*I+AGG, chosen because they yielded a good amount of variation for our species of interest in a previous study (Eckstein et al. 2006*b*). Finally, the amplification products were loaded on a LI-COR automated sequencer (4300 DNA Analysis System, LI-COR Biotechnology). Scoring of the presence and absence of bands was done using AFLP-Quantar version 1.0 (Keygene Products BV, Wageningen, The Netherlands).

The AFLP data were analysed using a Principal Coordinate (PCO) analysis with Jaccard Coefficient using NTsys-pc 2.02k (Rohlf, 1997). Neighbour Joining (NJ) and Maximum Parsimony (MP) analyses of the AFLP data were done using PAUP* 4.0b10 (Swofford 2003). Phylogenies were obtained using the heuristic search option, with 100 random sequence additions and TBR branch swapping. After each sequence addition, a maximum of 500 trees was saved. Bootstrap support (BS) (Felsenstein 1985) was calculated with 2,000 bootstrap replicates, using only ten random sequence additions in each bootstrap replicate. After every random sequence addition replicate a maximum of 250 trees was saved.

A model based approach for phylogenetic analyses was also performed using MrBayes 3.1.2 (Huelsbeck and Ronquist, 2001). Currently only one model of evolution implemented in MrBayes can be used for restriction site data such as AFLPs. This restriction site model is an F81-like model designed for restriction site data and other binary data, such as gapcoding data (Felsenstein, 1981), but can only take into account the rate at which bands are gained and lost (Ronquist et al., 2005). Luo et al. (2007) argued that this model hugely oversimplifies the evolutionary processes that result in the presence or absence of AFLP fragments and they therefore presented a more elaborate model of

evolution especially designed for AFLP data. This model is, however, not yet implemented in MrBayes and has the major drawback that it runs 40,000 times slower than the F81-like model, making it inoperable for the computational hardware currently at hand (Koopman et al., 2008).

Bayesian Inference analyses (BI) using the F81-like model were done using MrBayes 3.1.2 (Huelsbeck and Ronquist, 2001). Markov Chain Monte Carlo analyses (MCMC) were run for 23 million generations. We used two separate runs each containing 15 chains. The temperature was set to 0.0035. Furthermore, we set the swap frequency to 5 and the number of swaps to 4. Finally, the appropriate amount of burn-in was identified as 30% using the program Tracer 1.3 (Rambaut and Drummond, 2004). For assessment of support for individual branches in the Bayesian trees, Posterior Probabilities Index values (PPI) were calculated. The analyses were repeated three times to assure sufficient mixing to confirm that the program converged to the same PPI values.

Morphology

Morphological measurements and anatomical observations were done on both herbarium material and living plants collected in the wild. From these plants, herbarium vouchers were made and stored at L. In total, 15 morphological characters, 9 reproductive and 6 vegetative, were scored or measured (Appendix 2). Thirteen characters were quantitative and the remaining 2 were qualitative and scored as binary and multistate, respectively. The reported differences in stigma shape (den Held, 1977) were much more variable than initially reported and stigma shape was therefore excluded from the analyses. Morphological similarities between the different samples were analyzed with SPSS 15.0.1 statistical analysis software (2006, SPSS inc, Chicago, Illinois, USA). Principal Component Analysis (PCA) was used to create biplots for the morphometric data. Canonical Discrimant Analysis (CDA) was used to see which characters could best be used to separate the species used in this study, and to identify which characters differentiate the two morphs of *V. stagnina* most effectively. A stepwise selection method was used, and at each step the character that minimized Wilks' Lambda was entered. Characters with a significance level of its F value less than 0.05 were entered into the model, while characters with a significance level greater than 0.1 were removed. A Levene test was performed to test for equality of variance between the characters of the *V. stagnina* morphs analyzed, after which a Student-T test was carried out to determine which characters were significantly different between the two morphs.

Results

AFLP

In the PCO analysis the first two components together explained 73% of the variation (Fig. 11). Accessions of the different species each formed their own distinct group. However, the accessions from the *V. stagnina* morphs completely overlap with each other, and the *V. canina* × *stagnina* accessions all fall within the *V. canina* cluster.

The NJ analyses shows that all species form their own, well supported clusters, except for the accessions of *V. elatior* and *V. pumila*, of which the clusters collapse in

the BS consensus (Fig. 12). Within the *V. stagnina* cluster, several moderately to highly supported groups of different geographic origin can be recognized. However, no highly supported clusters are present for the *lacteoides* morph.

