Evolution of *Viola stagnina* and its Sisterspecies by Hybridization and Polyploidization

Violen

... ze zijn dapper; fragiel aan alle kanten, maar toch staan ze op wacht. Ze bewaken als het ware het voorjaarsgevoel. Ze symboliseren nederigheid en trouw.

Martin Bril - Volkskrant - 5 april 2006

Dedicated to the memory
of
Ruud van der Meijden

Kevin van den Hof

Evolution of *Viola stagnina* and its Sisterspecies by Hybridization and Polyploidization

Cover design and lay-out : René Glas (www.reneglas.com)
Drawings, fig. 9 : Esmée Winkel
Photograph, fig.17 : Bertie-Joan van Heuven
Printed by : Offsetdrukkerij Nautilus, Leiden

Chapter 2 : Published in Molecular Biology and Evolution 25: 2099–2108 (2008). van den Hof K, van den Berg RG, Gravendeel B. Chalcone synthase gene lineage diversification confirms allopolyploid evolutionary relationships of European rostrate violets.
Chapter 3 : Submitted to Taxon
Chapter 4 : Submitted to Taxon
Chapter 5 : Submitted to Conservation Genetics
Chapter 6 : Submitted to Plant Ecology and Evolution

ISBN/EAN: 978-90-71236-71-6

Evolution of *Viola stagnina* and its Sisterspecies by Hybridization and Polyploidization

Proefschrift

ter verkrijging van de graad van Doctor
aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden,
volgens het besluit van het College van Promoties
te verdedigen op woensdag 9 juni 2010
klokke 16:15 uur
door

Kevin van den Hof

Geboren te Geleen
in 1980

Promotiecommissie

Promotor •	Prof. Dr. E.F. Smets
Copromotor •	Dr. B. Gravendeel Dr. R.G. van den Berg
Referent •	Prof. Dr. H.E. Ballard Jr.
Overige leden •	Prof. Dr. P. Baas Dr. T. Marcussen Dr. M.E. Noordeloos Prof. Dr. M. Schilthuizen

Contents

Chapter

1

General introduction

In this thesis the patterns resulting from hybridization and polyploidization in a group of closely related *Viola* species were investigated. The status of the two infraspecific taxa within *V. stagnina* was studied in detail, and the nomenclature of a number of taxa was investigated.

Species concept

A never ending discussion in the field of biology is that of the species concept. Numerous papers and books have dealt with this subject, but no consensus about this definition exists among biologists. The fact that so many have discussed this subject is probably because a species is considered to be the most fundamental unit of comparison in all fields of biology and it is therefore the most important term used (de Queiroz, 2005).

Before the publication of Charles Darwin's book on the origin of species (1859), taxonomists had discussions about what a species defines. In those days, there was a more or less essentialist view on what a species was. Species were considered to be fixed entities that could not change over time. Discussions on species definition were in fact taxonomic puzzles about whether one was dealing with a species or a variety (Hey, 2006). This can be illustrated with an example in *Viola*. Some 19th century botanists treated *V. lactea*, *V. pumila* and *V. stagnina* as variations within a single species (e.g. Reichenbach, 1823), while others treated them as three separate species (e.g. Koch, 1836). A discussion about the species concept itself however did not exist.

After Charles Darwin presented his theory of evolution, the definition of a species became more prevalent and complicated, because the theory made biologists realize that all living organisms are subjected to evolution by means of adaptation and natural selection. This meant that varieties could now change into species over time. The boundary between a variety and a species became therefore not only vague, as it was for the 19th century essentialists, but it also became dynamic. Classifying species now became subjective and arbitrary (Hey, 2001; 2006).

The species concept had become a dilemma. It is in human's nature to classify the surrounding world, in the case of biologists this means classifying organisms. But biologists have a problem because the items they are classifying are changing over time, they are evolving. Biologists therefore started treating species as a "group of organisms enjoined by evolutionary processes that go on within it, and that is separate from other groups because of the absence of shared evolutionary processes with those other groups" (Hey, 2001). The focus on evolutionary processes within has led to the development of at least two dozen species concepts where species are defined by referring to evolutionary processes (e.g.

Cracraft, 1983, Cronquist, 1988; Kornet and McAllister, 1993; Mayden, 1997; Mayr, 1969; Templeton 1989; Van Valen, 1976; Wiley, 1978). These species concepts, however, are not applicable to all living organisms since it is seemingly impossible to incorporate the multitude of evolutionary processes driving speciation in one comprehensive definition. These evolutionary processes are just different ways used to describe what a species is, which shows that the species concept essentially is a human construct. It is therefore very unlikely that there ever will be a comprehensive definition for a species (Dobzhansky, 1955).

Still, biologists use species every day. They have to, because species are the most fundamental units of comparison in biology. It is therefore necessary to keep in mind that the concept one chooses to use is just a practical hypothesis. The working hypothesis used in this thesis is based on the phylogenetic species concept (Cracraft, 1983; Nixon and Wheeler, 1990). This concept defines species as the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals. A character state is an inherited attribute distributed among all comparable individuals of the same historical population, clade, or terminal lineage (monophyletic group).

In this thesis, we also encountered variation below the species level. Definitions of infraspecific ranks are an even bigger hornets' nest of contradicting opinions and concepts than that of the species concept itself (McDade, 1995; Stuessy, 1990) and most practical systematists and taxonomists try to avoid these ranks whenever possible. In some cases, however, complex patterns observed within a species demand using infraspecific ranks. In this thesis, two different infraspecific ranks are recognized below the species level: i.e. subspecies and variety. Subspecies differ from each other by at least one diagnosable character and are geographically separated from each other. The same definition is used for a variety, except that varieties are not geographically separated from each other (Stuessy, 1990). By recognizing infraspecific taxa, we acknowledge the existence of deviating populations. We feel that these populations deserve attention 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. In our view, though, the recognition of infraspecific taxa should be based on analyses of both molecular data and morphology in combination with common garden experiments.

Speciation by hybridization and polyploidization

Interspecific hybridization is seen as a common process and important mechanism for speciation in flowering plants (Grant, 1981; Ellstrand and Schierenbeck, 2000; Hegarty and Hiscock, 2004). Two forms of hybrid speciation are commonly recognized: homoploid speciation and alloploid speciation. Homoploid speciation involves the hybridization between two closely related taxa without a change in ploidy, resulting in more or less fertile offspring (Rieseberg, 1997; Rieseberg et al., 2003; Abbott et al., 2005). Alloploid speciation on the other hand usually involves hybridization between more distantly related taxa, which produces sterile offspring. The hybrid offspring then regains its fertility by doubling its chromosomes, which is called allopolyploidy. The resulting polyploid hybrid can have two or more sets of chromosomes derived from different parental species (Stebbins, 1971; Song et al., 1995; Bennett, 2004; Hegarty and Hiscock, 2004).

As a consequence of hybridization, allopolyploidy, but also autopolyploidy (doubling of chromosomes without hybridization) have been important factors in the evolutionary history of plants (Grant, 1981; Soltis and Soltis, 2000). Almost all flowering plants and ferns have experienced at least one polyploidization event in their evolutionary history (Soltis et al., 2009). It is estimated that approximately 15% of the speciation events in flowering plants and 31% of the speciation events in ferns are accompanied by an increase in ploidy (Wood et al., 2009).

The study species: Viola stagnina *and relatives*

The violet family (Violaceae) consists of about 900 species divided in ca. 22 genera (Tokuoka, 2008). *Viola* is the largest genus with approximately 500 species. In contrast to most other genera of the Violaceae, which have a subtropical and tropical distribution, *Viola* species mainly have a northern temperate distribution (Ballard et al., 1999). The primary centers of taxonomic and morphological diversity can be found in the Alps and the Mediterranean, the Himalayas, montane eastern Asia, Patagonia and the South American Andes from where the genus is believed to have originated (Clausen, 1929; Valentine, 1962; Ballard et al., 1999).

Viola species are usually herbaceous plants with zygomorphic flowers. The flowers that fully open i.e. chasmogamous flowers possess adaptations to a wide range of temperate pollinators such as solitary bees, bumblebees, bombyliids and butterflies (Beattie, 1974). Next to these insect pollinated flowers, many species also produce self-pollinating (cleistogamous) flowers later in the season, as an extra reproductive assurance when insects are scarce (Redbo-Torstensson and Berg, 1995). Having developed this reproductive strategy during evolution is probably one of the key aspects responsible for the successful distribution of *Viola* (Clausen, 1929; Valentine, 1962).

Two other key aspects explaining the evolutionary success of *Viola* are hybridization and polyploid evolution and numerous reports have described such events in *Viola* (e.g. Valentine, 1958; Moore and Harvey, 1961; Harvey, 1966; Ballard, 1993; Røren et al, 1994; Erben, 1996; Neuffer et al., 1999; Jonsell et al., 2000; Marcussen and Borgen, 2000; Marcussen et al., 2001; Marcussen et al., 2005). In fact, the first report of an infrageneric series of polyploid levels was from *Viola* (Miyaji, 1913).

Viola stagnina (Fen violet) is a widespread but rare plant species occurring throughout Europe with the exception of the Mediterranean, the southeast and north of Europe (Fig. 10). It favours wet and temporarily flooded, sunny habitats such as floodplains, fens and marshes. (Valentine et al., 1968; Eckstein et al., 2006*a*; Weeda, 2002). Within *Viola*, *V. stagnina* is placed in sect. *Viola* subsect. *Rostratae* Kupffer (also known as section *Trigonocarpea* Godr.). This subsection consists of approximately 50 species with a northern temperate distribution in North America and Eurasia. Subsection *Rostratae* is characterized primarily by primitive characters. Previous phylogenetic studies using nrITS sequences have shown that the subsection is paraphyletic with respect to a number of other north-temperate groups (Ballard et al. 1999; Yoo et al. 2005). In Europe, where subsection *Rostratae* is morphologically most diverse, the subsection has traditionally been subdivided into four morphologically defined groups, here referred to as series. These series are the *Arosulatae*, *Mirabiles*, *Repentes*, and *Rosulantes*.

Viola stagnina is placed in the *Arosulatae* series. This series consists of a group of five western Eurasian species. Species of the series are adapted to temporarily flooded habitats, rather than woodland, and are easily characterized by their leaf and stipule characters and the lack of a leaf rosette. The basal chromosome number of subsection *Rostratae* is x = 5, and since no diploid species (2n = 10) are known for this subsection, *V. stagnina* is considered to be a paleotetraploid with 2*n*=20 chromosomes (Marcussen and Nordal, 1998). *Viola canina*, *V. elatior*, and *V. pumila* are octoploids with 2*n*=40 and *V. lactea* is a subdodecaploid with 2*n*=58 chromosomes (Moore and Harvey, 1961). Cytological studies have shown that *V. stagnina* is involved as one of the parental species in the autoploid and alloploid origin of the other arosulate Violets (Fig. 1). *Viola canina*, *V. pumila*, and *V. lactea* are all alloploids, which have *V. stagnina* as one of the parental contributors to their alloploid genome (Moore and Harvey, 1961), while *V. elatior* is considered to be an autoploid derivative of *V. stagnina* (Clausen, 1927). The other parental species contributing to the alloploid genomes of the arosulate violets are likely to be extinct.

The varieties within V. stagnina

In the Netherlands, two morphs of *V. stagnina* have been described: *V. stagnina* var. *stagnina* and *V. stagnina* var. *lacteoides* W. Becker and Kloos (1924). The second variety was mentioned for the first time by Kloos (1924). He reported finding specimens resembling *V. stagnina* but being smaller in habit and having darker colored and thicker leaves. After having consulted Becker he concluded that he had found a new morph which he named *V. persicifolia* var. *lacteaeoides*. Dutch botanists after Kloos, however, had different opinions about the subdivision of *V. stagnina* into two infraspecific taxa, and after appearing in the flora of Heimans et al. (1924) and in the Heukels' Schoolflora voor Nederland (1927) the variety disappeared from subsequent Dutch floras until 1977. The varieties were mentioned again in the Heukels' flora (van Oostroom, 1977), this time as subspecies. Den Held described subsp. *lacteoides* in the addenda and added that its stigma is straight as compared to hooked in *V. stagnina* subsp. *stagnina*, and that the spur of subsp. *lacteoides* exceeds the appendices on the calyx, whereas the spur of *V. stagnina* subsp. *stagnina* normally does not exceed these. 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 (1990) no infraspecific taxa were recognized for *V. stagnina* anymore because Van der Meijden considered the differences between the morphs too small. 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 both morphs of *V. stagnina* have a different geographical distribution with only a small overlap. The common *stagnina* morph is found in the Holocene part of The Netherlands where it mainly grows 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. There it is mainly found in the valley of the IJssel river on the lower parts of wet heath lands on loamy and peaty soil (Weeda, 2001). Since the *lacteoides* morph has not been found outside The Netherlands, this is probably the first endemic plant for the Netherlands. Investigating its

taxonomic status with molecular biological techniques is therefore not only interesting from a scientific point of view, but also important for conservation management, since the *lacteoides* morph has a very limited distribution area and needs active conservation management for its preservation.

Research questions

In this thesis, infraspecific variation within *V. stagnina* and hybridization and polyploidization between *V. stagnina* and its closest relatives were investigated to answer the following research questions:

1. Which species are most closely related to *V. stagnina*?
2. Can reticulate patterns of evolution between *V. stagnina* and its closest relatives be determined by using the low copy nuclear Chalcone Synthase (CHS) marker?
3. How many duplication events of CHS have taken place during the evolution of *Viola*?
4. Are *V. persicifolia* and *V. montana* the appropriate scientific names to use?
5. Is *V. stagnina* var. *lacteoides* genetically distinct from the more common *V. stagnina* var. *stagnina*?
6. Are there morphological traits separating the two morphs of *V. stagnina* from each other?

Thesis Goal & Outline

In chapter 2, the results of a phylogenetic study are presented in which the closest relatives of *V. stagnina* are determined including their reticulate relationships by using sequences of the CHS gene. This study also presents the evolutionary history of the CHS gene itself within the angiosperms.

In chapter 3, the nomenclatural history of the scientific names *V. persicifolia* Schreb. (1771) and *V. montana* L. (1753) are discussed. In order to give priority to the names *V. stagnina* and *V. elatior*, we propose to reject the older name *V. persicifolia* and *V. montana* respectively in chapter 4.

In chapter 5, we aim to determine the taxonomic status of the *lacteoides* morph of *V. stagnina* by studying the morphological and genetic variation of different populations. In chapter 6, a common garden experiment, a crossing experiment and a chromosome count of both varieties are described. Also the nomenclature of the *lacteoides* morph for both the scientific and vernacular epithets is discussed.

Chapter

2

Chalcone Synthase Gene Lineage Diversification confirms Allopolyploid Evolutionary Relationships of European Rostrate Violets[1]

K. van den Hof, R.G. van den Berg
and B. Gravendeel

Phylogenetic relationships among and within the subsections of the genus *Viola* are still far from resolved. We present the first organismal phylogeny of predominantly western European species of subsection *Rostratae* based on the plastid *trn*S-*trn*G intron and intergenic spacer and the nuclear low-copy gene Chalcone Synthase (CHS) sequences. CHS is a key enzyme in the synthesis of flavonoids, which are important for flower pigmentation. Genes encoding for CHS are members of a multigene family. In *Viola,* three different CHS copies are present. CHS gene lineages obtained confirmed earlier hypotheses about reticulate relationships between species of *Viola* subsection *Rostrata*e based on karyotype data. Comparison of the CHS gene lineage tree and the plastid species phylogeny of *Viola* reconstructed in this study indicates that the different CHS copies present in *Viola* are the products of both recent and more ancient duplications.

Key words: Chalcone synthase, gene lineage diversification, phylogeny, *Viola* subsection *Rostratae*, allopolyploidy, *trn*S-*trn*G.

[1]Published as: van den Hof et al., 2008. Mol. Biol. Evol. 25: 2099-2109.

Introduction

Speciation through hybridization is considered a common process in higher plants. Although hybrids between distantly related taxa are usually sterile, they can become fertile again by doubling their chromosome numbers. The resulting chimeric species can have two or more sets of chromosomes derived from different parental species; this is called allopolyploidy (Stebbins, 1971; Song et al., 1995; Bennett, 2004; Hegarty and Hiscock, 2005). In contrast with allopolyploidy, which may occur in connection with hybridization between taxa that are not very closely related, hybrid speciation without a change in chromosome number may occur in cases where the parental species are closely related and their primary hybrid is somewhat fertile; this process is called homoploid hybrid stabilization (Rieseberg, 1997; Rieseberg et al., 2003; Abbott et al., 2005).

Polyploid evolution has been an important factor in the evolutionary history of land plants, and continues to be so also in extant lineages such as the plant genus *Viola* (Violaceae). In fact, the first report of an infrageneric series of polyploid levels was from *Viola* (Miyaji, 1913). The base chromosome number of *Viola* is believed to be $x=6$ or $x=7$, but the vast majority of north-temperate taxa have been shown to be paleo-allotetraploid with secondary base numbers of $x=10$ or $x=12$ (Nordal and Jonsell, 1998; Marcussen and Nordal, 1998; Karlsson et al., 2009). These are hereafter referred to as secondary diploids. Further polyploidy based on these secondary diploid chromosome numbers has been demonstrated especially within the species-rich subsections of section *Viola* (Karlsson et al., 2009).

Within Section *Viola* subsection *Rostratae* Kupffer (sometimes treated as the separate section *Trigonocarpea* (Godr.) Vl. V. Nikitin), most species have retained the secondarily diploid chromosome number of $2n=20$. However, subsequent polyploidization events have led to the formation of higher-ploids with chromosome numbers of $2n=40$ (octoploid), 60 (dodecaploid) or even 58 (sub-dodecaploid); these are hereafter referred to as secondary tetraploids and (sub-)hexaploids, respectively. Nearly all of these secondary polyploids, a total of ten species, are native to western Eurasia, and their relatively recent polyploid parentages have been investigated in a series of cytological studies in the late 1950s and early 1960s (fig. 1) (Valentine 1950, 1958; Moore and Harvey 1961; Harvey, 1966).
The subsection consists of about fifty species with a northern temperate distribution in North America and Eurasia. Most species have white to dark lilac flowers and grow in woodlands. Subsection *Rostratae* is characterized primarily by primitive characters. Phylogenetic analyses based on nuclear ribosomal Internal Transcribed Spacer (nrITS) sequences have recovered that the subsection is paraphyletic with respect to a number of other north-temperate groups (Ballard et al., 1999; Yoo et al., 2005). In Europe, where subsection *Rostratae* is morphologically most diverse, the subsection has traditionally been subdivided in a variable number of morphologically defined groups, usually at the series level. Series *Rosulantes* is characterized by having a basal rosette and flowers produced only from the lateral aerial shoots; this growth form is found also in other sections and may be considered as primitive within the genus. Series *Mirabiles* differs from the *Rosulantes* in producing flowers also from the basal leaf rosette, series *Arosulatae* in lacking the basal rosette altogether, and series *Repentes* in being stoloniferous and producing flowers from the rosettes. However, the recognition of series is problematic for

two main reasons. First, the series typically define small groups of species by a very limited number of autapomorphies, thereby rendering the remaining groups paraphyletic and defined by synplesiomorphies only. Second, several of these series cannot be considered monophyletic because of the alloploid relationships between taxa of different series.

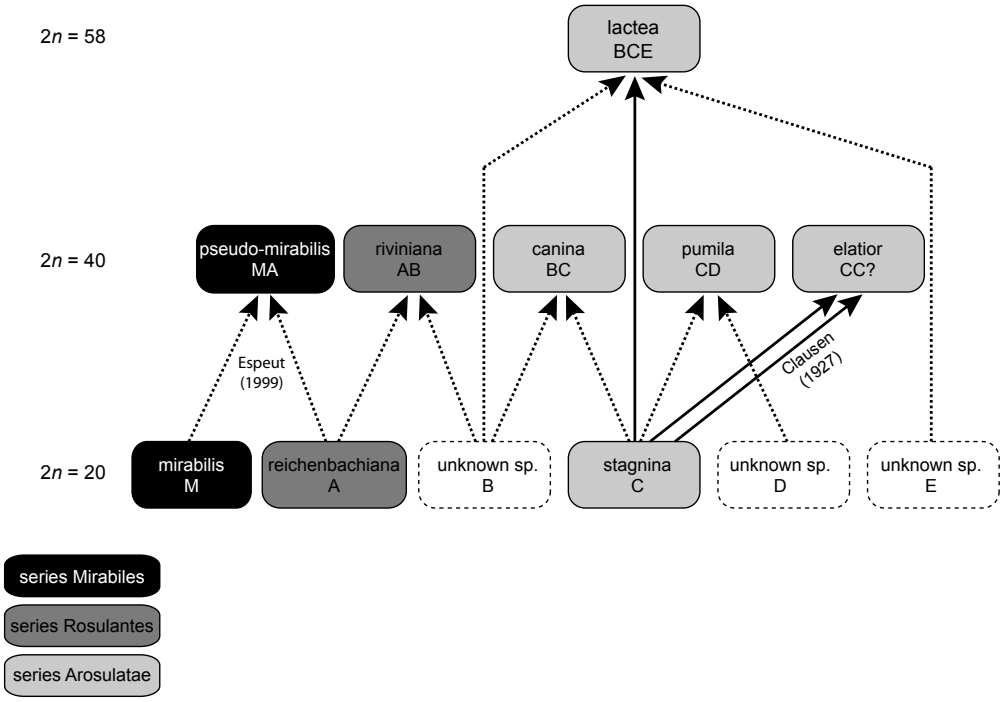


Fig. 1. Hypotheses of relationships between different genome types (A, B, C, D, E and M) in species of *Viola* subsection *Rostratae*. Series affinity is indicated with shades of gray: series *Mirabiles* black; series *Rosulantes* dark grey; series *Arosulatae* light gray. Presumably extinct taxa are indicated with dashed lines. Data from Moore and Harvey (1961) except where indicated (Clausen, 1927; Espeut, 1999).

Series *Arosulatae* defines a small group of five western Eurasian species. Species of the series are specialists of temporarily flooded habitats, rather than woodland, and are easily characterized by lacking leaf rosettes and by their leaf and stipule characters. Traditionally seven species have been recognized, but two (the East Asian *V. acuminata* and the submediterranean *V. jordanii*) must be omitted on the basis of having a leaf rosette and the quite different choices of habitat. The remaining five species are all Central European. *Viola stagnina* is the only secondary diploid with 2*n*=20, whereas three species are secondary tetraploids with 2*n*=40 (*V. canina*, *V. elatior*, *V. pumila*) next to a secondary sub-hexaploid with 2*n*=58 (*V. lactea*) (Moore and Harvey, 1961).

Especially the study by Ballard et al. (1999) indicated that the taxonomy of the genus *Viola* needs revision and that more molecular phylogenetic studies are called for. Although the nrITS region used by Ballard et al. (1999) was useful for recognizing infrageneric groups of the genus *Viola*, nrITS is generally not useful for examining evolutionary relationships among polyploid lineages. This is because recombination and concerted evolution between orthologous nrITS copies often lead to retention of only one copy type and erasion of the

other parental copy (Wendel et al., 1995; Álvarez and Wendel, 2003). This is usually also the case in *Viola*, as nearly all investigated species have retained only one nrITS copy type regardless of ploidal level (Ballard et al., 1999; Malécot et al., 2007). The nrITS as a phylogenetic marker is therefore not suitable for recovering the reticulate relationships within the genus. Plastid markers generally also have the problem of retention of a single parental copy as these are usually uniparentally inherited in plants; furthermore, sequence variation in plastid markers is usually low (Corriveau and Coleman, 1988; Taberlet et al., 2007). Again, reticulate relationships therefore remain obscure.

Álvarez and Wendel (2003) suggested using single or low copy nuclear markers to circumvent the problem of concerted evolution causing misleading phylogenetic reconstructions of polyploid species. Phylogenetic analysis of paralogous and orthologous copies of single or low copy genes in alloploid species is also a good method to reveal the parental contributors to alloploid genomes. This method has been successfully applied in numerous studies (e.g. Popp and Oxelman, 2001; Smedmark et al., 2005).

We utilized the low copy nuclear Chalcone synthase (CHS) gene as a phylogenetic marker in *Viola* subsection *Rostratae*. As an independent dataset we chose the *trn*S-*trn*G intergenic spacer and intron as plastid phylogenetic marker, as this region proved to be sufficiently informative in *Viola* to assess interspecific relationships.

CHS is the first enzyme in the flavonoid synthesis pathway and is encoded by a small gene family (Durbin et al., 1995). Flavonoids are important secondary metabolites responsible for a multitude of tasks in plants, ranging from flower and fruit coloration and protection against UV radiation to pathogen defense and pollen development (Harborne, 1994). In *Viola cornuta*, three different CHS gene copies were found to be expressed from early stages of flower coloration onwards (Farzad et al., 2003). In general, genes of the CHS family consist of one intron flanked by two exons. There is high variation in the number of CHS copies among angiosperms. In asterids the number of CHS copies ranges from a single copy in *Antirrhinum* (Sommer and Saedler, 1986) to six copies in *Ipomoea* (Clegg and Durbin, 2003) and eight in *Petunia* (Koes et al., 1987). Similarly for the rosids, both *Arabidopsis* (Wang et al., 2007) and *Populus* (Tuskan et al., 2006) have two CHS copies, whereas both *Vitis* (Sparvoli et al., 1994; Jaillon et al., 2007) and *Viola cornuta* cultivars (Farzad et al., 2003; 2005) have three CHS copies.

We collected different CHS paralogues in species of *Viola* subsection *Rostratae* and analyzed these phylogenetically to 1) test earlier hypotheses about reticulate relationships of several allopolyploid taxa based on karyotype data in subsection *Rostratae* (e.g. between *V. stagnina*, a possible Dutch endemic, and its closest relatives), 2) make a comparison with a species phylogeny of *Viola* subsection *Rostratae* based on sequences of the plastid *trn*S-*trn*G intron and intergenic spacer to infer how many duplications of CHS took place during the evolution of *Viola*.

Materials and Methods

Taxon sampling

In total, 30 *Viola* taxa with a predominantly western European origin were sampled, of which 21 taxa belong to *Viola* subsection *Rostratae*. The nine taxa outside subsection *Rostratae* represent sections *Andinium*, *Boreali-Americanae*, *Chamaemelanium*, *Erpetion*,

and *Melanium* and subsection *Viola* of section *Viola*. These species appeared to be either closely or more distantly related to the species of subsection *Rostratae* in a previous molecular phylogenetic study of *Viola* (Ballard et al., 1999). DNA was obtained from freshly collected material from the field and from herbarium collections.

For reconstruction of the CHS gene lineage tree, two different parts of the gene were sampled, the intron and exon 2. Exon 2 lineages available in Genbank from representatives of major Angiosperm clades were included in the analysis to find out whether the different CHS copies present in *Viola* are the products of recent or more ancient duplications. The following lineages were sampled: Gymnosperms: *GbCHS* (*Ginkgo biloba*, AY647263) and *PsCHS* (*Pinus sylvestris*, X60754); Monocots: *IhCHS* (*Iris x hollandica*, AB232914), *HvCHS* (*Hordeum vulgare*, X58339), and *ZmCHS* (*Zea mays,* AY728478, X60204); Core eudicots: *VvCHS* (*Vitis vinifera*, AB015872, AB066275, EF192464, AM 454341, X75969); Rosids: *GmCHS* (*Glycine max*, AY262686), *PsCHS* (*Pisum sativum*, D88263, D88262, D88261, D88260, X63333), *PtCHS* (*Populus* spp., DQ371804, EF147137, EF147091, DQ371802), *VcCHS* (*V. cornuta* cultivar, AY497407, AY497414); Asterids: *AmCHS* (*Antirrhinum majus*, X03710*), DcCHS* (*Daucus carota*, D16255), and *PhCHS* (*Petunia hybrida*, X14597).

The phylogenetic analyses performed were all rooted differently. There were several reasons for this. First of all, plastid sequences of non-Violaceae were not used for phylogenetic analyses. The Angiosperm Phylogeny Group topology was used to constrain the analyses instead (see below). For the plastid phylogeny, closely related genera of *Viola* were used as outgroups. Second, CHS intron sequences outside *Viola* could not be aligned with CHS intron *Viola* data because of too high sequence divergence. For the CHS intron analyses, we therefore tentatively used *Viola CHS3* as outgroup. Third, CHS gene duplication events could only be assessed with a broad taxonomic sampling. For the CHS exon 2 analyses, we therefore used gymnosperm lineages for rooting.

DNA Extraction, Polymerase Chain Reaction Amplification, Cloning, and Sequencing

Total genomic DNA was extracted using the Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) and the cetyltrimethylammonium bromide (CTAB) method of Doyle JJ and Doyle JL (1987) with some modifications. Leaf material was ground using a Ratch Mill. In total, 750 µl CTAB buffer (Doyle and Doyle, 1987) was added to the ground material together with proteinase K and RNase. After incubating for 30 minutes at 60°C, 750 µl of chloroform-isoamyl alcohol (24:1) was added. The samples were briefly vortexed and then centrifuged for 10 minutes at 12,000 rpm. The upper aqueous layer was transported to a clean 2 ml tube. A total of 500 µl chloroform-isoamyl alcohol (24:1) was added and the samples were again centrifuged at 12,000 rpm. After 5 minutes spinning, the upper phase was transferred to a new 2 ml tube. The DNA was then precipitated by adding cold 500 µl isopropanol. The samples were shaken 5 to 10 minutes and subsequently centrifuged for 15 minutes at 12,000 rpm. The supernatant was removed and 70% ethanol was added. The samples were subsequently shaken vigorously for 2 minutes, after which the ethanol was poured off. The remaining ethanol was removed by evaporation. The resulting DNA pellet was dissolved in 200 µl 0.1x Tris-EDTA buffer.

In total, one plastid region (*trn*S-*trn*G spacer and intron) and one nuclear region (CHS intron and exon 2) were amplified and sequenced. Polymerase chain reaction (PCR) amplification of the plastid spacer and intron was performed with primers designed by

Shaw et al. (2005). For the *trn*S spacer, the primers trnS^{GCU} (5′-AGA TAG GGA TTC GAA CCC TCG GT-3′) and trnG2S (5′-TTT TAC CAC TAA ACT ATA CCC GC-3′) were used. The *trn*G intron was amplified with the primers trnG^{UUC} (5′-GTA GCG GGA ATC GAA CCC GCA TC-3′) and trnG2G (GCG GGT ATA GTT TAG TGG TAA AA).

The primers CHSX1F (5′-AGG AAA AAT TCA AGC GCA TG-3′) and CHSX2RN (5′-TTC AGT CAA GTG CAT GTA ACG -3′) designed by Strand et al. (1997) were used for amplifying the CHS intron. The primers CHS forward (5′-TAY CAR CAR GGN TGY TTY GC-3′) and CHS reverse (5′-GGR TGD GCD ATC CAR AAV A-3′) from Farzad et al. (2003) were used to amplify exon 2 of the CHS gene (fig. 2a). The generated intron and exon sequences did not overlap as the intermediate part turned out to be too large and too heterogeneous for this. PCR fragments for several *Viola* species of the CHS intron are shown in fig. 2b. Per individual, 12 clones were analyzed and consensus sequences were compiled from 3-7 individual clones.

Fig. 2. Amplified regions of chalcone synthase (CHS):

Fig 2a. Map of the two exons and interjected intron of the CHS gene. Primers and their binding sites are indicated for the CHS intron data set (black triangles) and the CHS exon 2 data set (white triangles).

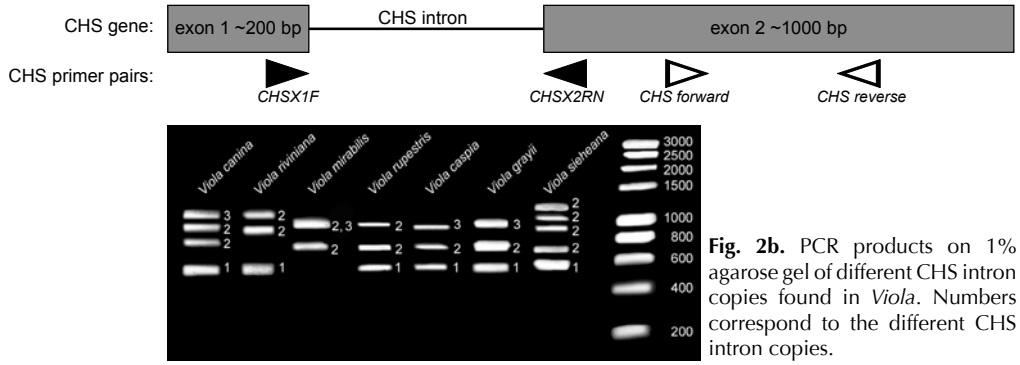


Fig. 2b. PCR products on 1% agarose gel of different CHS intron copies found in *Viola*. Numbers correspond to the different CHS intron copies.

PCR amplification conditions for the *trn*S spacer consisted of denaturing for 50 s at 95°C, annealing for 1 min at 53°C, and extension for 2 min at 72°C. This cycle was repeated 35 times. The *trn*G intron was amplified with the same conditions except for the annealing temperature which was 56°C. PCR amplification conditions for the CHS intron consisted of denaturing for 1 min at 95°C, annealing for 90 s at 53°C, and extension for 2 min at 72°C. This cycle was repeated 35 times. The conditions for amplifying the CHS exon 2 consisted of denaturing for 45 s at 95°C, annealing for 1 min at 55°C and extension for 1 min at 72°C. This cycle was repeated 40 times.

PCR products were purified using the Promega Wizard Purification System, cloned using the pGEM®-T Easy Vector System and sequenced using the M13 primers with 30 s denaturing at 95°C, annealing for 30 s at 50°C, and extension for 1 min at 72 °C. This cycle was repeated 35 times. PCR products were purified and analyzed on an ABI 377 (Applied

Biosystems Inc., Foster City, CA) or a MegaBACE Sequence Analyzer 4.0 (Amersham Biosciences, Uppsala, Sweden) automated sequencer using the manufacturers' protocols.

Phylogenetic analyses

DNA sequences were aligned using McClade 4.06 (Maddison DR and Maddison WP, 2003) with the pairwise alignment option and manual adjustment where necessary. Individual insertion and deletion events were manually added as additional binary characters.

MrModeltest version 2.2 (Nylander, 2004) was used to find the best model of sequence evolution (Posada and Crandall, 1998). The models used for Bayesian analyses were the symmetrical model with separate gamma distributions and a separate proportion of invariant sites for CHS exon 2 (SYMIG model), the General Time Reversible model with gamma distribution for CHS intron (GTRG model), and the General Time Reversible model with gamma distribution and a separate proportion of invariant sites for *trn*S-*trn*G (GTRIG model). Maximum Parsimony (MP) analyses were carried out with PAUP* 4.0b10 (Swofford, 2003). Phylogenies were obtained using the heuristic search option, with twenty random sequence additions and Tree Bisection-Reconnection branch swapping. After each sequence addition, a maximum of 10,000 trees was saved.

For MP, bootstrap support (Felsenstein, 1985) was calculated with 2,000 bootstrap replicates, using only ten random sequence additions each bootstrap replicate. After every random sequence addition replicate a maximum of 2,500 trees were saved. Bayesian inference analyses were performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Markov Chain Monte Carlo analyses (MCMC) were run for eight million generations with five simultaneous MCMCs, saving one tree per 100 generations. The burn-in values were identified using the program Tracer 1.3 (Rambaut and Drummond, 2004).

To convert the CHS gene tree composed of multiple paralogous lineages from allopolyploid taxa into a species tree to assess gene duplications, GeneTree version 1.3 (Page and Charleston, 1997) was used. The analyses were run with default settings. GeneTree requires fully resolved organismal and gene trees as input. For the organismal tree, one of the 200,000 fully resolved most parsimonious trees (MPTs) of *trn*S-*trn*G data was chosen randomly. This analysis was constrained for all non violets to the latest angiosperm phylogeny topology as depicted on the Angiosperm Phylogeny Website (version 8, June 2007) (www.mobot.org/MOBOT/research/Apweb/). For the CHS exon 2 gene tree, the 95% most probable Bayesian tree was used.