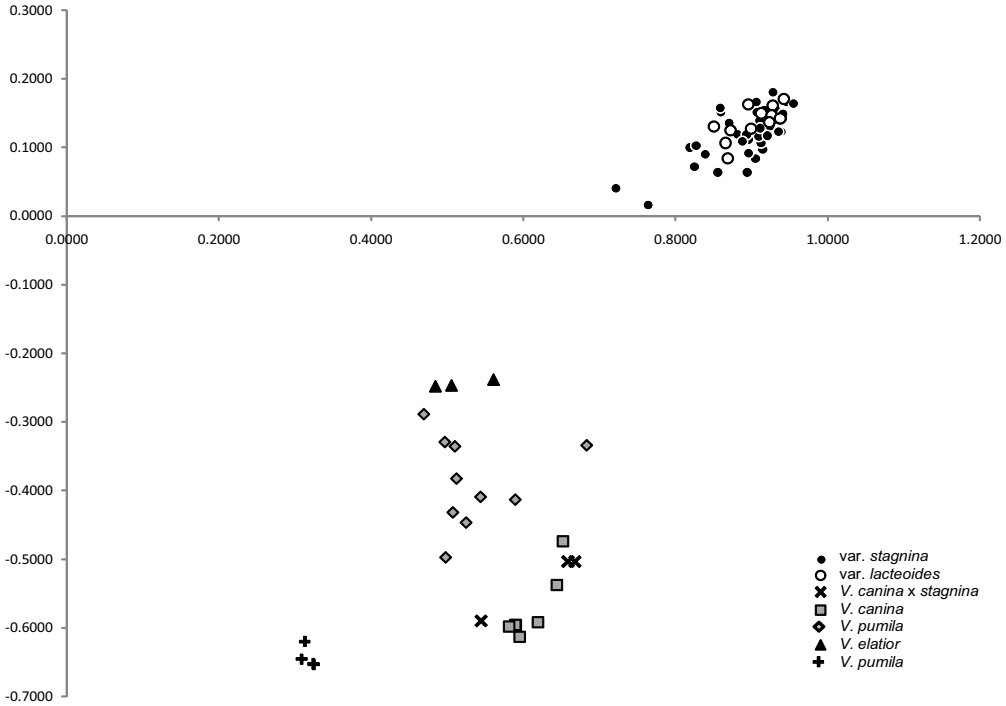


Fig. 11. Principal Coordinate Analysis (PCO) based on the presence/absence of the AFLP markers of all *Viola* accessions. PCO axes 1 and 2 extracted 64% and 9% of the variance, respectively.

MP analyses of the AFLP dataset produced a total of 48.000 MPTs with 545 steps (CI = 0.2844, RI = 0.7156). Of the 166 characters scored, 143 were parsimony informative. The MP strict consensus tree (Fig. 13) shows several weakly supported clades. One clade consists of all *V. canina* accessions and *V. canina* × *stagnina*, the natural hybrid between *V. stagnina* and *V. canina*. The accessions of *V. pumila* do not form a clade but are present in a grade instead. The accessions of *V. elatior* form a sistergroup to the polytomy of all the *V. stagnina* accessions. Inside the *V. stagnina* polytomy, several weakly supported clades can be recognized. These clades represent populations of different geographic origin. Two clades contain only Scandinavian accessions, one clade consists of French accessions only, two clades consist of Dutch accessions only, and one weakly supported clade contains a German and a Dutch accession. Although there is one clade of the *lacteoides* morph inside the *V. stagnina* polytomy, the BS for this clade is below 50%.

The BI tree (Fig. 14) shows strongly supported clades but also grades for the species analyzed. Accessions of *V. canina* and *V. pumila* form a grade and the accessions of *V. elatior* are part of a large *V. stagnina* polytomy. All the *V. canina* × *stagnina* accessions are found inside the *V. canina* grade. Similar to the *V. stagnina* polytomy of the MP strict consensus tree, the polytomy of this species in the BI tree consists of poorly supported

clades of different geographic origin. Five clades contain only Fennoscandian accessions, one clade consists of French accessions only, two clades of German accessions only, and five clades contain only Dutch accessions. The remaining accessions in the polytomy are individuals from both Dutch and German origin. The *V. stagnina* polytomy contains two clades of the *lacteoides* morph. Both clades are poorly supported with a PPI of 0.71 and 0.79, respectively.

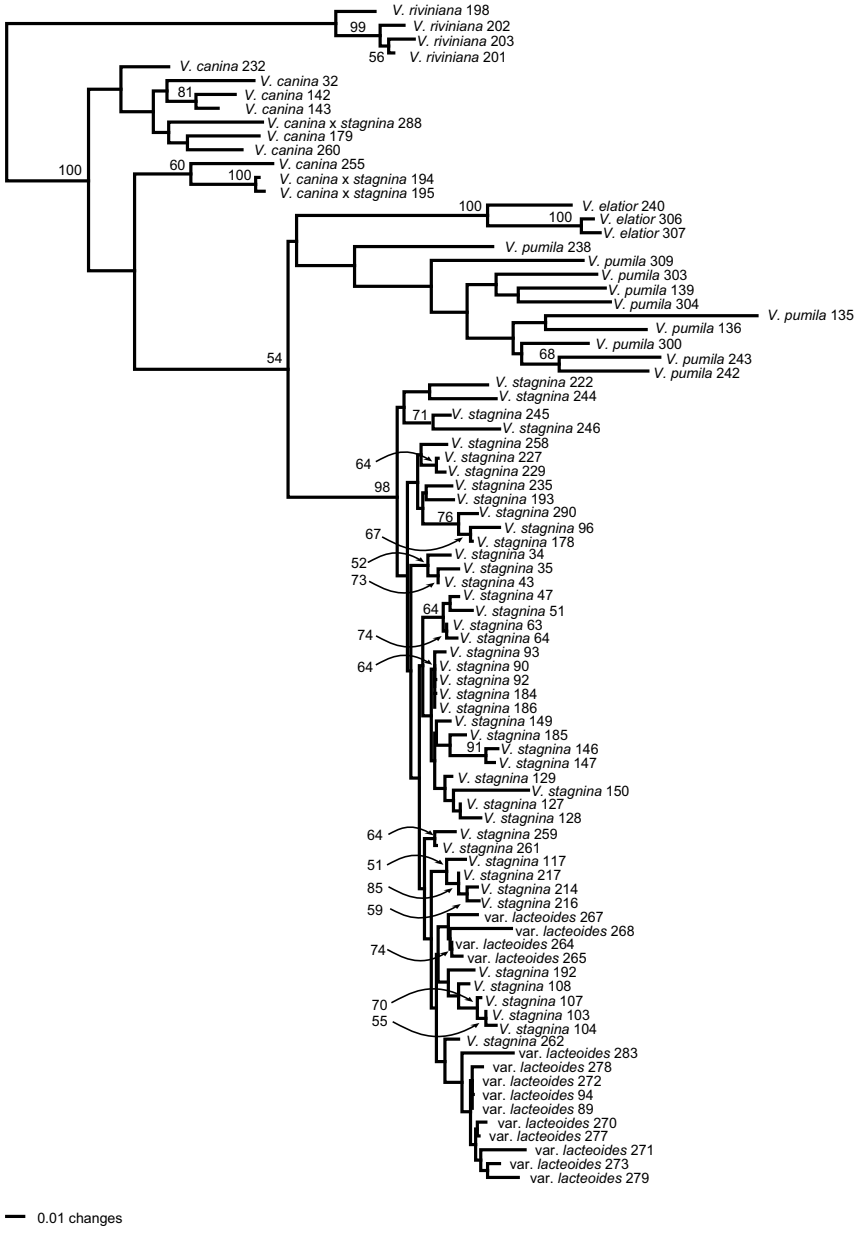


Fig. 12. NJ tree of AFLP markers of *Viola* accessions analysed. Bootstrap values >50 % are indicated above the branches.

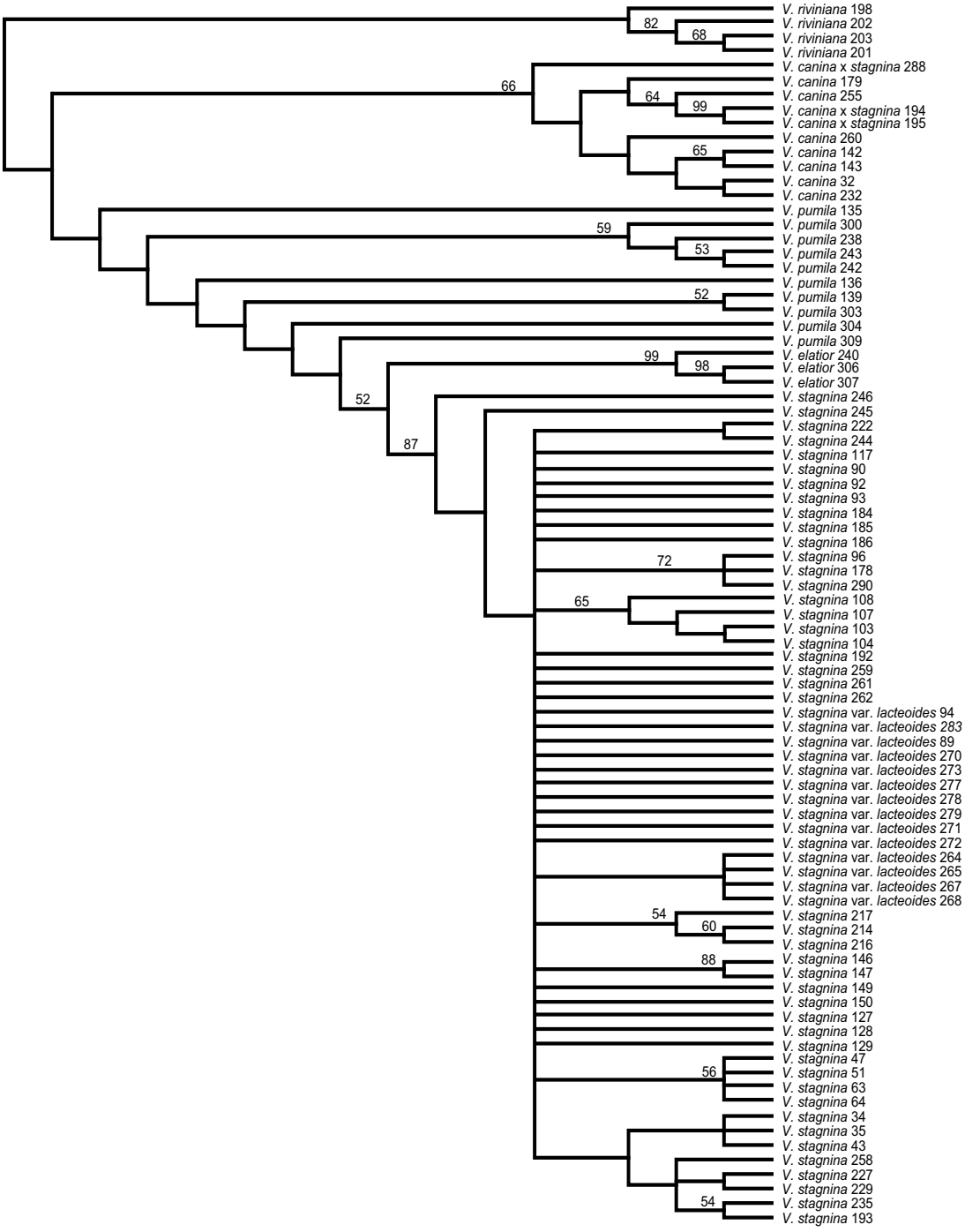


Fig. 13. MP strict consensus tree produced by analysis of AFLP markers of *Viola* accessions. BS values > 50% are indicated above the branches.