Homology Assessment of CHS Copies

The CHS lineages found were assigned to different copies based on size, sequence divergence and phylogenetic position (Helariutta et al., 1996; Doyle and Davies, 1998; Smedmark et al., 2005). CHS fragments within one species with only minor divergence and gaps were interpreted as alleles. The different CHS copies of *V. cornuta* published by Farzad et al. (2003) always ended up in a single clade in all analyses performed here. We therefore used a single representative sequence only. When size difference and sequence divergence were more apparent, e.g. by the presence of large indel events, the CHS fragment was treated as a paralogous copy. Our classification of alleles and paralogous copies was further confirmed by topological positions in the phylogenies obtained.

Results

*trn*S-*trn*G

MP analyses of the *trn*S-*trn*G alignment produced a total of 200,000 MPTs with 537 steps (consistency index [CI] = 0.8239; retention index [RI] = 0.7312). The majority rule consensus tree (data not shown) has a similar topology as the Bayesian tree (fig. 3). We plotted both the Bootstrap Support values (BS) and Posterior Probability Index values (PPI) on the latter. All species sampled of *Viola* subsection *Rostratae* ended up in five different, poorly to well supported subclades (<50–98% BS; 0.56–1.00 PPI). The largest subclade consists of *V. stagnina*, *V. elatior*, *V. lactea*, *V. canina*, *V. sieheana*, *V. jordanii*, *V. oligyrtia*, *V. rupestris*, and *V. pumila*.

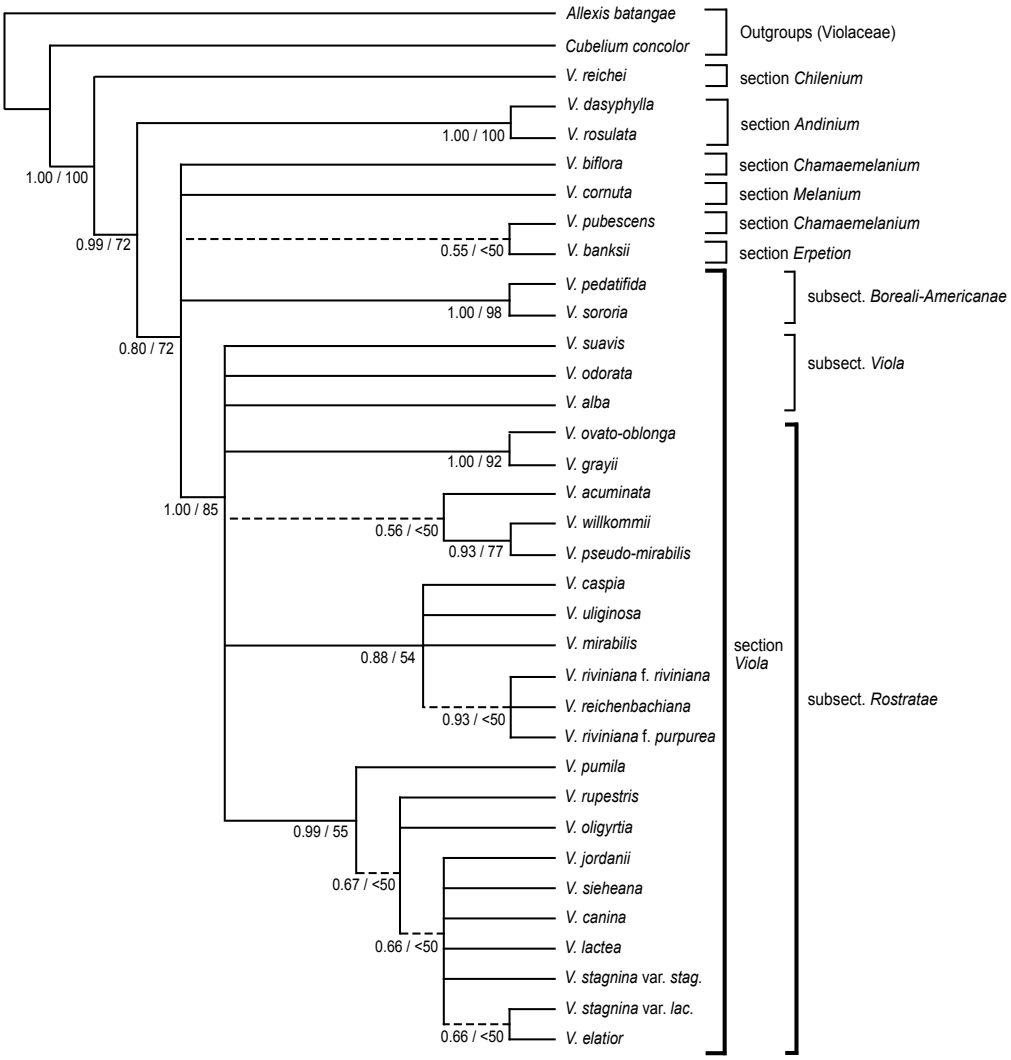


Fig. 3. Plastid *trn*S-*trn*G phylogeny of *Viola*.
(MP majority rule consensus of 200,000 trees; ci = 0.8239, ri = 0.7312, 537 steps. Numbers on branches refer to PPI and BS values).

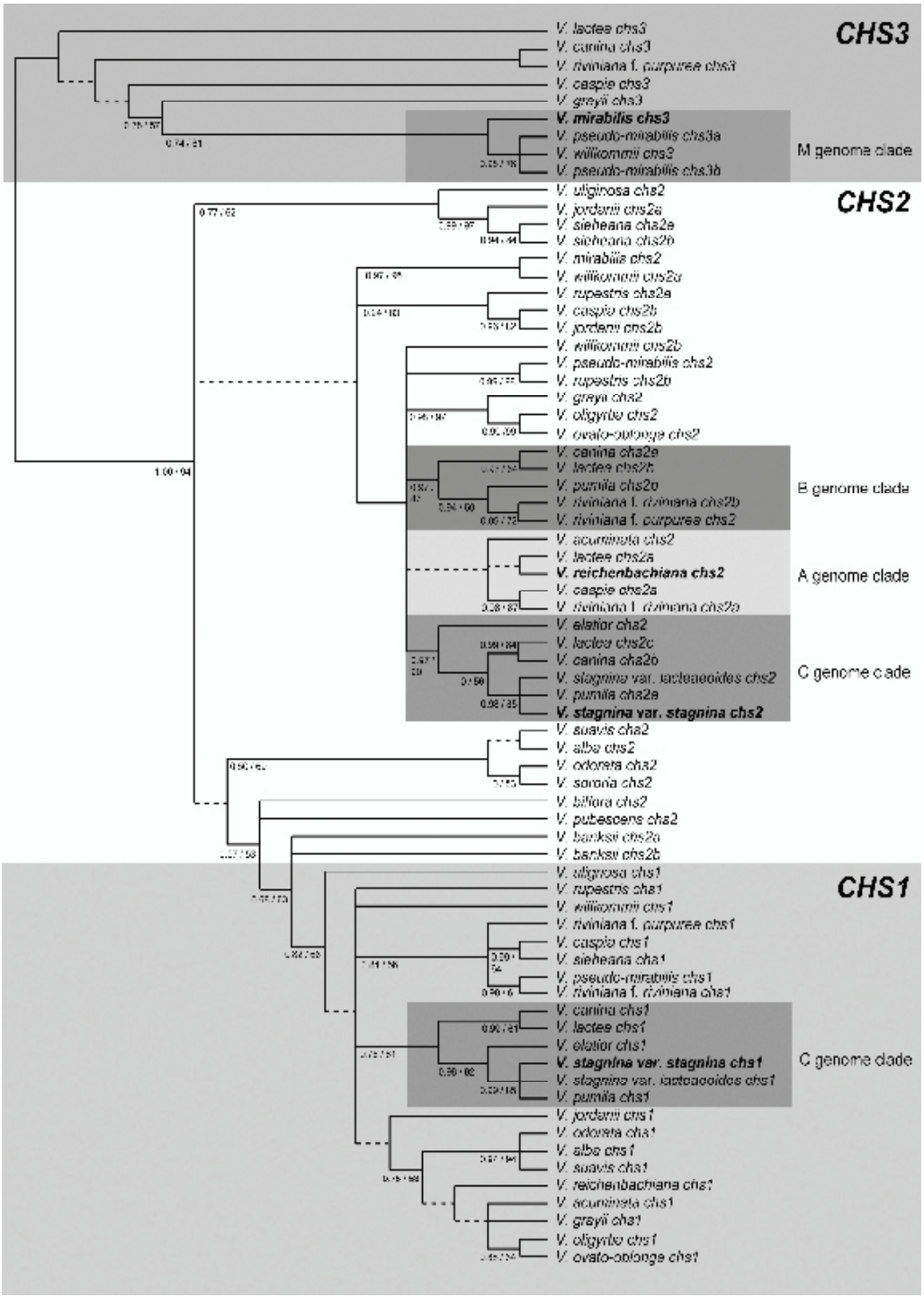


Fig. 4. CHS intron gene lineage tree.
(MP majority rule consensus of 200,000 trees; ci = 0.7828 ri = 0.8824, 921 steps. Numbers on branches refer to PPI and BS values) The genomes described in Figure 1 which could be recognized are indicated. Taxa in bold refer to extant secondary diploids.

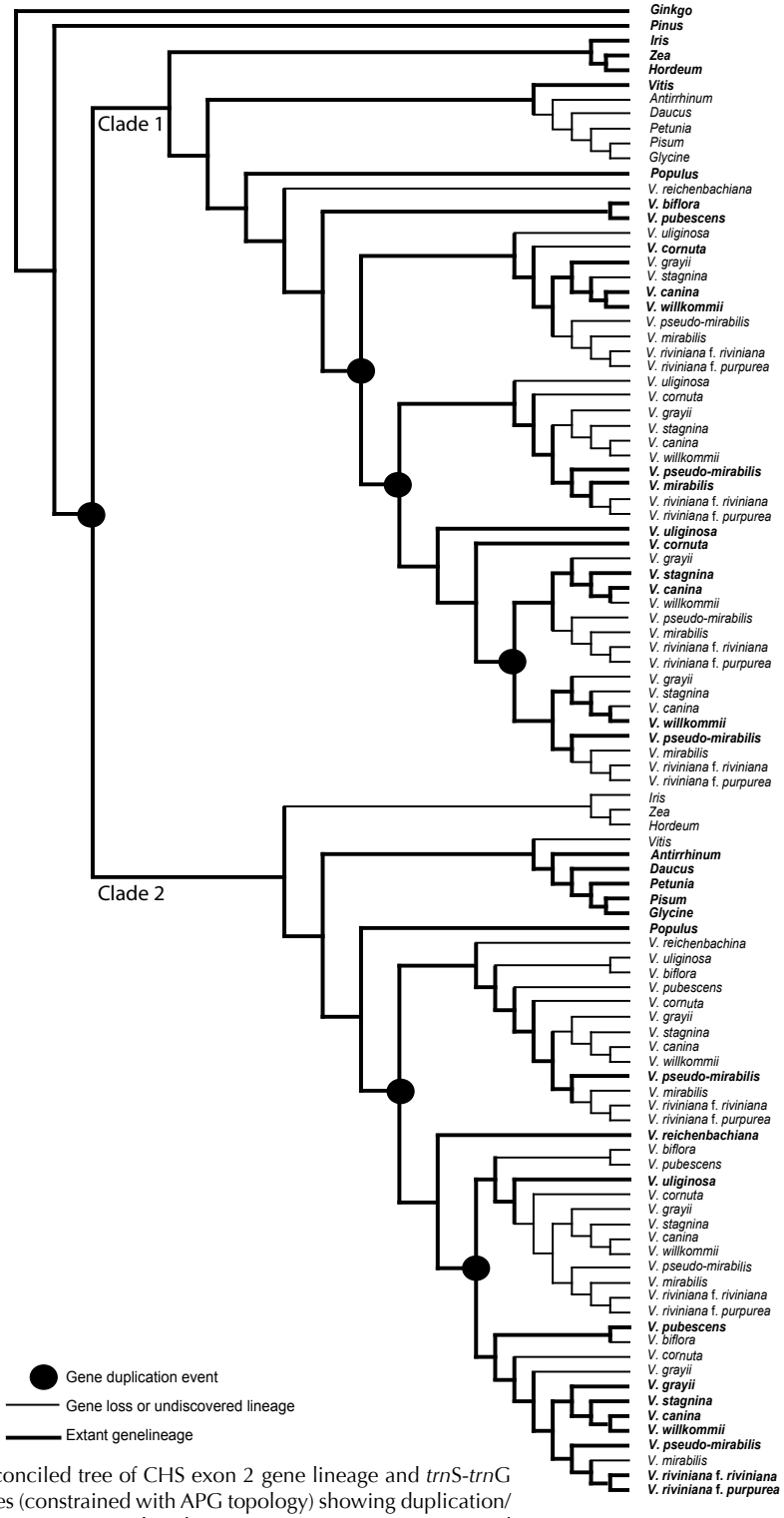


Fig. 5. Reconciled tree of CHS exon 2 gene lineage and *trn*S-*trn*G phylogenies (constrained with APG topology) showing duplication/loss events, reconstructed with GeneTree version 1.3 (Page and Charleston, 1997).

CHS intron and exon 2

Three copies of the CHS intron were found in the sampled species of *Viola* subsection *Rostratae* (figs. 2b and 4). Copies 1 (*CHS1*, 600 base-pairs [bp]) and 2 (*CHS2*, 735–1100 bp) have a relatively similar sequence identity and were found in all *Viola* species sampled with the exception of *V. biflora*, *V. banksii*, and *V. pubescens*. The third copy was not found in all species and was the largest sized (*CHS3,* 775–1,160 bp). The *CHS3* copy is probably present in more taxa, but amplification failures probably led to an under sampling of this particular copy. It differed quite substantially from the other two copies in size and sequence similarity. In contrast with *CHS1*, multiple paralogs/orthologs were found in *CHS2* and *CHS3*.

The complete CHS intron alignment consisted of 2,980 bp after exclusion of a 64 bp segment that was too variable for proper alignment. A total of 302 characters were phylogenetically informative, of which 12 were indel characters (indels varying in size between 5 and 422 bp). Two indels, found in *CHS1* and *CHS2*, seemed to be the result of slip strand mispairing as many repeats were found in these regions. The first (TGATTT) and second repeat (TGTT) were repeated up to four times. The other ten indels lacked a repetitive structure. Most of the indels occurred in *CHS2*. In *CHS3*, two large indels were found of 182 and 422 bp, respectively.

MP analyzed of CHS intron sequences produced 200,000 MPTs (921 steps, CI = 0.7828, RI = 0.8824). The majority rule consensus tree (fig. 4) had a similar topology as the Bayesian tree (data not shown).

At least two different copies of CHS exon 2 were found in *Viola*. Both copies had a similar size (984 bp) and were retrieved from almost all *Viola* species analyzed. They differed substantially in sequence similarity. Of the 984 bp retrieved, 498 were phylogenetically informative. One autapomorphic gap was found. Bayesian analyses of CHS exon 2 sequences produced a topology similar to MP (data not shown), in which two main clades were present comprising the different copies of CHS exon 2.

Reconciliation of Gene Tree and Species Tree

A reconciled tree (fig. 5) reconstructed with the program GeneTree (Page and Charleston 1997) was used to visualize CHS exon 2 duplications during the evolution of *Viola*. It seems that assuming six CHS gene duplication events is sufficient to make the gene and species tree congruent.

Discussion

Polyploidy in Viola Subsection *Rostratae*

The internal topology of the *CHS2* intron clades (fig. 4) is in general agreement with previously inferred relationships between parental species and their allopolyploid hybrids in *Viola* subsection *Rostratae* (Moore and Harvey, 1961). Moore and Harvey (1961) could recognize the parental karyotypes in the genomes of artificially constructed allopolyploid *Viola* hybrids by the unique size and shape of the chromosomes. Subsequently, they

used observations on chromosome pairing to formulate hypotheses regarding the origin of allopolyploids (fig. 1). In their study, five different types of genomes (A–E) could be recognized, each referring to the secondary diploid level (2*n*=20). Only the A and C genome occurred in extant secondary diploids, namely in *V. reichenbachiana* (A) and *V. stagnina* (C). The other four genome types were found only in combination with other genome types in the secondary tetraploid (2*n*=40) or sub-hexaploid taxa (2*n*=58). From this, they concluded that *V. stagnina* (C) contributed a C genome to *V. canina* (BC) and its close relative *V. lactea* (BCE) and possibly also to *V. pumila* (CD). Similarly, *V. reichenbachiana* (A) would have contributed an A genome to *V. riviniana* f. *riviniana* (AB). Thus, the species possessing the B genome was involved in the origin of both *V. riviniana* f. *riviniana* (AB) and *V. canina* (BC). The authors attributed the three "missing" genomes (B, D, and E) to secondary diploids species that might have become subsequently extinct, at least in Europe. *Viola elatior* was not included in this study.

In the *CHS2* intron tree, *V. canina* was found to have one orthologue in common with *V. stagnina* (corresponding to genome C) and a second in common with *V. riviniana* (corresponding to genome B). The second orthologue of *V. riviniana* was found to be closely related to *V. reichenbachiana* (corresponding to genome A). Like *V. canina*, *V. pumila* had one orthologue in common with *V. stagnina* (genome C) while its second copy was found to be closely related to *V. canina* and *V. riviniana* suggesting that genome D could have been derived from genome B.

The phylogenetic position of the gene lineages retrieved from *V. elatior* suggests that this particular species probably contains the C genome because its *CHS2* intron copy ended up close to *V. stagnina*. The chromosome number of *V. elatior* (2*n*=40) suggests that it is a secondary tetraploid, but we were not successful in detecting more than one orthologue in this species. Unpublished isozyme studies also reveal a lower number of allozymic bands than usual in species of this ploidal level. These findings, together with earlier observations of quadrivalents in the meiosis of the species (Clausen, 1927) indicate that *V. elatior* may be an autopolyploid derivative of some *stagnina*-like ancestral species possessing the C genome.

In contrast with the *Arosulatae* series, which was found to be monophyletic for *CHS1* and part of *CHS2*, CHS lineages retrieved from species assigned to the *Rosulantes* and *Mirabiles* series did hardly ever end up in the same clades. This is probably caused by the fact that the morphological characters used to delimit these series are phylogenetically uninformative. Unknown hybridization and polyploidisation events within and between these series probably also cause paraphyly.