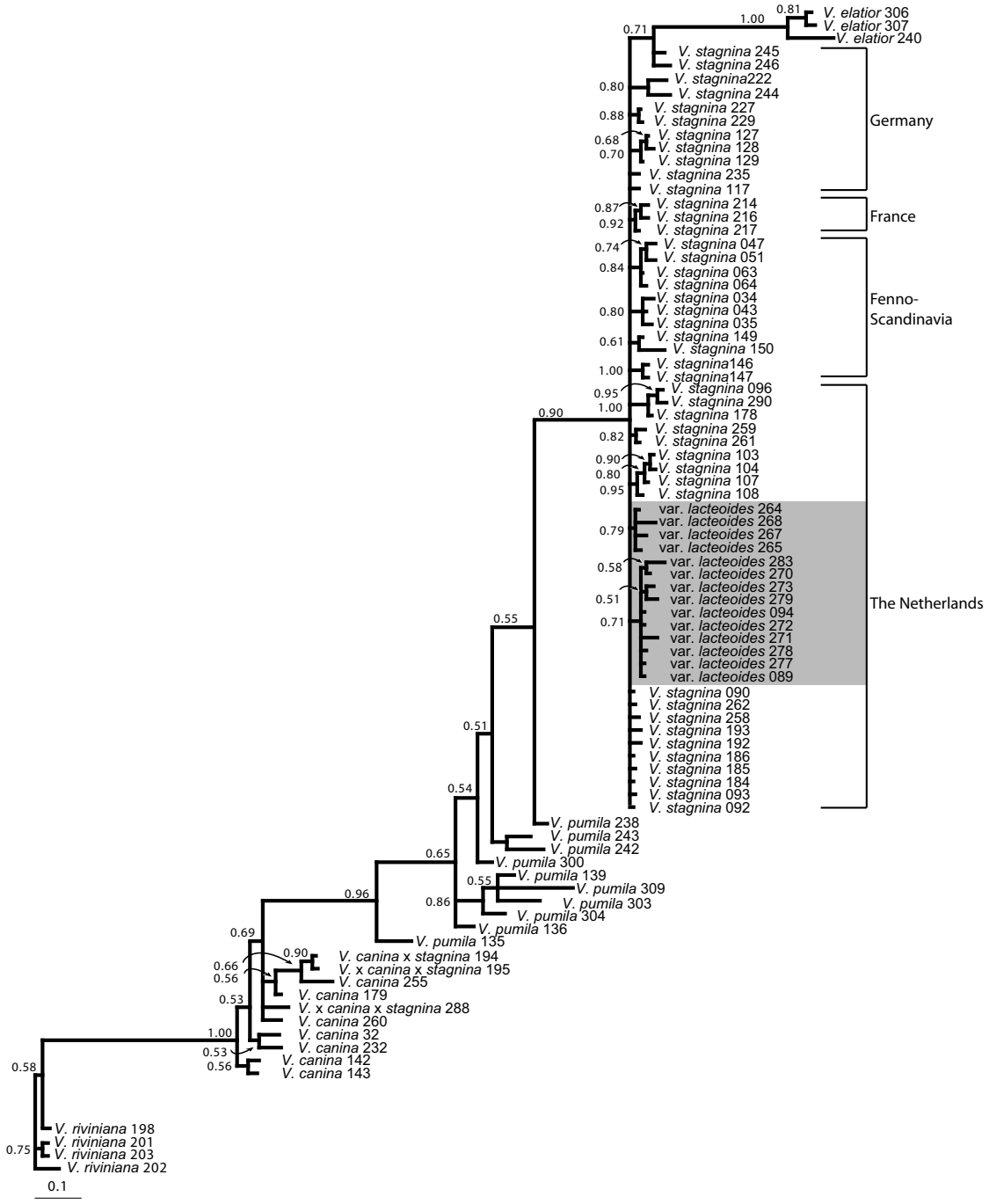


Fig. 14. BI tree produced by analysis of AFLP markers of *Viola* accessions. Posterior probabilities are indicated above the branches.

Morphology

The first component of the PCA of all morphological characters explained 25.6 % of the variation observed and correlated most strongly with leaf length (Table 2). The second component of the PCA explained 16.5% of the variation. Leaf length/petiole length ratio correlated most strongly with this component. The PCA plot based on these first two components showed that the examined species group in several overlapping clusters (Fig. 15). The accessions of the *stagnina* morph only partly overlapped with those of the *lacteoides* morph. Accessions of *V. canina* and *V. pumila* only slightly overlapped with both *V. stagnina* morphs, while the hybrid *V. canina* × *stagnina* mainly fitted on the edge of the *V. canina* cluster. The four accessions of *V. elatior* fell outside the more or less overlapping clusters of the other species analyzed.

Table 2. Correlations of the morphometric characters with the first two components of the PCA.

	All characters		**Reproductive characters**		**Vegetative characters**	
	Comp.1	**Comp.2**	**Comp.1**	**Comp.2**	**Comp.1**	**Comp.2**
Reproductive characters						
Flower Color	-0.010	-0.422	0.160	0.315		
Spur/ventral petal length ratio	-0.207	-0.363	0.120	-0.023		
Dorsal petal length/width ratio	-0.091	0.537	-0.475	0.688		
Lateral petal length/width ratio	0.084	0.533	-0.402	0.715		
Ventral petal length/width ratio	0.194	0.548	-0.257	0.660		
Sepal length	0.767	-0.456	0.849	0.358		
Sepal length/width ratio	0.470	-0.284	0.703	0.226		
Sepal /sepal appendage length ratio	-0.358	0.278	-0.680	-0.038		
Upper bract length	0.807	-0.209	0.548	0.452		
Vegetative characters						
Plant height	0.804	-0.108			0.674	0.670
Lamina length	0.846	-0.067			0.765	0.560
Lamina length/width ratio	0.539	0.424			0.731	-0.126
Lamina length/petiole length ratio	0.157	0.600			0.450	-0.571
Stipule length/Petiole length ratio	-0.439	-0.412			-0.579	0.564
Leaf base shape	-0.529	-0.396			-0.686	0.297

The first component of the PCA of reproductive characters explained 27.5 % of the variation observed and correlated most strongly with sepal length (Table 2). The second component of the PCA explains 21.2% of the variation and correlated most strongly with the length/width ratio of the lateral petal. Here, the two morphs of *V. stagnina* and *V. canina* overlapped almost completely as compared to the analysis of all characters (data not shown). *Viola pumila* still only slightly overlapped with both *V. stagnina* morphs. The *V. elatior* accessions now slightly overlapped with accessions of *V. pumila*.

When only vegetative characters were included in the PCA, the first component explained 43.0 % of the variation observed and correlated most strongly with lamina length. The second component explained 25.2 % and correlated most strongly with plant height. The PCA plot (data not shown) of these two components clearly separated *V. elatior* from the other taxa. The clusters of the two *V. stagnina* morphs only slightly overlapped. Also, *Viola canina*, *V. canina* × *stagnina*, and *V. pumila* accessions only slightly overlapped with both those of both *V. stagnina* morphs.

We also performed the same three PCAs with accessions of the *V. stagnina* morphs

only. PCA plots of the first two components (not shown) demonstrated the same patterns for the *V. stagnina* morphs as in the plots where all species were included. Characters correlating with each component for the three different analyses are mentioned in Table 3.

We also examined if any patterns would become visible when the accessions analyzed were not labeled by taxonomic name but by habitat type, instead. For the Dutch and German accessions analyzed, this additional information was available. The accessions could be divided into two groups: wet moorlands and floodplain grasslands. The variation in all groups was very large and no distinct clusters could be recognized (data not shown).

The CDA with accessions of all species showed that leaf base shape, plant height, stipule length/petiole length ratio, sepal length, sepal appendage/sepal length ratio, and ventral petal length/width ratio separate the species most effectively (Fig. 16). In total, 89.5% of all accessions (88.2 % for the *stagnina* morph, 93.8 % for the *lacteoides* morph, 25% for *V. canina* × *stagnina*, and 100% for *V. canina*, *V. pumila* and *V. elatior*) were identified correctly when these characters were used. A similar analysis with accessions of the two *V. stagnina* morphs only showed that leaf length, upper bract length, sepal appendage/sepal length ratio, and stipule length/petiole length ratio separate the two morphs most effectively. Of all *V. stagnina* accessions 92% (91.2% of the *stagnina* morph and 93.8% of the *lacteoides* morph) were identified correctly with these characters.

The results of the Student-T test indicate that 10 out of the 13 characters analyzed are significantly different for the two morphs of *V. stagnina* (Table 4). Descriptive statistics of the morphological dataset are summarized in Table 5.

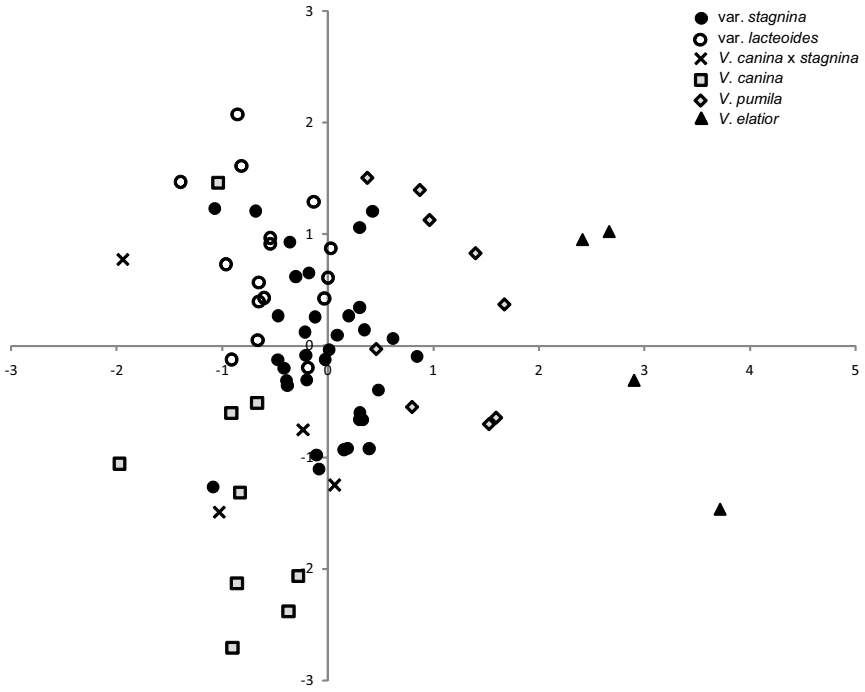


Fig. 15. Principal Component Analysis (PCA) of all morphological characters.

Table 3. Correlations of the morphometric characters with the first two components of the PCA for *V. stagnina* accessions only.