The small series *Mirabiles* consists of one secondarily diploid (2*n*=20) species, *V. mirabilis*, and two secondary tetraploids (2*n*=40). The local endemic *V. pseudo-mirabilis* of Les Grands Causses in southern France has been variously interpreted on morphological grounds as an intermediate between *V. mirabilis* and *V. riviniana* (Valentine et al., 1968) or as a polyploid derivative of *V. mirabilis* and *V. reichenbachiana* (Espeut, 1999). The latter view has later been confirmed by own unpublished isozyme data and a chromosome count of 2*n*=40 (Verlaque and Espeut, 2007). In the present study, *V. pseudo-mirabilis* ends up as sister to *V. riviniana* (61% BS; 0.99 PPI), which is not in contradiction to the previous findings because *V. riviniana* is itself a polyploid derivative of *V. reichenbachiana*. The other secondary tetraploid *Mirabiles* species, *V. willkommii*, endemic to northern Spain, is morphologically rather similar to *V. mirabilis* but differs considerably in choice of habitat. This particular species is a secondary tetraploid and has been supposed to have

originated from *V. mirabilis* and *V. rupestris* (Marcussen, personal communication). Our CHS data confirm the parentage of *V. mirabilis* but the second parent of *V. willkommii* remains unclear. Two paralogues of the *CHS2* intron were retrieved from *V. willkommii*, of which one ended up in a strongly supported clade (96% BP; 0.97 PPI) with *V. mirabilis* and the other in an unresolved polytomy. The *CHS1* intron fragment retrieved from *V. willkommii* ended up in a basal dichotomy with the lineage of *V. rupestris*. Future genome type data should be collected of the *Mirabiles* series to confirm hypotheses about reticulate relationships suggested by the CHS gene lineages obtained here.

Closest Relatives of Viola stagnina

Viola subsection *Rostratae* as mentioned before has been taxonomically subdivided in series *Arosulatae*, *Mirabiles*, *Repentes* and *Rosulantes* (Valentine, 1958). *Viola stagnina* is considered to be a member of the *Arosulatae* series together with *V. canina*, *V. elatior*, *V. lactea*, and *V. pumila*, based on the lack of a basal leaf rosette. This taxonomic placement is supported by the fact that in our study, two *Arosulatae* clades are present in the CHS intron gene lineage tree (fig. 4), one consisting of *CHS1* intron lineages and one of *CHS2* intron lineages. These lineages were all retrieved from species assigned to the *Arosulatae* series.

Based on previous morphological and karyological studies and our results, we can conclude that the closest relatives of *V. stagnina* are *V. pumila*, *V. elatior*, *V. canina* and *V. lactea*. Fingerprinting techniques are currently being applied to assess gene flow between different European populations of *V. stagnina* and its close relatives to determine whether a Dutch variety of *V. stagnina* deserves a different taxonomic status.

CHS Lineage Diversification in Viola

In the reconciled tree (fig. 5), two main clades are present. Unfortunately, GeneTree does not provide statistical support for individual nodes. The congruence in topology with Huang et al. (2004) and Yamazaki et al. (2001) indicates that a general phylogenetic signal was recovered, though. The first clade consists of monocot, core eudicots and *Viola* representatives. This clade indicates that at least one duplication event in the CHS gene family took place before the split between the monocots and the eudicots. The second main clade consists of rosid, asterid and *Viola* representatives. This indicates that another duplication event in the CHS gene family took place during the split between the core eudicots and rosids/asterids. Similar results were also found by Huang et al. (2004).

Yang and Gu (2006) also describe multiple rounds of CHS gene duplications during the evolution of the angiosperms. According to these authors, the most ancestral gene lineages originated during the divergence of different plant families, such as Solanaceae, Convolvulaceae and Asteraceae in a first round of duplications. Derived CHS genes further duplicated and diverged, which led to the occurrence of various CHS plant family specific genes in subsequent rounds of duplication.

The CHS intron gene lineage tree suggests that three CHS copies are present in *Viola*, whereas the CHS exon 2 gene lineage tree (data not shown) only contains two copies. The oldest duplication event in clade I (fig. 5) suggests the possibility of the presence of a third copy of CHS in *Viola*. This might also explain why CHS intron copies 1 and 2 have a close resemblance. The fact that not all copies were retrieved does not mean they are not there

but could explain why taxa from different sections end up nested in section *Viola* in the reconciled tree. Our interpretation of the CHS paralogues in *Viola* is different from Farzad et al. (2003, 2005). We consider the CHS paralogues in *V. cornuta* to be different alleles whereas the latter study identified them as different copies. The plant analyzed by Farzad et al. (2003, 2005) was a garden cultivar of hybrid origin. The nominal species is in itself a high-polyploid (Marcussen et al., forthcoming). Furthermore, our analyses of a larger sample of different *Viola* lineages showed that the interpretation of Farzad et al. (2005) was incomplete. Farzad et al. (2005) showed that the three CHS paralogous in *V. cornuta* are all still expressed and fully functional. Expression patterns were found to be slightly different, which might indicate subfunctionalization. Subfunctionalization of duplicate CHS genes in angiosperms appears to have happened by differentiation of their regulatory elements (Yang and Gu, 2006). It would be interesting to further investigate the mechanisms of subfunctionalization in a wider array of *Viola* species.

Acknowledgements

We would like to acknowledge T. Marcussen for providing material and for his valuable feedback on earlier versions of this manuscript. Furthermore, we like to acknowledge P. van Beers, R.L. Eckstein, H. den Held, F. Hellberg, W.J. Holverda, the late R. van der Meijden, R. van Moorsel, I. Nordal, P.B. Pelser, N. Schidlo, E.J. Weeda, and B. Wijlens for providing material and assistance in the field; Kew Botanical Gardens for providing DNA samples; and M.C.M. Eurlings, B.J. van Heuven, L. McIvor, S. Bollendorff, and D.L.V. Co for technical support. We would like to acknowledge the following organizations and departments for providing access to plant material and collecting permits: Staatsbosbeheer, Natuurmonumenten, Overijssels Landschap, and Kalmar county environmental department.

Chapter

3

Viola montana and *V. persicifolia* (*Violaceae*): two names to be rejected[2]

J. Danihelka, K. van den Hof, T. Marcussen and B. Jonsell

The taxonomic and nomenclatural histories of *Viola elatior* Fries (1828), *V. pumila* Chaix (1785) and *V. stagnina* Kit. ex Schult. (1814) in central and western Europe are discussed. The names *V. stagnina* and *V. elatior* are lectotypified with specimens corresponding to the current use of these names. The neglected lectotypification of *V.montana* L. (1753) from 1988 with a specimen referable to *V. elatior* is briefly reviewed. The name *V. persicifolia* Schreb. (1771), used in some floras instead of *V. stagnina*, is analyzed in detail, and we conclude that it should be interpreted as referring to *V. elatior* as well. The use of *V. persicifolia* and *V. montana*, representing the correct name for the species widely known as *V. elatior*, has been notoriously confused for two centuries, and we herein recommend to reject these two names in order to assure nomenclatural clarity and stability.

Keywords: Europe, nomenclature, typification, *Viola elatior, V. stagnina, Viola* subsect. *Rostratae*

[2]Danihelka et al., submitted to Taxon (in review).

Introduction

Viola subsect. *Rostratae* Kupffer (= *V*. sect. *Trigonocarpea* Godron) is represented in Europe by five arosulate species, often referred to as *V*. ser. *Arosulatae* Borbás (van den Hof et al., 2008). *Viola canina* L. (2*n* = 40), the commonest one, has a wide distribution range reaching from the Iberian Peninsula in the west to Lake Baikal in the east. It is extremely morphologically variable, and its intraspecific classification is still in dispute. *Viola lactea* Sm. (2*n* = 58), in contrast, is a strongly oceanic species confined to the British Isles, the northern parts of the Iberian Peninsula, western France, and Belgium. The three remaining species, in recent literature known as *V. elatior* Fries (2*n* = 40), *V. pumila* Chaix (2*n* = 40), and *V. stagnina* Kit. ex Schult. (or *V. persicifolia* Schreb.; 2*n* = 20), have wide distribution ranges reaching from the British Isles and eastern France eastwards to western or central Siberia. In central Europe they are often confined to the floodplains of the large lowland rivers. The taxonomy and ecology of the three floodplain violets in Central Europe was recently reviewed by Eckstein et al. (2006a). In the course of our studies, we have encountered nomenclatural difficulties that will be dealt with herein.

Viola montana

Herbarium specimens of *V. elatior* collected in the late 18th and early 19th centuries have been frequently identified as *V. montana* L. (Sp. Pl. 2: 935. 1753), which is in conflict with the prevailing current use of this Linnean name for certain morphotypes of *V. canina*. These different interpretations can be traced back to a redefinition of *V. montana* in the second edition of Flora suecica (Linnaeus, 1755) and subsequently in the second edition of Species Plantarum. The use of the name *V. montana* has been repeatedly discussed. Some authors have suggested that the name *V. montana* originally referred mainly to the plant currently known as *V. elatior* (e.g. Fries, 1828; Neilreich, 1859; Borbás, 1892; Wilmott, 1916; Lindberg, 1958). Nikitin (1988) reviewed the nomenclatural history of *V. montana* and proposed a lectotype (Herb. Linn. No. 1052.13, LINN) referable to *V. elatior*. This lectotypification is in accordance with the protologue and should not be overruled. However, only a few authors apart from Nikitin seem to have accepted its consequences (e.g., Chen Zousheng et al., 2007) and replaced *V. elatior* by *V. montana,* while many other national checklists and floras published after 1988 preferred nomenclatural stability and clarity to correctness, and continued using *V. elatior*. The replacement of a well established name by another name that was only rarely used in its original sense after the 1820's is undesirable and would destabilize nomenclature. Therefore we have decided to propose *V. montana* for rejection, as already announced by Kirschner and Skalický (1989).

Viola montana L., Sp. Pl. 2: 935. 1753, *nom. utique rej. prop.* (van den Hof et al., Taxon: in review[3]).
Ind. loc.: "Habitat in Alpibus Lapponiae, Austriae, Baldo."
Lectotypus (vide Nikitin in Bot. Žurn. 73: 1541. 1988): "Viola 10 / montana" (Herb. Linn.
No. 1052.13, LINN, vide http://www.linnean-online.org/11110/).

[3]Chapter 4 of this thesis.

Viola persicifolia - Taxonomic history.

The name *Viola persicifolia* was published by Schreber (1771) with a reference to a description in a pre-Linnean flora of Leipzig (Boehmer, 1750; Fig. 7). However, this publication remained neglected for long or the name was considered illegitimate due to the putative lack of description. For these reasons or due to contemporary nomenclatural practice, the name was ascribed to later authors, initially to Roth (1789; e.g. Schultes, 1814; Mertens and Koch, 1826; Reichenbach, 1823) and later to Schkuhr (1803; e.g. Reichenbach 1832, 1839–1840). Even now, more than two centuries after its publication, the name *V. persicifolia* is subject to controversy: in some floras, mainly those from western Europe, it has been used for the species otherwise known as *V. stagnina* (Valentine et al., 1969; Guinochet and Vilmorin, 1982; Stace, 1997; Haeupler and Wisskirchen, 1998; Elven, 2005; van der Meijden, 2005), while others argued that it refers to the species known as *V. elatior* Fries and should be proposed for rejection in the terms of the Code to prevent further confusion (Mansfeld, 1939; Hylander, 1945; Rauschert, 1983; Kirschner and Skalický, 1990; Eckstein et al., 2006a).

The interpretation of the name *V. persicifolia* has been connected with difficulties from the very beginning. Both Roth (1789) and Schkuhr (1803) recognized two arosulate *Viola* species from this group, *V. persicifolia* and *V. montana*, the latter in its original concept and including at least partly *V. elatior*. Still, Schkuhr (l.c.) clearly expressed his uncertainty about their delimitation (see also p. 6 of Nachtrag), and his contemporary Willdenow (1798) did not consider them different at all (though he referred to Flora Suecica; Linnaeus, 1755) and treated them collectively as *V. montana*. However, it is probable that the description of another arosulate violet, *V. lactea* Sm. (Smith, 1798), provided an incentive for Willdenow to later recognize more than one species in this group. To our knowledge this was never published by Willdenow himself, who died in 1812, but his herbarium (Röpert, 2000 onward) contains one folder labeled *V. montana* and a second labeled *V. lactea*. His delimitation of the two, however, makes no sense in our point of view. The former folder contains two sheets of what is now known as *V. elatior* and one of *V. pumila*, and the latter folder two sheets of what is now known as *V. elatior*, three of *V. pumila*, and two of *V. stagnina*.

Schultes (1814) was the first to recognize more than two species of floodplain violets. His three species were *V. lactea*, based on a specimen collected by P. Kitaibel in Hungary and whose description roughly corresponds to *V. pumila* (represented by two Kitaibel's specimen in herbarium Willdenow; see Röpert, 2000 onward), *V. persicifolia* "Roth", referable to *V. elatior*, and *V. stagnina* that he described as a new species based on a specimen collected by P. Kitaibel in Croatia. In addition to these three species, Schultes (l.c.) further kept *V. montana* (with a question mark and only general information about its distribution and habitat); the fifth species, *V. lancifolia*, reported from the surroundings of Berežany in southwestern Ukraine, is difficult to interpret but it may refer to a specimen of *V. canina*.

Nine years later, Reichenbach (1823) paid a great deal of attention to *Viola*. Based on Wahlenberg's opinion, he coined the concept of *V. montana* redefined by Linnaeus (1755) in Flora Suecica, i.e. as a species similar to *V. canina*. The taxonomy of the floodplain violets was discussed on pp. 86–88 as comments on his Plates XCIX (*Viola lactea*. Sm.) and C (*Viola persicifolia*. Roth.). Reichenbach recognized two species, *V. lactea* Smith, with all leaves glabrous and oblong-lanceolate (sometimes ovate- or cordate-lanceolate),

and *V. persicifolia* "Roth", with ovate-lanceolate leaves, pubescent when young. The latter clearly corresponds to *V. elatior*; a chasmogamously flowering specimen and the upper part of a fruiting specimen with capsules from cleistogamous flowers were drawn after plants collected in Leipzig (Fig. 6). Here and in the synonymy Reichenbach explicitly refers to a violet treated in two pre-Linnean floras as occurring near Leipzig (Ruppius, 1726; Boehmer, 1750). He also explained that Roth, as not being familiar with this violet, created a new name based on Ruppius's phrase name. Also *V. lactea* in Plate XCIX was drawn after a plant collected in Leipzig. In his comments Reichenbach stated that it is a widespread species collected from a major part of Europe, but at the same time often confused with other *Viola* species. Variation in leaf and stipule shape were, according to Reichenbach, merely plastic responses to differences in humidity and soil conditions, and thus not worth noting. To prove his point, he drew along with the whole plant a series of laminas and stipules as figures c–n of Plate XCIX. In our opinion, while the whole plant is clearly referable to *V. stagnina*, the detailed leaf and stipule drawings belong to *V. pumila*. Reichenbach also associated *V. pumila* "Vill." with his *V. lactea*, but with some degree of uncertainty, while the choice of the younger name *V. lactea*, based on British plants, was supported by comparison of his specimens with the drawing in Smith (1798).

The second volume of Röhlings Deutschlands Flora (Mertens and Koch, 1826) brought important novelties. Its authors accepted *V. persicifolia* "Roth" as circumscribed by Reichenbach (1823) but almost excluded *V. lactea* Smith (with *V. lancifolia* Thore, Essai Chloris, 1803 as synonym) from the flora of Germany, referring only to a single specimen collected by Wallroth near Wendelstein in Thuringia. They were the first to recognize that Reichenbach's *V. lactea* consisted of two species, *V. stagnina* and the newly described *V. pratensis*, i.e. *V. pumila*. The characters given in their descriptions delimitates the two from each other as well as from *V. canina* s. lat. They further discussed the appearance of plants with capsules and cleistogamous flowers and also noted, in the synonymy of *V. stagnina*, that plants identified as *V. persicifolia* by Schreber in his herbarium correspond to *V. stagnina*.

Two years later, Fries (1828) also accepted three species and with similar concepts, but under completely different names. First he argued that the plant found by Ruppius (1745) near Leipzig was *V. stagnina* rather than *V. elatior*, referring also to the description in Haller (1768, species no. 562), and that the description provided by Roth (1789) would apply better to *V. stagnina* than to *V. elatior* (treated by Roth under *V. montana*). For these reasons, he used the name *V. persicifolia* ("*V. persicaefolia*") for *V. stagnina* and proposed a new name, *V. elatior*, to replace *V. persicifolia* as used especially by Roth (1789) and afterwards. In contrast to Mertens and Koch (1826), he kept the name *V. lactea* (instead of *V. pratensis*) for *V. pumila*, based on the opinion of O. Swartz, who had declared Fries's specimens to be the genuine *V. lactea* of Smith.

Reichenbach (1832) may be understood as a polemic with Mertens and Koch (1826). He insisted that only two species of floodplain violets should be recognized in Germany, i.e. *V. lactea* Sm., consisting of our *V. pumila* and *V. stagnina*, and *V. persicifolia* "Schk.", corresponding to *V. elatior*. He further argued that "*V. stagnina* Kit. nil est nisi status post florescentiam" of his *V. lactea*. However, he accepted Fries's opinion that "*V. persicaefoliis*" of Ruppius, Schreber, and Roth is conspecific with his *V. lactea* and not with *V. persicifolia* as described and drawn by Schkuhr (1803). Instead of accepting *V. elatior* as the correct name, he kept *V. persicifolia* and ascribed it to Schkuhr. This may have been in accordance with contemporary nomenclatural practice but it only further deepened the

nomenclatural confusion.

In the first edition of the Synopsis, Koch (1836) kept the concept of the three species as proposed ten years earlier (Mertens and Koch, 1826) but, following Fries (1828), he replaced the name *V. persicifolia* with *V. elatior*. The diagnoses were precise and distinguished well among the three. Referring to Plate XCIX in Reichenbach (1823), Koch assigned the main figure to *V. stagnina* but the leaf drawings c–f to his *V. pratensis*. He further definitely excluded *V. lactea* (as *V. lancifolia*) from the flora of Germany.