	All characters		Reproductive characters		Vegetative characters	
	Comp.1	**Comp.2**	**Comp.1**	**Comp.2**	**Comp.1**	**Comp.2**
Reproductive characters						
Spur/ventral petal length ratio	0.148	0.245	0.320	-0.670		
Dorsal petal length/width ratio	-0.420	0.595	-0.559	0.699		
Lateral petal length/width ratio	-0.163	0.565	-0.366	0.698		
Ventral petal length/width ratio	-0.488	0.079	-0.665	0.174		
Sepal length	0.800	0.307	0.752	0.323		
Sepal length/width ratio	0.430	0.641	0.575	0.646		
Sepal /sepal appendage length ratio	-0.020	-0.723	-0.286	-0.598		
Upper bract length	0.824	-0.098	0.788	-0.073		
Vegetative characters						
Plant height	0.820	0.006			0.924	0.056
Lamina length	0.813	-0.146			0.745	0.316
Lamina length/width ratio	0.218	-0.346			0.258	0.840
Lamina length/petiole length ratio	-0.489	-0.229			-0.640	0.620
Stipule length/Petiole length ratio	0.480	-0.071			0.749	-0.143

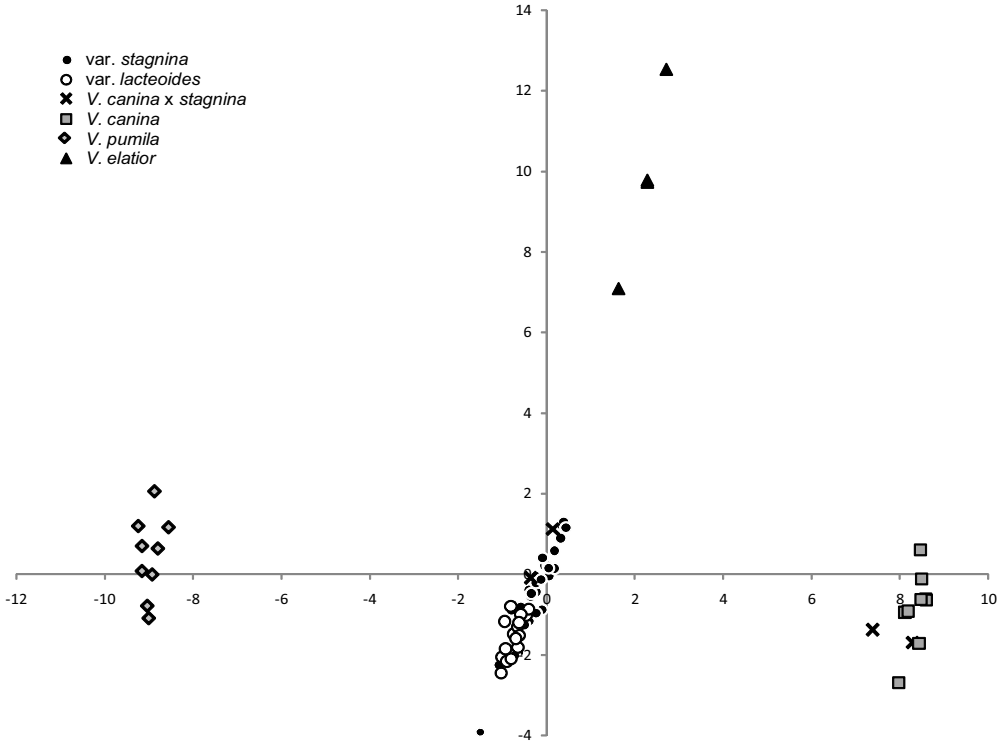


Fig. 16. Canonical Discriminant Analysis (CDA) of the first two axes of all morphological characters.

Table 5. Descriptive statistics for all characters for both varieties of *V. stagnina*.

Characters	Morph	Mean	Median	Mode	Std. Deviation	Variance	Range	Minimum	Maximum	Quartiles 25	50	75
Spur length/ventral petal length ratio	*stagnina*	0.47	0.46	0.47	0.05	0.00	0.23	0.38	0.60	0.42	0.46	0.50
	acteoides	0.47	0.45	0.45	0.05	0.00	0.18	0.41	0.59	0.42	0.45	0.50
Dorsal petal length/ width ratio	*stagnina*	1.54	1.54	1.54	0.24	0.06	1.10	1.03	2.13	1.40	1.54	1.70
	acteoides	1.76	1.73	2.00	0.21	0.04	0.63	1.50	2.13	1.57	1.73	2.00
Lateral petal length/ width ratio	*stagnina*	1.49	1.50	1.50	0.21	0.04	1.00	1.00	2.00	1.33	1.50	1.60
	acteoides	1.58	1.60	1.71	0.19	0.04	0.63	1.25	1.88	1.44	1.60	1.74
Ventral petal length/ width ratio	*stagnina*	1.11	1.12	1.00	0.11	0.01	0.54	0.86	1.40	1.05	1.12	1.20
	acteoides	1.22	1.20	1.20	0.15	0.02	0.64	0.92	1.56	1.13	1.20	1.29
Sepal length	*stagnina*	4.85	5.00	5.00	0.89	0.80	4.50	2.00	6.50	4.00	5.00	5.50
	acteoides	3.78	4.00	4.00	0.77	0.60	3.00	2.00	5.00	3.13	4.00	4.00
Sepal length/width ratio	*stagnina*	2.50	2.51	2.50	0.51	0.26	2.67	1.33	4.00	2.18	2.51	2.77
	acteoides	2.45	2.50	2.00	0.69	0.48	2.50	1.50	4.00	2.00	2.50	2.92
Sepal length /sepal appendage length ratio	*stagnina*	0.39	0.39	0.33	0.07	0.00	0.33	0.17	0.50	0.36	0.39	0.42
	acteoides	0.33	0.32	0.25	0.10	0.01	0.32	0.25	0.57	0.25	0.32	0.35
Upper bract length	*stagnina*	3.53	3.40	4.00	0.67	0.45	3.00	2.00	5.00	3.15	3.40	4.00
	acteoides	2.39	2.50	2.50	0.54	0.29	1.50	1.50	3.00	2.00	2.50	3.00
Plant height	*stagnina*	100.82	95.00	55.00	39.74	1579.42	153.00	37.00	190.00	68.50	95.0	123.25
	acteoides	50.13	50.00	24.00	16.69	278.65	62.00	24.00	86.00	39.00	50.00	60.00
Lamina length	*stagnina*	30.13	31.00	31.00	6.21	38.54	25.20	15.00	40.20	26.00	31.00	34.00
	acteoides	17.69	16.00	16.00	4.56	20.76	16.00	12.00	28.00	14.25	16.00	21.75
Lamina length/width ratio	*stagnina*	2.53	2.50	3.50	0.53	0.28	2.64	1.36	4.00	2.16	2.50	2.77
	acteoides	2.40	2.23	2.00	0.50	0.25	1.43	1.71	3.14	2.00	2.23	2.96
Lamina length/petiole length ratio	*stagnina*	1.67	1.72	1.78	0.42	0.18	1.77	0.77	2.54	1.31	1.72	1.95
	acteoides	1.93	1.89	1.50	0.41	0.17	1.38	1.43	2.80	1.53	1.89	2.16
Stipule length/Petiole length ratio	*stagnina*	2.23	2.04	1.29	0.96	0.91	3.45	1.11	4.56	1.43	2.04	2.69
	acteoides	1.48	1.29	1.00	0.48	0.23	1.50	1.00	2.50	1.11	1.29	1.74