Reichenbach returned to the topic with two plates (Reichenbach, 1838–1839) and a long accompanying text (Reichenbach, 1839–1840). He was very critical about the treatment of floodplain violets in the Synopsis (Koch, 1836) and used strong words bordering on personal attacks. Like in his earlier work, Flora Germanica Excursoria (Reichenbach, 1832), he recognized only two species, *V. persicifolia* "Schkuhr" and *V. lactea* Smith. The latter consisted of populations classified now as *V. pumila* and *V. stagnina*, and Reichenbach considered them one taxon conspecific with the British populations of *V. lactea* (but different from *V. lancifolia* described from north-western France). He repeated his arguments against the species rank of *V. stagnina* and *V. pratensis*, at the same time recognizing as a separate taxon 4507b *V. lactea* var. *humilior* Fries (with *V. pratensis* in synonymy); the corresponding figure in Plate XVII (labeled as 4507.b. *pratensis* M.K) represents a typical *V. pumila*. However, Koch (1843) apparently ignored Reichenbach's strong criticism and only added a few reasons for not using the names *V. lactea* and *V. persicifolia*.

Uechtritz (1871) adopted the same taxonomy as proposed by Koch (1836, 1843). However, he was probably among the first to replace *V. pratensis* by the priority name *V. pumila*. He interpreted *V. persicifolia* as originally referring to *V. stagnina* but recommended to "remove" this notoriously misapplied name. Borbás (1892), adopting the same classification, paid a lot of attention to nomenclature: he suggested to return to the original Linnean concept of *V. montana* and recommended to use this name instead of *V. elatior*, and, based on the description by Roth (1789), he replaced the name *V. stagnina* with *V. persicifolia* "Roth".

Becker (1910) accepted the taxonomy coined by his immediate predecessors but preferred to use the unambiguous name *V. stagnina* instead of *V. persicifolia*. However, in his monograph on Asian and Australian species (Becker, 1917), he reintroduced *V. persicifolia* "Roth" to replace *V. stagnina*. Becker's last important monograph seems to have influenced the interpretation of the name *V. persicifolia* until present. Becker's reasoning reads as follows: "Ich habe für diese Art die Bezeichnung, *V. persicifolia* Roth wieder verwandt, da es keinem Zweifel unterliegt, daß Roth unter diesem Namen obige (= *V. stagnina*) Pflanze verstanden hat. Roth hat die Art nach der Phrase Ruppius' in der Fl. Jenens. (1726, 1745) benannt: ‚Viola palustris, angustis Persicae foliis mucronatis et serratis, nondum descripta. Rupp gibt seine Art von Sumpfwiesen, bei Leipzig, nicht weit von der Funkenburg an. Roth zitiert nicht nur die Ruppsche Pflanze, sondern auch Boehmer Fl. Lipsiae indigena (1750), welcher auch als Standort die Funkenburg angibt und gut beschreibt. Hier kam die Art, die von Rupp l. c. als häufig bezeichnet wird, noch zu Reichenbachs Zeiten vor (Reichenbach, 1839–1840)." How convincing this may sound it is, however, incorrect. Although it is true that Reichenbach (1839–1840) discussed the identity of the Funkenburg violet and attributed it to *V. lactea* (i.e. *V. stagnina* or *V. pumila*), there is no evidence that any of the plants depicted as "4507. Viola lactea Smith" in Icones (Reichenbach, 1839–1840: plate XVI & XVII) were collected near the Funkenburg. Actually, Reichenbach

published a drawing made after the Funkenburg plant 16 years earlier in the Plantae criticae (Reichenbach, 1823; Fig. 6), and the drawings unambiguously represent *V. elatior*. Already Gerstlauer (1943) pointed to this error but this publication has been neglected by some botanists.

A simplified survey of taxonomical and nomenclatural treatments in floras described above and some other monographs is given in Table 1.

Fig. 6. Plate C (Reichenbach, 1823) depicting *Viola persicifolia* "Roth" drawn by Reichenbach himself after plants collected near the Funkenburg in Leipzig.

Table 1. Taxonomy and nomenclature of floodplain violets and *V. lactea* in major Central European floras and monographs between 1771 and 1917

	Currently accepted species			
Author	*Viola elatior*	*Viola stagnina*	*Viola pumila*	*Viola lactea*
Schreber, 1771	*V. persicifolia*			
Roth, 1789	*V. persicifolia* (*V. montana*)			
Willdenow, 1798	*V. montana*			
Schkuhr, 1803	*V. persicifolia* (*V. montana*)			
Schultes, 1814	*V. persicifolia* "Roth" *V. montana?*	*V. stagnina*	*V. lactea*	
Reichenbach, 1823	*V. persicifolia* "Roth"	*V. lactea*		
Mertens & Koch, 1826	*V. persicifolia* "Roth"	*V. stagnina*	*V. pratensis*	*V. lactea* (syn. *V. lancifolia*)
Fries, 1828	*V. elatior*	*V. "persicaefolia"* Schreber	*V. lactea* *V. l.* var. *humilior*, *V. l.* var. *pratensis*	
Reichenbach, 1832	*V. persicifolia* "Schkuhr"	*V. lactea* (different from *V. lancifolia*)		
Koch, 1836	*V. elatior*	*V. stagnina*	*V. pratensis*	*V. lactea* (syn. *V. lancifolia*)
Reichenbach, 1839–1840	*V. persicifolia* "Schkuhr"	*V. lactea* (incl. *V. l.* var. *humilior* but different from *V. lancifolia*)		
Koch, 1843	*V. elatior*	*V. stagnina*	*V. pratensis*	
Uechtritz, 1871	*V. elatior*	*V. stagnina*	*V. pumila*	*V. lactea*
Borbás, 1892	*V. montana*	*V. persicifolia* "Roth"	*V. pumila*	*V. lactea* (syn. *V. lancifolia*)
Becker, 1910	*V. elatior*	*V. stagnina*	*V. pumila*	*V. lactea*
Becker, 1917	*V. elatior*	*V. persicifolia* "Roth"	*V. pumila*	*V. canina* subsp. *lactea*

Nomenclatural analysis

The name *Viola persicifolia* was published with an extremely short protologue (Schreber 1771). It consisted solely of a number "456", in the right column, representing a reference to species 456, *Viola caule erecto, foliis ovato lanceolatis, serratis*, in Flora Lipsiae indigena (Boehmer, 1750 cf. Schreber, 1771). This has to be considered indirect reference to a previously published description as described in Art. 32.6, required for valid publication of a name by Art. 32.1.(c) of the ICBN (McNeill et al., 2006). The species' treatment in Boehmer (Fig. 7) consists of a phrase name, another phrase name used in the third edition of an earlier flora of Jena and its surroundings (Ruppius, 1745), locality information, and a description. As no herbarium specimens collected by Boehmer or Ruppius are known to be extant (Stafleu and Cowan, 1976, 1983), those four elements are the only base for the interpretation of the name. In principle, *V. persicifolia* could refer to any or all of *V. elatior*, *V. pumila*, and *V. stagnina* because all three are known to have occurred in the surroundings of Leipzig at least until the 1850s (Reichenbach, 1823; Petermann, 1838; Hardtke and Ihl, 2000; P. Gutte, in litt.).

Boehmer's description is rather ambiguous and contains only little information. "Stipulae duae minores" may be interpreted as a character of *V. stagnina* or merely as a comparison to the size of the lamina and petiole. The erect stem is typical of *V. elatior*, while pale corolla (in comparison with *V. odorata*) applies better to *V. elatior* and *V. stagnina* than to *V. pumila*. However, the fact that the species was cultivated in gardens applies best to *V. elatior* and to lesser extent to *V. pumila*. *Viola elatior* is relatively easy to cultivate and certainly has an interesting habit and some decorative value. The treatment in Ruppius (1745) is even shorter "Viola palustris, angustis Persicae foliis mucronatis, & serratis, nondum descripta. Ist häuffig auf sumpfigten Wiesen bey Leipzig, nicht weit von de Funcken-Burg, floret Aprili." and does not offer much additional information. In general, the informative value of such old diagnoses should not be overestimated: in this case, the phrase names from Ruppius and Boehmer are also cited in the validating description of a species in the *V. canina* group, *V. ruppii* All. (Haller, 1768; Fries, 1828; Dandy, 1970). Further, Haller (l.c., species 562), editor of the third edition of Ruppius's flora (Ruppius, 1745), mentioned that he collected it in Jena, in Suevia (Schwaben, Germany), and not far from Scaphusia (Schaffhausen), but not in Leipzig. His collections, now preserved at P, correspond to neither *V. stagnina* nor *V.*

456. Viola caule erecto, foliis ouato lanceolatis, ſerratis.

Viola paluſtris anguſtis Perſicae foliis mucronatis *Rupp. 289.*

In paſcuis, *auf der Funckenburg bey Gonnewitz*, floret Aprili, perennis.

Radix eſt fibroſa, ex qua caulis ſimplex ſurgit, qui folia longe petiolata, anguſta, ex ouatis acuta, ſerrata obtinet; ſtipulae duae minores ad petiolum poſitae inueniuntur. Pedunculus vel eſt caulis continuatus, vel aliquando peculiaris ex alis foliorum exit, longus atque tenuis, flos ſolitarius, qui cum Viola vulgari martia conuenit, pallidior modo eſt et quodammodo minor. Haec dum in hortis colitur, licet inſigniter mutetur, et omni parte longe major euadat, ſingularem tamen et ipſi proprium habitum ſemper oſtendit retinetque.

Fig. 7. Validating description of *Viola persicifolia* Schreb. (Boehmer, 1750)

elatior, but to *V. ruppii* as understood today (Kirschner and Skalický, 1989). This indicates that he had himself not seen the Funkenburg violet. In contrast to some other violet species in Ruppius's flora, he did not add any comments behind the species treatment adopted from the second edition, which further supports this assumption.

Both Boehmer (l.c.) and Ruppius (l.c.) referred to the same site, variously spelled as Funckenburg bei Gonnewitz or Funcken-Burg, now part of the city Leipzig and not far from its centre. Adjacent to the Funkenburg, hard-wood forest (Leipziger Auenwald) and wet meadows were found in the 19th century. Leipzig floras from this period (P. Gutte, in litt.; Petermann, 1836; Reichenbach 1823) reported only *V. elatior* from this site but not *V. pumila* or *V. stagnina*. The former presence of *V. elatior* at this site is confirmed by an undated specimen from the herbarium Reichenbach fil. "Funkenburg Lips." (sine coll.) now deposited at W as no 1889/305915 (Fig. 8). Still, we cannot rule out that also *V. pumila* and/or *V. stagnina* occurred there as well, but we have not seen any specimens. The probability that more collections from the Funkenburg will be discovered is very low because the Leipzig university herbarium was completely destroyed by fire during World War II (P. Gutte, in litt.). The fact that *V. elatior* had been known from the Funkenburg was used as base for the interpretation of *V. persicifolia* by Reichenbach (1823) and later by Gerstlauer (1943) and Rauschert (1983). In contrast, Fries (1828) argued that the Funkenburg violet was *V. stagnina* because Ruppius (1726) considered it as not described yet ("nondum descripta"), whereas *V. elatior* had been repeatedly described and illustrated by early authors ("planta tum temporis notissima, in quovis libro picta"). Petermann (1836) also concluded that Ruppius had *V. stagnina* in mind because of its "frequent" occurrence in wet meadows.

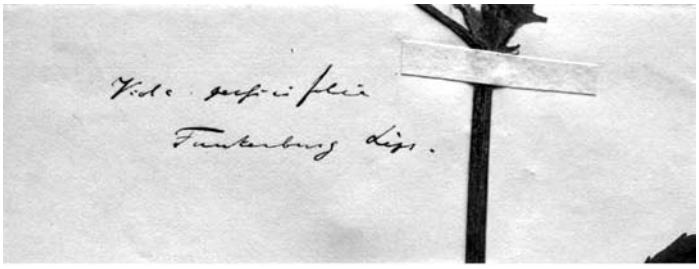


Fig. 8. Label of a *V. elatior* specimen from the herbarium Reichenbach fil. (W1889/305915) collected near the Funkenburg in Leipzig.

In our opinion, there is one circumstance neglected before: Ruppius (1726, 1745), Boehmer (1750) and Schreber (1771) all recognized only one species of floodplain violets in spite of the fact that three species grew around the contemporary Leipzig. From this point of view the speculations about what species these early authors had in mind are less important. Further, there are reports (Reichenbach, 1839–1840) that plants identified by Schreber as *V. persicifolia* are referable both to *V. stagnina* and to *V. elatior*. However, when we investigated the material of the Schreber herbarium deposited at M, we found that all collections identified as *V. persicifolia* can be considered as *V. stagnina*. This corresponds to what Mertens and Koch (1826) and Fries (1828) reported. Also Schweigger (1804), disciple of Schreber, probably used the name *V. persicifolia* when referring to *V. stagnina* (see Koch, 1843); he accepted the phrase name from Boehmer (1750) and added: "Pro varietate violae montanae habetur." In contrast, the specimens of *V. elatior* from the herbarium Schreber (now at M) were identified as *V. montana*, *V. canadensis* or *V. sibirica*.

The first botanist who clearly linked the name *V. persicifolia* to *V. elatior* was Schkuhr (1803). He was later followed by Schultes (1814) and especially Reichenbach (1823), who

published an illustration based on plants from the Funkenburg site and clearly distinguished between *V. persicifolia* (= *V. elatior*) and *V. lactea* "Sm." (= *V. pumila* and *V. stagnina*). These descriptions and plates may be considered informal emendations and tradition to follow. This point of view was already presented by Neilreich (1859), though he referred only to Reichenbach. The later note in the Specimen florae erlangensis (Schweigger, 1804) is less clear but may be interpreted as indirect emendation in favor of our *V. stagnina*.

Under the provision of the Code, no lectotypification is possible in the absence of any original material or an illustration. The only way to fix the use of *V. persicifolia* remains a neotypification (Art. 7.7, 9.2 and 9.6, McNeill et al., 2006). Here, in our opinion, a pragmatic solution may be offered by selecting a type referable to either *V. elatior* or *V. stagnina*. In the first case the specimen number W 1889/305915 from the Funkenburg site (*locus classicus*) or a modern specimen may be proposed, in the second case a modern specimen is the only option. Each of the neotypifications would be in conflict with a part of the protologue, but we believe that the choice of the well preserved Funkenburg plant from the herbarium Reichenbach fil., referable to *V. elatior*, would be more evidence-based than the choice of any *V. stagnina* specimen. However, any neotypification is potentially reversible (Art. 9.17, McNeill et al., 2006) if some original material is discovered, and it should not be used to resolve a long-lasting dispute like this. Conservation of *V. persicifolia* with a conserved type (Art. 14.9, McNeill et al., 2006) referable to *V. stagnina* would make it possible to retain this name instead of *V. stagnina* but it would bring about an undesirable nomenclatural change in some national floras (mainly in central European countries), which is in conflict with the aim of conservation as stated in the Code (Art. 14.2, McNeill et al., 2006). Further, we do not think that it is reasonable to use this option provided by the ICBN for such a notoriously confused name still in dispute. For these reasons we decided not to designate a neotype but to propose the name *V. persicifolia* for rejection (Art. 56, McNeill et al., 2006) in a rejection proposal published simultaneously.

Viola persicifolia Schreb., Spic. Fl. Lips.: [163]. 1771, *nom. utique rej. prop.* (van den Hof et
al., Taxon: in review[2]).
Ind. loc. (Boehmer, 1750: 190): [Germania. Saxonia, urbs Lipsia.] "In pascuis, auf der Funckenburg bei Gonnewitz …"
Typus: non designatus.

Typification of *Viola stagnina*

The name *Viola stagnina* was published by Schultes (1814). The original description is brief and poor in diagnostic characters, and it refers to a plant with developed capsules and cleistogamous flowers, collected in late spring or early summer. A comparison with the descriptions of other violet species described there (see above) makes it possible to link this description to *V. stagnina* as understood today. The name has to be cited as Kit. Ex Schult. because only the name is ascribed to Kitaibel but not the diagnosis and description (Art. 46.4, McNeill et al., 2006); this is different, however, in the case of e.g. *Cerastium eriophorum* Kit. (Schultes, 1814). The corresponding *Viola* specimen sent by Kitaibel to

Schultes is still deposited at M as M-0111205. It bears the original label "*Viola stagnina* mihi. In Croatiae locis depressis in quibus aqua stagnat.", glued on a newer label of the Royal Munich Herbarium with a note "A Kitaibelio ipso". There is also a revision label of L. Gerstlauer on the sheet: "*Viola stagnina* Kit., Originalstück (Cotypus) von Kitaibel selbst. Rev. Gerstlauer, 1941". The plants (two stems) represent a late spring or summer collection of *V. stagnina* as understood today, with cleistogamous flowers and capsules. It may be selected as lectotype. As Kitaibel used to send duplicates also to Willdenow (Z. Barina, in litt.), we searched also in the herbarium Willdenow; however, Kitaibel's collections found under *V. lactea* (B-W04916-07) and *V. montana* (B-W04915-03) represent *V. pumila* (see above; Röpert, 2000 onward). There is also a sheet of *V. stagnina* in the herbarium Kitaibel (fascicle IX, nr 191) at BP. It is labelled "*stagnina* mihi ignota Willdenowio. In pratis humidis ad Brezovicam, integras plagas ita occupat, ut plantas reliquas fere omnes extendat" (Z. Barina, in litt.; Jávorka, 1936). The (unmounted) plants were revised by J. Kirschner in 1984. The collection consists of two species: the unbranched plant with large laminae and stipules is referable to *V. elatior*, whereas the branched small-leaved plants correspond to *V. stagnina*. Kirschner marked one of the *V. stagnina* specimens as lectotype but this lectotypification has never been effectively published. The plants were probably collected during Kitaibel's journey to Croatia in 1794 (Z. Barina, in litt.). As reported by Harmatta (1962), P. Kitaibel collected plants in Brezovica near Zagreb in Croatia in the second half of May 1794. However, there is no direct evidence that the plant in M represents the same collection as sheet IX/191 in the herbarium Kitaibel, so the latter should not be considered iso(lecto)type. Curiously, Croatian floras do not report *V. stagnina* (cf. Schlosser and Vukotinovic, 1869; Domac, 1994).

***Viola stagnina* Kit.** ex Schult., Oestr. Fl., ed. 2, 1: 426. 1814. Ind. loc.: "In Morästen, in Sümpfen in Kroatien fand sie Herr Professor Kitaibel."
Lectotypus (hic designatus): "*Viola stagnina* mihi. In Croatiae locis depressis in quibus aqua
stagnat." (Kitaibel s.a. M 0111205!).

Typification of *Viola elatior*

Viola elatior was described by Fries (1828) after plants from Öland. The diagnosis and description clearly apply to *V. elatior* as understood today. This is also supported by the fact that Fries at the same time distinguished *V. lactea* (= *V. pumila*) and *V. persicifolia* (= *V. stagnina*). He found *V. elatior* during his visit to Öland in 1818 (cf. p. 276) and immediately noted the distinctive tall stature of this species: "Statura elatiori mox dignoscitur; nomen a primo Clusio sumtum & mihi a primo inventionis momento in mentem venit." In the protologue two collections are cited, the first made by Fries himself and the second by A. Ahlquist. The corresponding specimens are found at UPS, labeled "*Viola persicifolia*. Rstn 18" (with later remarks "Runsten Ahlqvist" in a different handwriting; UPS 220503) and "*Viola elatior*. Ölandia ad Allgutsrum 1824. E. Fries scripsit." (UPS 220505), both stamped "Herb. Hartman". Also the third specimen found at UPS and labeled "*Viola elatior* Fries. Ölandia. 1818. Haec sunt duo specimina prima in Suecia a me detexta" (UPS 220509), stamped "Herb. E. Fries", may be considered original material. Plants on all three sheets

represent *V. elatior* as currently understood.

Nikitin (1988) analyzed the protologue of *V. elatior* (Fries, 1828) and argued that this name has to be considered illegitimate because Fries included in its synonymy *V. montana* L. (cited from the second edition of Species Plantarum in accordance with contemporary practice) without excluding the type (Art. 52.1, McNeill et al., 2006). However, in the same work by Fries, *V. montana* served as basionym for *V. canina* (= var.) *montana* (L.) Fries; here, *V. montana* was cited from Flora suecica (Linnaeus, 1755). As already shown by Kirschner and Skalický (1989), Nikitin's reasoning is not correct because Fries (l.c.) excluded the type of *V. montana* by implication, as described in Art. 52.2. Ex. 8 (McNeill et al., 2006). The fact that Fries cited *V. montana* from different Linnean works is unimportant because a name refers to the same type regardless of the work from which it is cited. A later lectotypification of *V. montana* by Nikitin (1988) is not retroactive (Art. 52.2. Note 2, McNeill et al., 2006); in other words, it cannot make a name published in 1828 nomenclaturally superfluous and, consequently, illegitimate.

Nikitin (1995) disagreed and repeated his arguments against the legitimate status of *V. elatior* and added another reason: in the synonymy (Fries, 1828), "*V. stipulacea* Hartm., 1820 and *V. elatior* Link, 1821" are included, both earlier and validly published, and therefore impossible to reject. "Therefore, if somebody does not agree yet that it is necessary to return to *V. montana* in its original sense, he will have to refrain from the use of *V. elatior* Fries and use the priority name *V. stipulacea* Hartman instead. The name *V. elatior* ascribed to Link, 1821, not to Fries, 1828, should be included in its synonymy". However, neither of these statements are correct. Link (1821) only wrote in comments on his no 2314. *V. persicifolia* "Roth": "Differt a *V. elata* (sic) Fries foliis latioribus, ovata basi, non scabris, bracteis minutis sub flore." This is by no means a valid publication of a name, as already noted by Hylander (1945). Further, what Fries actually did was to include "V. stipularis. *Fr. Hall. p. 47. Hartm.! Scand.*", not *V. stipulacea*, in the synonymy of *V. elatior*. Indeed, *Viola stipularis* was published by Fries (1817), but it is illegitimate due to homonymy with the South American *V. stipularis* Sw. (Prodr.: 117. 1788). The name *V. stipulacea* ascribed to Fries (it may be interpreted as a reference to Flora hallandica) was used by Hartman (1820). However, the epithet "stipulacea" was used by mistake instead of "stipularis"; it was clearly not intended as a replacement (avowed substitute; see Art. 33.3, McNeill et al., 2006). The epithets "stipularis" and "stipulacea" are confusingly similar and they may therefore be treated as homonyms (Art. 53.3, McNeill et al., 2006); the three subsequent mistakes by Hartman, Fries and Nikitin described above support our opinion. These facts demonstrate that Nikitin's conclusions are wrong, and that *V. elatior* indeed does represent a legitimate name.

Viola elatior Fries, Novit. Fl. Suec. Alt.: 277. 1828.Ind. loc.: "In Ölandiae tractu silvatico inter Algutsrum & Tveta uberrime legi; Ad Runstens Canal rariorem Rev. Ahlquist detexit."
Lectotypus (hic designatus): "*Viola elatior*. Ölandia ad Allgutsrum 1824. E. Fries scripsit" (UPS 220505!).
= *Viola stipularis* Fr., Fl. Hall.: 48. 1817, nom. illeg. (non *V. stipularis* Sw., Prodr.: 117.1788).
= *Viola stipulacea* Hartm., Handb. Skand. Fl.: 110, 1820, nom. illeg. (Art. 53.3, McNeill et al., 2006; non *V. stipularis* Sw., Prodr.: 117. 1788).

Acknowledgements

We are grateful to Franz Schuhwerk, Hajo Esser (both M), Zoltán Barina (BP), and Ernst Vitek (W) for their help during our search for nomenclaturally important herbarium specimens and additional information. Peter Gutte informed us about the former flora of Leipzig and localities of floodplain violets in the city and its surroundings. Walter Gutermann with his unpublished manuscript "Über unnötige und notwendige Namensänderungen der mitteleuropäischen Flora" provided an important incentive to this study. The work done by JD was supported by the Ministry of Education of the Czech Republic (grants MSM0021622416 and LC06073) and by the long-term research plan AV0Z60050516 of the Institute of Botany, Czech Academy of Sciences. The work done by TM was supported by the Norwegian Research Council (grant 170832: "Allopolyploid evolution in plants: patterns and processes within the genus *Viola*").

Chapter

4

Proposal to reject the names *Viola montana* and *V. persicifolia* (*Violaceae*)[4]

K. van den Hof, J. Danihelka, T. Marcussen, B. Jonsell, R.G. van den Berg and B. Gravendeel

Viola montana L., Sp. Pl. 2: 935. 1 Mai 1753 [*Dicot.: Violac.*], *nom. Utique rej. prop.* Typus (vide Nikitin in Bot. Žurn. 73: 1541. 1988): "Viola 10 / montana" (Herb. Linn. No. 1052.13, LINN, vide http://www.linnean-online.org/11110/).

Viola persicifolia Schreb., Spic. Fl. Lips.: [163]. 1771 [*Dicot.: Violac.*], *nom. utique rej. prop.* Typus: non designatus.

The nomenclatural history of *Viola montana* L., a name referring to a violet species of *Viola* sect. *Viola* with a wide Euro-Siberian distribution range, was briefly reviewed by Danihelka et al. (Taxon: in review[5]). As shown by Wilmott (in J. Bot. 54: 257–262. 1916) and Nikitin (in Bot. Žurn. 73: 1536–1542. 1988), this name was misinterpreted soon after its publication, and since the 1820s, it was only exceptionally used in its original sense. After 1800, the name *V. persicifolia* Schreb. was often used for the species under consideration, while the name *V. montana* was applied for some morphotypes conspecific with *V. canina* L. However, starting from the 1830s, *V. persicifolia* was gradually replaced by *V. elatior* Fries (1828), i.e. by a name that has been widely accepted over large part of its distribution range. There have been a few attempts to restore *V. montana* in its original sense, including Borbás (in Koch, Syn. Deut. Schweiz. Fl., ed. 3, 1: 213. 1892) and Wilmott (l.c.), whereas Burnat and Briquet (in Annuaire Conserv. Jard. Bot. Genève. 6: 143–153. 1902) and Hylander (in Uppsala Univ. Årsskr. 7: 242. 1945) argued that *V. montana* should be typified with a specimen representing plants related to *V. canina*. Despite the latter opinions, Nikitin (in Bot. Žurn. 73: 1541. 1988) formally lectotypified *V. montana* with a specimen referable to the taxon currently known as *V. elatior*, and we consider his lectotypification correct and in full accordance with

[4]van den Hof et al., submitted to Taxon (in review)
[5]Chapter 3 of this thesis

the ICBN. Soon after this lectotypification, Kirschner and Skalický (in Preslia 61: 318. 1989) argued that the reintroduction of *V. montana* in its original sense would be contraproductive, and they announced a formal rejection proposal to be submitted. However, such a proposal was never written.

We have reviewed the more important floras and taxonomic papers published in the last 20 years, since the lectotypification of *V. montana*, and covering the whole range of the species. Our survey shows that this name has been used instead of *V. elatior* only by a few authors, including Nikitin himself (in Bot. Žurn. 83/3: 130. 1998; in Tzvelev, Fl. Russia 9: 291. 2006), Cerepanov (Sosudistye Rast. Ross. Sopredel'nyh Gosudarstv: 956. 1995), Mosyakin and Fedoronchuk (Vasc. Pl. Ukraine: 325. 1999), and Chen Zousheng et al. (in Wu Zhengyi and Raven, Fl. China 13: 79. 2007; co-authored by Vl. V. Nikitin). In contrast, other floras, many of which published after 1988, accept *V. elatior* as the correct name, but sometimes with a note that *V. montana* should be proposed for rejection. These floras include Valentine et al. (in Tutin et al., Fl. Eur. 2: 275. 1968), Guinochet and Vilmorin (Fl. France 4: 1216. 1982), Lambinon et al. (Nouv. Fl. Belgique du Grand-Duché de Luxembourg, du Nord de la France et des Régions voisines, ed. 4: 207. 2004), Haeupler and Wisskirchen (Standardliste Farn- und Blütenpfl. Deutschl.: 545. 1998), Jäger and Werner (Exkursionsfl. Deutschl., ed. 9, 4: 244. 2002), Heß et al. (Fl. Schweiz 2: 749. 1970), Pignatti (Fl. d'Italia 2: 117. 1982), Mossberg and Stenberg (Den nya nordiska floran: 402. 2003), Marcussen et al. (in Jonsell and Karlsson, Fl. Nordica 6 [in review, scheduled for publication in 2009]), Fischer (Exkursionsfl. Österreich, Liechtenstein Südtirol, ed. 3: 433. 2008), Suda (in Kubát et al., Klíc Kvet. Ceské Republ.: 212. 2002), Mirek et al. (Flow. Pl. Pterid. Poland: 186. 2002), Martincic (Mala Fl. Slovenije, ed. 3: 363. 1999), Domac (Fl. Hrvatske: 136. 1994), Mereda et al. (in Goliašová and Šípošová, Fl. Slov. 6/1: 141. 2008), Simon (Magyar. Edény. Fl. Határoz., ed. 4: 474. 2001), Diklic (in Josifovic, Fl. SR Srbije 3: 150. 1972), Beldie (Fl. Român. 1: 356. 1977), Kuusk et al. (Fl. Balt. Resp. 2: 194. 1996), Delipavlov and Cešmedžiev (Opredelitel Rast. Balgarija, ed. 3: 110. 2003), and Zuev (in Peškova, Fl. Sibiri 10: 89. 1996). At the same time, some of these authors use the names *V. montana* or *V. canina* subsp. *montana* (L.) C. Hartm. for plants of the *Viola canina* group; e.g., Valentine et al. (in Tutin et al., Fl. Eur. 2: 275. 1968), Stace (New Fl. Brit. Isles, ed. 2: 221. 2001), Guinochet and Vilmorin (Fl. France 4: 1216. 1982), Haeupler and Wisskirchen (Standardliste Farn- und Blütenpfl. Deutschl.: 545. 1998), Jäger and Werner (Exkursionsfl. Deutschl., ed. 9, 4: 244. 2002), Heß et al. (Fl. Schweiz 2: 748. 1970), Pignatti (Fl. d'Italia 2: 117. 1982), Mossberg and Stenberg (Den nya nordiska floran: 401. 2003), Koistinen (Retkeilykasvio: 129. 1984), Mirek et al. (Flow. Pl. Pterid. Poland: 186. 2002), Domac (Fl. Hrvatske: 136. 1994), Simon (Magyar. Edény. Fl. Határoz., ed. 4: 474. 2001), Diklic (in Josifovic, Fl. SR Srbije 3: 149. 1972), Beldie (Fl. Român. 1: 356. 1977), Kuusk et al. (Fl. Balt. Resp. 2: 194. 1996), and Delipavlov and Cešmedžiev (Opredelitel Rast. Balgarija, ed. 3: 109. 2003). Apart from Nikitin (in Tzvelev, Fl. Russia 9: 293. 2006), among the floras checked only Muñoz Garmendia et al. (in Castroviejo et al., Fl. Iber. 3: 292. 1993), Marcussen et al. (in Jonsell and Karlsson, Fl. Nordica 6 [in review, scheduled for publication in 2009]), Suda (in Kubát et al., Klíc Kvet. Ceské Republ.: 212. 2002), Mereda et al. (in Goliašová and Šípošová, Fl. Slov. 6/1: 141. 2008), and Cerepanov (Sosudistye Rast. Ross. Sopredel'nyh Gosudarstv: 955. 1995) indicate that the name *V. montana* was actually misapplied when used for plants of the *V. canina* group. Finally, only Elven (in Lid and Lid, Norsk Fl.: 549. 2005) took the consequence of this misapplication and proposed another name to replace it, *V. canina* subsp. *nemoralis* (Kütz.) ined.; however this combination has

not been validly published.

Our review demonstrates that even twenty years after Nikitin's typification of *V. montana*, the nomenclatural consequences have been accepted only by a few authors. These floras, however, treat an important part of the species' range. Authors of other floras, however, including those who paid a lot of attention to nomenclatural issues and who were aware of the typification, deliberately continued using the name *V. elatior* instead of *V. montana*. They clearly preferred nomenclatural stability and clarity to correctness. Based on our analysis of the topic and related nomenclatural and taxonomic questions, we decided to follow these authors and propose the notoriously misapplied name *V. montana* for rejection. If this proposal is accepted, a clear and never misapplied name (*V. elatior*) will remain in use. Apart from a nomenclatural change in three countries (in fact restoration of the previous situation), we cannot see any disadvantage of this rejection. However, if this proposal is rejected, a name (*V. montana*) will necessarily come into general use that will have to be accompanied for decades with a note that it actually refers to a plant previously known as *V. elatior*, not to *V. canina* s.l. As *V. elatior* is red-listed and/or protected by law in most central European countries, the replacement of this name by *V. montana*, which is usually associated with a common species within the same region, would also have undesirable effects for nature conservation and legislation. As shown in our analysis (Danihelka et al., Taxon: in review), *Viola persicifolia* Schreb. represents most probably the second-earliest name for the plant recently known as *V. elatior*. This use prevailed in the first half of the 19th century. However, following the opinion of Fries (Fries, Novit. Fl. Suec. Alt.: 275–276. 1828), the name was reinterpreted as referring to a species in some other national floras still known under *V. stagnina* Kit. ex Schult. (1814). This interpetation was supported by the authority of W. Becker, who accepted *V. persicifolia* instead of *V. stagnina* in his last major monograph (Becker in Beih. Bot. Centralbl. 34/2: 393–395. 1917); unfortunately, his most important argument is erroneous (Gerstlauer in Ber. Bayer. Bot. Ges. 26: 45–46.1943; Danihelka et al., Taxon: in review), and the name most probably refers to *V. elatior* (apart from Gerstlauer also Rauschert in Feddes Repert. 83: 647–648. 1972; W. Gutermann, in litt.). The name *V. persicifolia* has been accepted as correct (usually with *V. stagnina* as a synonym) by Valentine et al. (in Tutin et al., Fl. Eur. 2: 275. 1968), Stace (New Fl. Brit.Isles, ed. 2: 221. 2001), Guinochet and Vilmorin (Fl. France 4: 1217. 1982), Lambinon et al. (Nouvelle Flore de la Belgique du Grand-Duché de Luxembourg, du Nord de la France et des Régions voisines, ed. 4: 207. 2004), van der Meijden (Heukels' Fl. Nederland: 342. 2005), Haeupler and Wisskirchen (Standardliste Farn- und Blütenpfl. Deutschl.: 546. 1998), Elven (in Lid and Lid, Norsk Fl.: 551. 2005), Mossberg and Stenberg (Den nya nordiska floran: 401. 2003), Koistinen (Retkeilykasvio: 129. 1984), Diklic (in Josifovic, Fl. SR Srbije 3: 149. 1972), Beldie (Fl. Român. 1: 357. 1977), Mosyakin and Fedoronchuk (Vasc. Pl. Ukraine: 325. 1999), Nikitin (in Bot. Žurn. 83/3: 130. 1998; in Tzvelev, Fl. Russia 9: 296. 2006), and Cerepanov (Sosudistye Rast. Ross. Sopredel'nyh Gosudarstv: 956. 1995). In contrast, the same species is referred to as *V. stagnina* by Heß et al. (Fl. Schweiz 2: 750. 1970), Jäger and Werner (Exkursionsfl. Deutschl., ed. 9, 4: 245. 2002), Marcussen et al. (in Jonsell and Karlsson, Fl. Nordica 6. 2009]), Fischer (Exkursionsfl. Österreich, Liechtenstein Südtirol, ed. 3: 433. 2008), Suda (in Kubát et al., Klíc Kvet. Ceské Republ.: 212. 2002), Mirek et al. (Flow. Pl. Pterid. Poland: 186. 2002), Mereda et al. (in Goliašová and Šípošová, Fl. Slov. 6/1: 133. 2008), Simon (Magyar. Edény. Fl. Határoz., ed. 4: 474. 2001), Delipavlov and Cešmedžiev (Opredelitel Rast. Balgarija, ed. 3: 110. 2003), Kuusk et al. (Fl. Balt. Resp. 2: 193. 1996), and Zuev (in

Peškova, Fl. Sibiri 10: 89. 1996). This survey shows that the number of national floras using *V. persicifolia* and those using V. *stagnina* for the same species is approximately equal. However, there seems to be a certain trend in favour of the latter in recent floras of Germany, Austria and most recently in the Nordic countries. The options for a typification, necessary to fix the use of the name, are discussed in a simultaneously published article (Danihelka et al., Taxon: in review). However, we think that neotypification or even conservation with a conserved type referable to *V. stagnina* is a worse solution than a rejection proposed here. In the first case, a notoriously confused name (*V. persicifolia*) would replace another name that has never been misinterpreted (*V. stagnina*), and the extent of the accompanying nomenclatural change will be similar to that caused by the rejection. In contrast, the rejection of *V. persicifolia*, informally proposed already by Koch (Syn. Fl. Germ. Helv.: 85. 1836), will bring to an end a long-lasting and rather unproductive nomenclatural dispute. It will also stabilise nomenclature, and attention will be paid to taxonomy and conservation. We also believe that if the names *V. montana* and *V. persicifolia* are rejected, floristic records under these names would be interpreted with more care.