Table 4. Levene test for equality of variance and Student-T test for equality of means for each character analyzed between the two *V. stagnina* forms. Significant results for the Levene test are in italic. Significant results for the Student-T test are in bold.

Characters	Levene's Test for Equality of Variances			T-test for Equality of Means		
		F	Sign.	t	Df	Sign. (2-tailed)
Spur length/ventral petal length ratio	Equal assumed	0.006	0.938	-0.311	48	0.757
Dorsal petal length/width ratio	Equal assumed	0.008	0.927	-3.130	48	**0.003**
Lateral petal length/width ratio	Equal assumed	0.076	0.783	-1.389	48	0.171
Ventral petal length/width ratio	Equal assumed	0.554	0.460	-2.771	48	**0.008**
Sepal length	Equal assumed	0.171	0.681	4.099	48	**0.000**
Sepal length/width ratio	Equal assumed	2.659	0.109	0.320	48	0.750
Sepal length /sepal appendage length ratio	Equal assumed	2.173	0.147	0.015	48	**0.015**
Upper bract length	Equal assumed	0.528	0.471	5.913	48	**0.000**
Plant height	*Equal not assumed*	11.510	*0.001*	6.344	31.3	**0.000**
Lamina length	Equal assumed	0.992	0.324	7.146	48	**0.000**
Lamina length/width ratio	Equal assumed	0.148	0.702	0.848	48	0.401
Lamina length/petiole length ratio	Equal assumed	0.088	0.768	-2.068	48	**0.044**
Stipule length/Petiole length ratio	*Equal not assumed*	4.791	*0.034*	3.692	31.3	**0.001**

Discussion

AFLP

No highly supported clades could be detected within *V. stagnina* based on the AFLPs analyzed here. Although some geographic structure could be detected in the NJ and BI trees, none of this could be traced back to a distinct ecology or morphology except for the two clades consisting of accessions of the *lacteoides* morph. Although not supported with high BS or PPI values, these clades did not merge with the other accessions of *V. stagnina* analyzed. Judging from the very short branch lengths, though, genetic exchange within *V. stagnina* still seems to take place regularly. This conclusion is also supported by the results of the PCO analysis where the morphs of *V. stagnina* did not differentiate into separate clusters, and by crossing experiments carried out between both morphs of *V. stagnina*, which produced fully viable seeds (Van den Hof et al., submitted[7]).

The MP strict consensus is different from the NJ and BI trees (Fig. 12-14) in the fact that only a single population of the *lacteoides* morph clusters separately from the other *V. stagnina* accessions analyzed. In addition, the *V. canina* accessions are not placed in a grade but in a clade. Although the majority of the topology is generally the same as the MP tree, the support for branches of the BI tree is slightly higher. This is to be suspected since the PPI in general is an overestimation as compared to the BS in MP and Maximum Likelihood analyses (Simmons et al., 2004). Branch lengths in both MP (not shown) and BI analyses clearly separate the different species included in this analyses.

The placement of *V. elatior* individuals in the MP tree is different from that in the BI tree. According to the MP analyses, the *V. elatior* clade is placed as sister group to the *V. stagnina* clade, whereas in the BI analyses the *V. elatior* clade is part of the *V. stagnina* polytomy. Although the placement of *V. elatior* is different in the two analyses, both suggest that this species is the closest relative of *V. stagnina*. *Viola elatior* is probably an ancient autoploid derivative of *V. stagnina* (Clausen, 1927; Van den Hof et al., 2008). The different placement of *V. elatior* might be caused by the fact that the accessions of this octoploid species produced approximately twice as many AFLP markers as the accessions of the tetraploid *V. stagnina*. It might therefore be expected that the octoploid species would be placed closer to each other than to the tetraploid *V. stagnina*, due to long branch attraction. This might explain the fact that *V. pumila* and *V. elatior* are closer related to each other in the MP as compared to the BI analyses than is expected from the reticulate relations described by Moore and Harvey (1961), Clausen (1927) and Van den Hof et al. (2008).