Acknowledgements

The research by Jirí Danihelka was supported by the Ministry of Education of the Czech Republic(grants MSM0021622416 and LC06073) and by the long-term research plan AV0Z60050516 of the Institute of Botany, Czech Academy of Sciences. The work done by Thomas Marcussem was supported by the Norwegian Research Council (grant 170832: "Allopolyploid evolution in plants: patterns and processes within the genus Viola"). Walter Gutermann encouraged us in a discussion to write these proposal.

Chapter

5

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

K. van den Hof, T. Marcussen, R.G. van den Berg and B. Gravendeel

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.

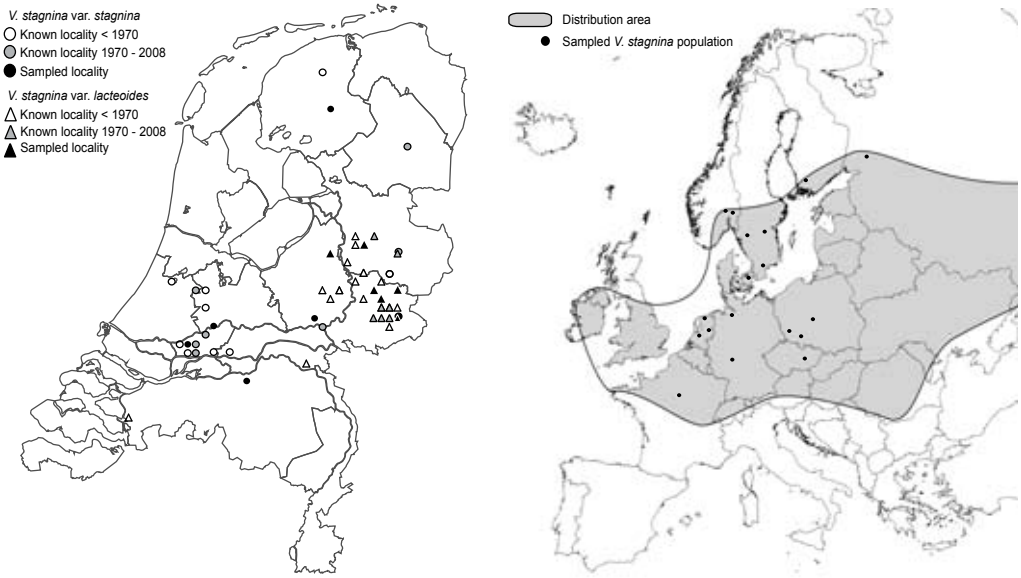
Key words: AFLP, Bayesian analysis, morphometrics, phylogeny, *Viola stagnina*

[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]).

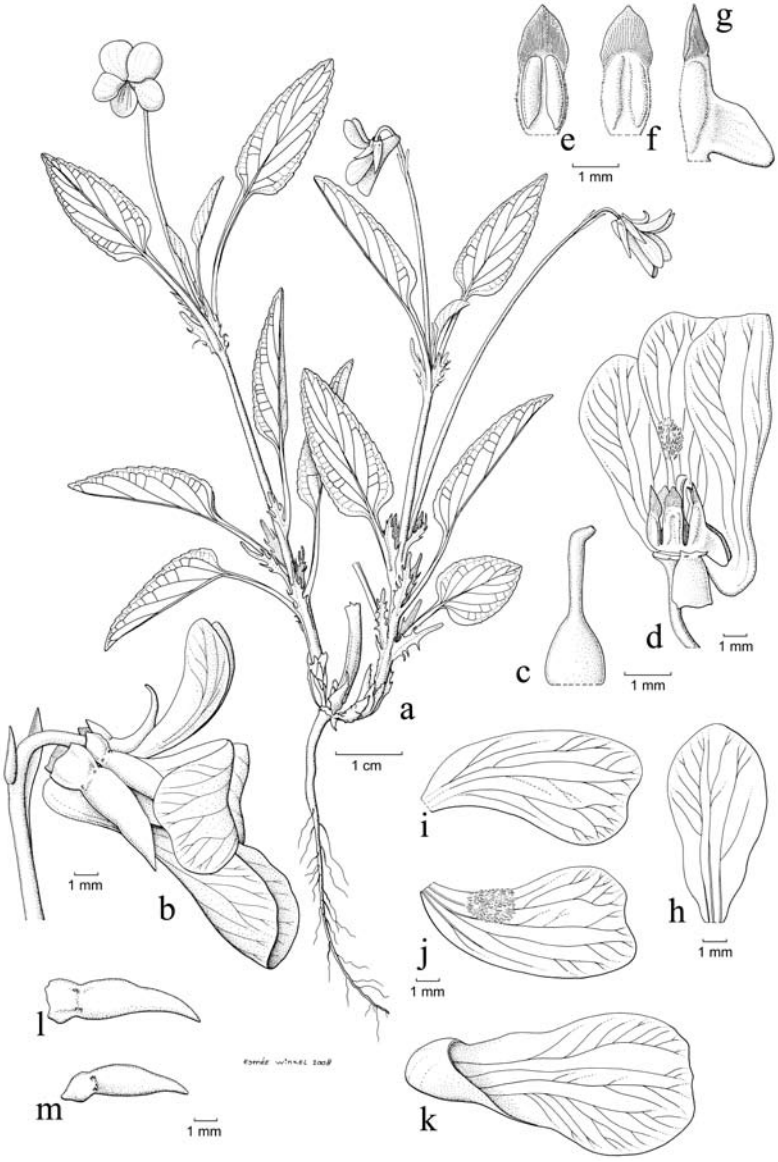


Fig. 9a. *Viola stagnina* var. *stagnina* a. Habit b. Lateral view of the flower c. Lateral view of the flower with male and female reproductive organs d. Gynoecium e. Adaxial view of the upper stamen f. Abaxial view of the upper stamen g. Side view of the spurred lower stamen h. Dorsal petal i. Lateral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

[7] Chapter 6 of this thesis.

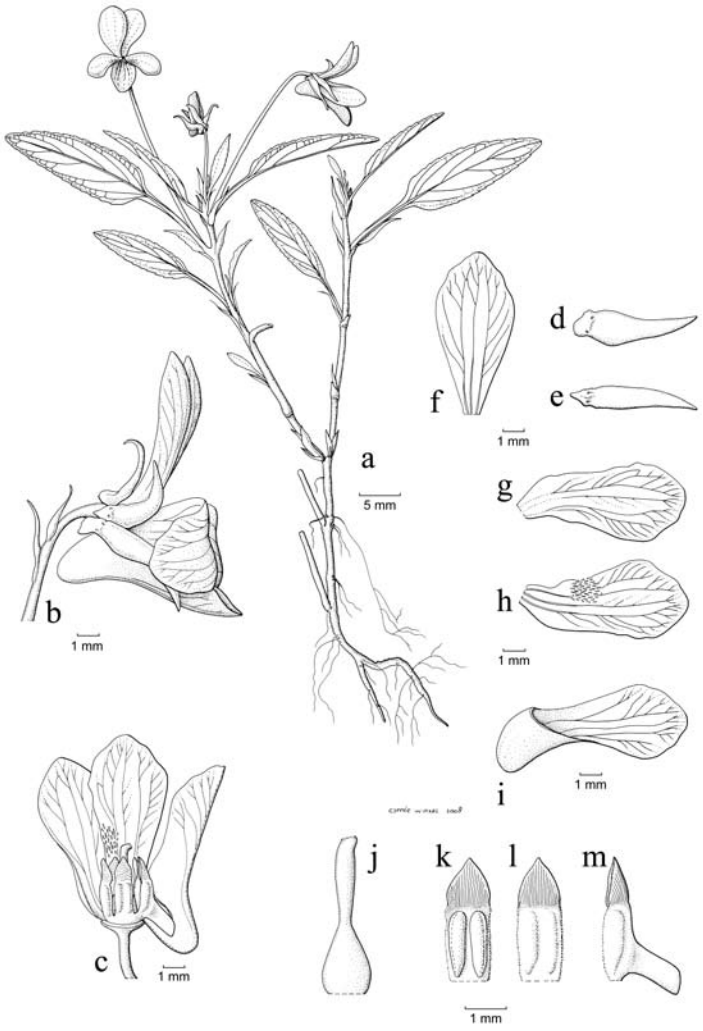


Fig. 9b. *V. stagnina* var. *lacteoides* a. Habit b. Lateral view of the flower c. Lateral view of the flower with male and female reproductive organs d. Lower sepal e. Upper sepal f. Dorsal petal g. Lateral petal h. Lateral petal with fimbriae i. Ventral petal with spur j. Gynoecium k. Adaxial view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

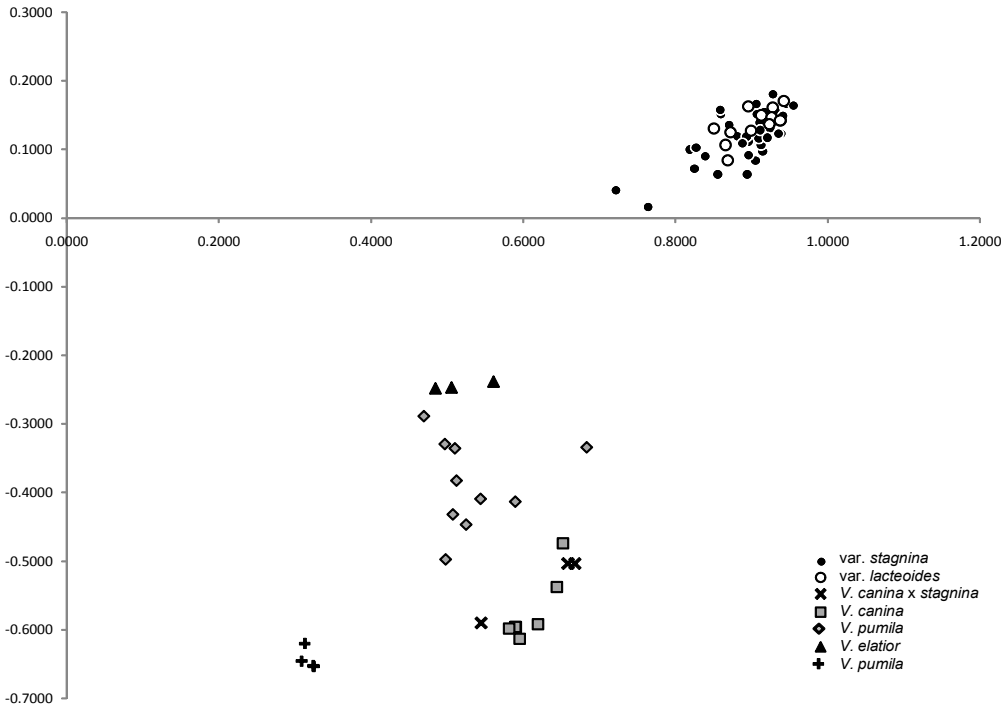


Fig. 11. Principal Coordinate Analysis (PCO) based on the presence/absence of the AFLP markers of all *Viola* accessions. PCO axes 1 and 2 extracted 64% and 9% of the variance, respectively.

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.

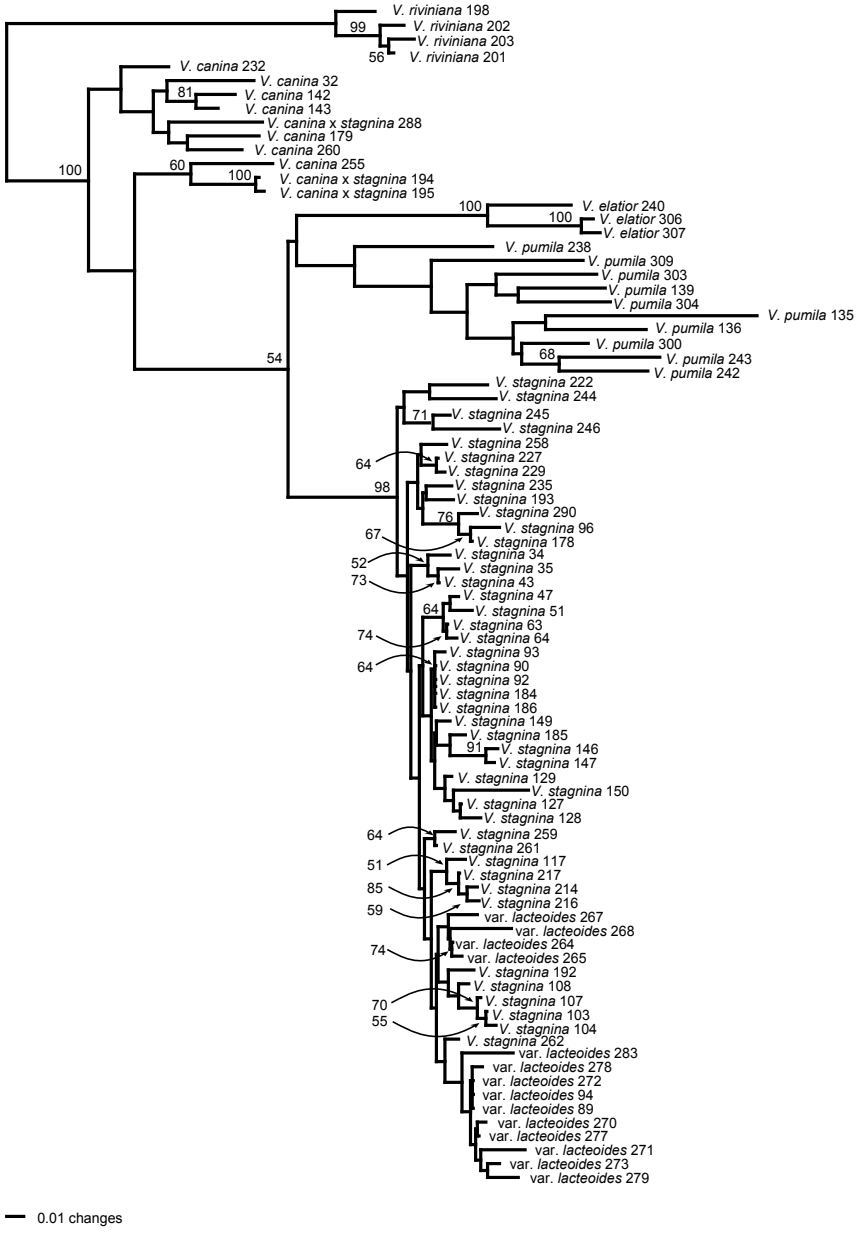


Fig. 12. NJ tree of AFLP markers of *Viola* accessions analysed. Bootstrap values >50 % are indicated above the branches.

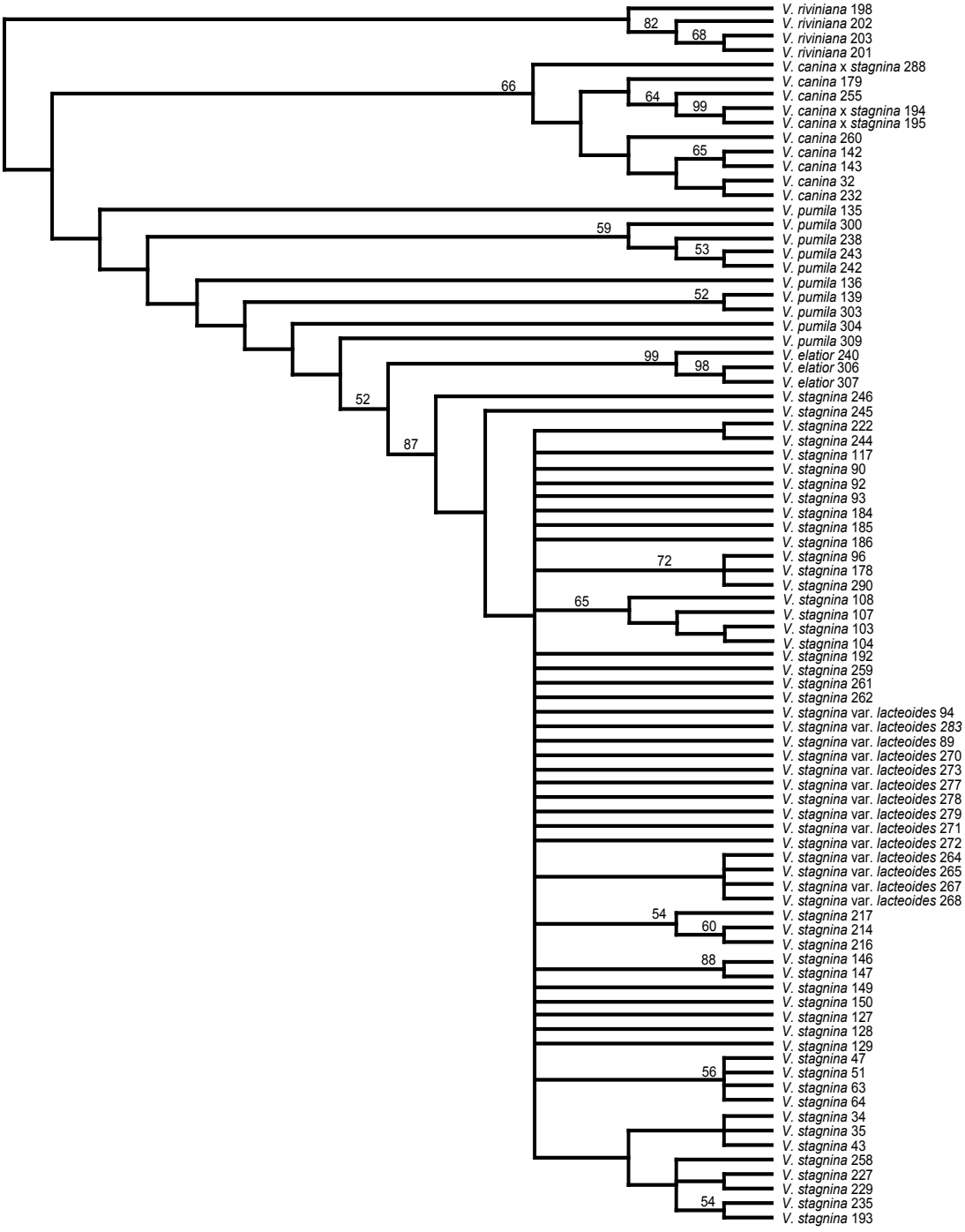


Fig. 13. MP strict consensus tree produced by analysis of AFLP markers of *Viola* accessions. BS values > 50% are indicated above the branches.