Taxa of hybrid origin are expected to end up as sister taxon to each parent in phylogenetic analyses when they have the same number of derived characters in common with each parent. Given the unequal branch lengths observed in most phylogenetic studies this is very unlikely to occur. The hybrid taxon will therefore generally be placed near the parent with which it has the most derived characters in common (McDade, 1995). The accessions of the hybrid *V. canina* × *stagnina* were placed near *V. canina* in all our analyses of the AFLP data. Due to the allopolyploid origin of the octoploid *V. canina* from the tetraploid *V. stagnina* and another tetraploid species, it is to be expected that *V. canina* × *stagnina* has more markers in common with *V. canina* than with *V. stagnina*.

Morphology

The PCA indicates that the vegetative characters explain most of the variation between the taxa analyzed. The vegetative characters correlating most with the variation between the two *V. stagnina* morphs are plant height and petiole length/stipule length ratio. Bract length and sepal length are the reproductive characters correlating most with the variation observed between the two morphs. The CDA of all accessions included in this study shows that only very few accessions of the two morphs of *V. stagnina* are misidentified. Accessions of the hybrid *V. canina* × *stagnina* are either identified as *V. stagnina* or *V. canina*. because two accessions had especially vegetative characters in common with, while the characters of the other hybrid accessions resembled those of *V. canina*. The accessions of the other three species are all correctly identified.

The discriminant analysis of only the *V. stagnina* accessions shows that leaf length, upper bract length, sepal appendage/sepal length ratio, and stipule length/petiole length ratio together correctly identify 91.2% of the *stagnina* morph and 93.8% of the *lacteoides* morph. These four characters were also highly significant in the Student-T test (Table 4),

suggesting that these are the best characters to distinguish both morphs. Re-examination of the misidentified *stagnina* morph accessions suggests that these plants had not properly developed because they suffered from drought. Precipitation during the spring of 2007, the year of collection, was extraordinary low. The misidentification of the *lacteoides* morph accession as *stagnina* morph is probably caused by the fact that this plant had unusual large stipules and leaves as compared to other accessions of the *lacteoides* morph analyzed. These characters are known to be plastic in *V. stagnina* (Bergdolt, 1932). All the other morphological characters and our AFLP data, however, indicate that the identification of this accession is correct.

The morphology of *V. stagnina* is known to be greatly influenced by abiotic factors such as moisture content, light exposure and soil type (Bergdolt, 1932). In a common garden experiment with non-flowering plants of both morphs, initial differences observed in the field, such as plant height and leaf color, disappeared over time. Lamina length and stipule length/petiole length ratio, however, remained significantly different between the two morphs (Van den Hof et al., submitted[7]).

Contrary to den Held (in van Oostroom, 1977), we did not find any difference in the spur length of the ventral petal between both morphs of *V. stagnina*. The length of the calycine appendages were, however, significantly longer in the *stagnina* morph causing the spur to exceed less than was the case in the *lacteoides* morph (Fig. 9). The spurred flowers of most temperate *Viola* species are adapted to a wide array of pollinating insects with medium to long sized tongues, primarily bumblebees, solitary bees, syrphids and bombyliids (Beattie 1971, 1974). The fact that the spur size is the same for both morphs of *V. stagnina* might indicate that there has been no shift in pollination strategy. The differentiation between the two morphs is therefore probably not caused by a shift in pollinator preference but by environmental factors linked to the different habitats.

Conclusions

With this study, we intend to settle an 80 year old debate among Dutch botanists about whether infraspecific taxa should be recognized within *V. stagnina*. AFLP fingerprints showed that there is little genetic differentiation present within this species. Separate clades for both morphs were found in NJ, MP and BI analyses, although none received very high statistical support. When looking at the morphological differences, 10 out of the 13 characters analyzed are significant different for both morphs, and a CDA showed that four of those characters together can identify 92% of both *V. stagnina* morphs correctly. PCA of morphology showed that especially the vegetative characters clearly separate the two morphs. A number of these characters remained significantly different in a common garden experiment.

Based on the genetic and morphological differences found and the unique distribution, we recommend recognition of the infraspecific taxon *V. stagnina* var. *lacteoides*. Because of the low genetic differentiation and small overlap in geographic distribution between both morphs of *V. stagnina*, we prefer to use the infraspecific rank of variety rather than subspecies (Stuessy, 1990; Hamilton and Reichard, 1992).

With our recommendation of recognizing yet another infraspecific taxon for the European flora, we might get accused of contributing to taxonomic inflation which hampers the conservation of real biological entities (Pillon and Chase, 2006). We feel that we do not contribute to this for several reasons. First of all, by recognizing infraspecific taxa we

acknowledge the existence of deviating populations. These populations deserve attention from conservation biologists because they might eventually evolve into new species. Because we cannot witness this process within a human lifetime, this does not mean we should not recognize and describe them already. Having said that, we like to stress that the recognition of infraspecific taxa should be based on phylogenetic and phenetic analyses of both molecular data and morphology in combination with common garden experiments. Secondly, implementation of conservation laws is not influenced by our recommendation as they act from the species level onward only. We are not satisfied with this particular aspect, though, since it makes these laws very unrealistic. The Bern Convention of 1981, for example, currently lists six protected plant species for The Netherlands of which two are already extinct for more than sixty years. The orchid species *Spiranthes aestivalis* has not been found in The Netherlands since 1936 and *Sisybrium supimum* (Brassicaceae) was last found in 1940. In our opinion, conservation laws should not apply to these kind of species occurring on the fringe of their distribution area. Instead, the focus of these laws should be on endangered infraspecific and specific taxa which occur in the centre of a geographically limited distribution range.

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