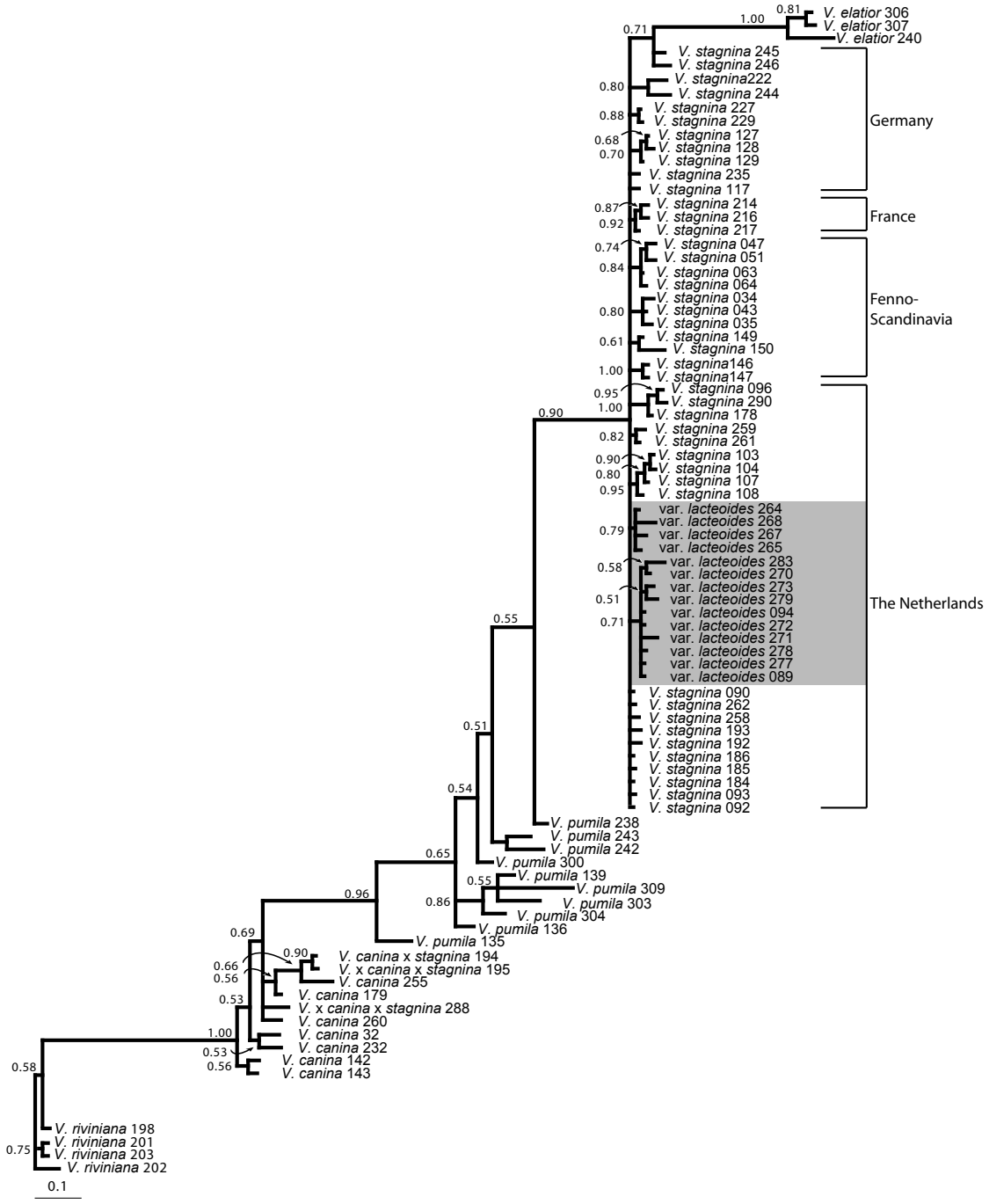


Fig. 14. BI tree produced by analysis of AFLP markers of *Viola* accessions. Posterior probabilities are indicated above the branches.

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.

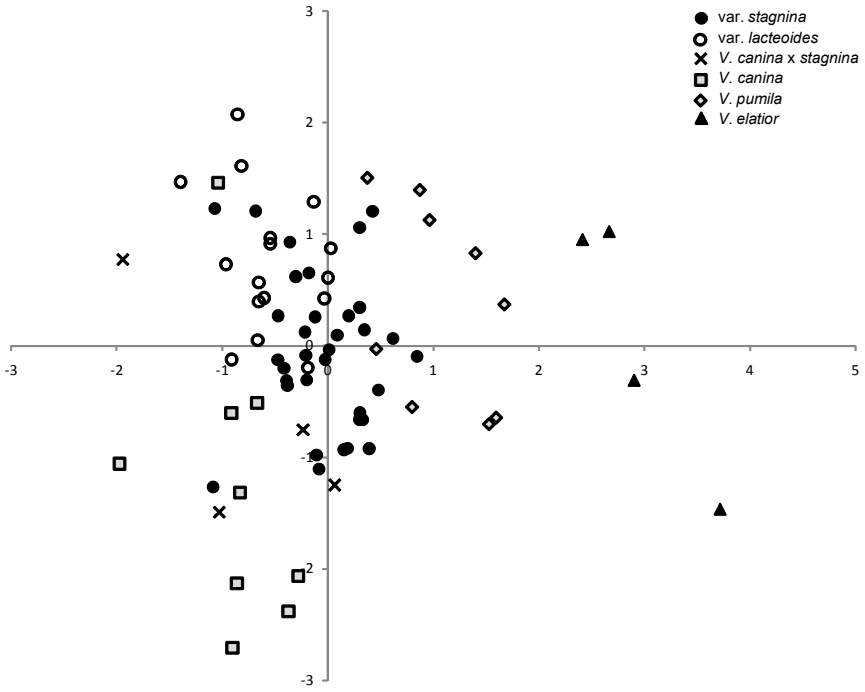


Fig. 15. Principal Component Analysis (PCA) of all morphological characters.

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

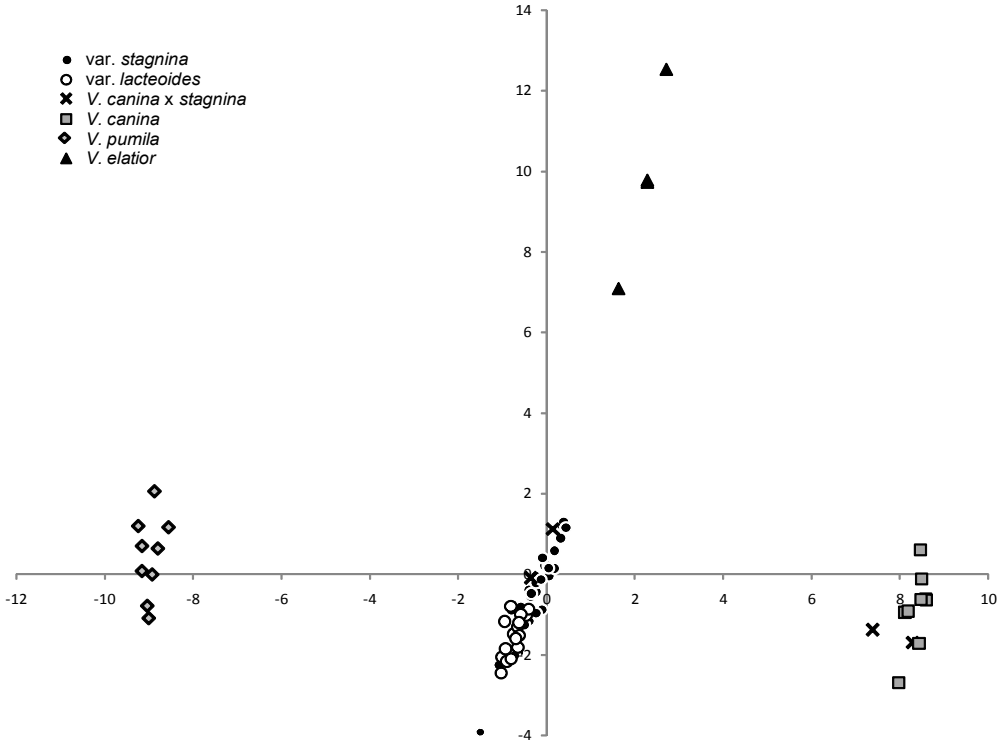


Fig. 16. Canonical Discriminant Analysis (CDA) of the first two axes of all morphological characters.

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.

Acknowledgements

Ria Vrielink-van Ginkel and Nynke Groendijk-Wilders (Leerstoelgroep Biosystematiek - Wageningen University) are thanked for her help with generating the AFLP data. Delia Co-David and André David are thanked for their help with the Baysian analyses. Kim Eshuis, Violet Houwelingen, Marije Stoops, and Mark Tordoir (biology students at Leiden University) helped collecting parts of the morphometric dataset. Jirí Danihelka is thanked for contributing plant material. Peter van Beers, Lutz Eckstein, Frank Hellberg, René van Moorsel and Ben Wijlens are thanked for their assistance in the field. Hanneke den Held, Eddy Weeda, and the late Ruud van der Meijden helped collecting plants in the field and also gave valuable comments to earlier versions of this manuscript.

Chapter

6

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

K. van den Hof, T. Marcussen, R.G. van den Berg and B. Gravendeel

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%.

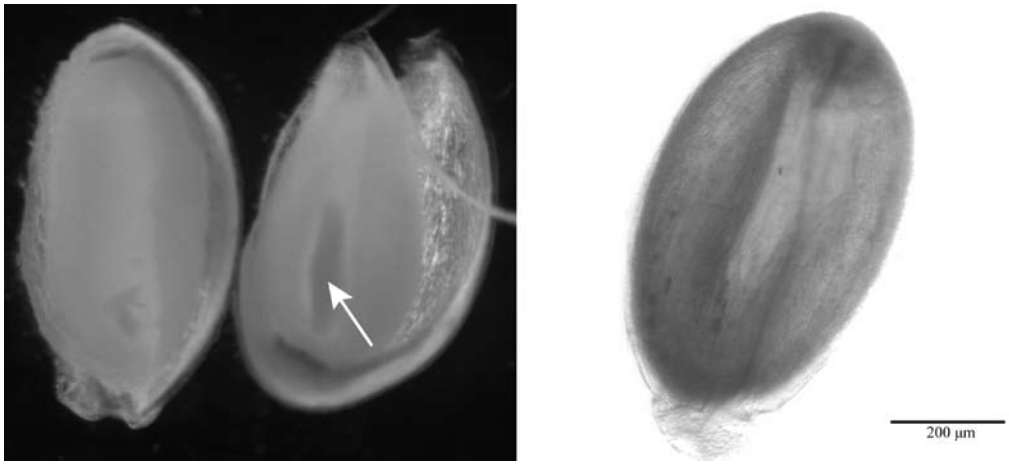


Fig. 17. SEM pictures of the stigma of a mature flower of *V. stagnina* var. *stagnina* (Veenmelkviooltje) at the right, and *V. stagnina* var. *lacteoides* (Heidemelkviooltje) at the left. The white lines indicate the amount of curving.

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).

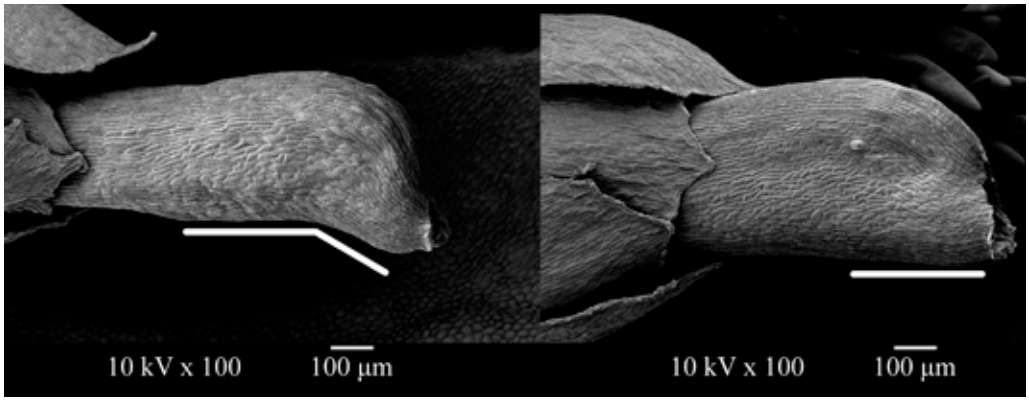


Fig. 18. LM pictures of seeds of crosses between *V. stagnina* var. *lacteoides* (Heidemelkviooltje) and *V. stagnina* var. *stagnina* (Veenmelkviooltje). At the right, a viable seed containing an embryo (indicated with arrow), at the left an aborted seed without embryo.

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.

Acknowledgements

We like to thank Hanneke den Held, Eddy Weeda, and the late Ruud van der Meijden for their help with collecting plants in the field and valuable feedback. Jan Frits Veldkamp (NHN - Leiden University), Jan Wieringa (NHN - Wageningen University), Kanchi Gandhi (Harvard University Herbaria) and Jiři Danihelka (Academy of Sciences of the Czech Republic) are thanked for their nomenclatural advice. We would also like to thank Natasha Schidlo and René van Moorsel for their assistance in the field, and Gerjo van Genderen for providing transportation during the field season. Bertie-Joan van Heuven, Hans Kruijer, and Peter Hovenkamp are thanked for their assistance in the lab. Natuurmonumenten and Staatsbosbeheer are acknowledged for providing access to plant material and collecting permits.

Chapter

7

Summary and Conclusions

Viola stagnina

The European Fen Violet (*Viola stagnina* Kit.) is a rare plant species despite its large distribution area, ranging from Western Asia to the Atlantic coast of Europe (Fig. 10). The species is placed on the Belgian and Dutch red lists which contains species that are threatened with local extinction. The genus *Viola* (Violaceae) is divided into several sections and subsections. *Viola stagnina* is a member of section *Viola* subsection *Rostratae*. The subsection contains approximately 50 species with a northern temperate distribution in North America and Eurasia, and is primarily characterized by primitive characters. Phylogenetic analyses suggest that the subsection is probably paraphyletic with other subsections of section *Viola*. The evolutionary relationships within subsection *Rostratae* are still only poorly understood because of hybridization and polyploidisation events. In Europe, where the subsection is considered morphologically most diverse, the subsection has been further divided into several series. *Viola stagnina* is placed in the *Arosulatae* series. This group of species is characterized by the lack of a basal leaf rosette. As a paleotetraploid ($2n = 20$), *V. stagnina* was involved in the alloploid origin of the other Arosulate species, *V. canina* ($2n = 40$), *V. lactea* ($2n = 58$), and *V. pumila* ($2n = 40$). *Viola elatior* ($2n = 40$) is most probably an autoploid derivative of *V. stagnina*.

At the beginning of the previous century two varieties have been described for *V. stagnina*: *V. stagnina* var. *stagnina* and *V. stagnina* var. *lacteoides* by A.W. Kloos Jr. and W. Becker. The latter variety was considered to be endemic for the Netherlands. Over the past 80 years, Dutch botanists disagreed among each other about its taxonomic status. Some considered the morphological variation within *V. stagnina* too small to distinguish separate varieties, while others recognized them as separate subspecies or even distinct species. With the molecular techniques now at hand, it is possible to study the genetic variation within *V. stagnina* in detail. The aim of this thesis was to investigate hybridization and polyploidisation events between *V. stagnina* and its closest relatives to gain insight in the role of these processes in speciation. The infraspecific variation within *V. stagnina* was also studied to settle the debate about the taxonomic status of its infraspecific morphs.

Chalcone Synthase and the reticulate relationships of the Arosulate Violets

Multicopy genes such as nrITS are usually not suited for studying the phylogenetic relationships of groups of (allo)polyploid species. Recombination and concerted evolution between orthologous copies often lead to retention of only one copy type and erasion of the other parental copy. With low copy nuclear genes it is usually possible to circumvent these problems and phylogenetic analysis of paralogous and orthologous copies of such genes in alloploid species is also a good method to reveal the parental contributors to alloploid genomes.

An example of a low copy gene is the Chalcone Synthase (CHS) gene family. These genes encode for the first enzyme in the flavonoid synthesis pathway. Flavonoids are secondary metabolites responsible for many tasks in plants, ranging from flower and fruit coloration and protection against UV radiation to pathogen defense and pollen development. In chapter 2, this gene is used for the phylogenetic reconstruction of the Rostrate Violets, in order to determine the closest relatives of *V. stagnina* and to determine the alloploid relationships of certain species within the subsection. The evolutionary history of the CHS gene family itself was also studied.

Phylogenetic analyses show that during the evolution one duplication event took place before the split between monocots and eudicots. A second duplication event of the CHS gene probably took place during the split between the core eudicots and the rosids and asterids. Similar results were also found in other studies. Finally, a third duplication event took place within *Viola* or the Violaceae. These findings are in congruence with other studies of the Chalcone Synthase gene family, where family specific duplication events of CHS have taken place.

Previous studies, using chromosome number and morphology recognized six different types of genomes (A-E, M; fig 2.1) for several species of subsection Rostratae. Three genome types are still present in the extant lineages of the tetraploid species (2n = 20) *V. reichenbachiana* (A), *V. stagnina* (C), and *V. mirabilis* (M). The other three genome types (B,D, and E) are only found in combination with other genome types in octoploids (2n = 20) or sub-dodecaploids (2n = 58). The alloploid relationships between the species that the previous studies inferred from these genome types were confirmed by the phylogenetic analyses of the CHS intron. The analyses of the CHS intron confirm that *Viola stagnina* is one of the parental species of the alloploid species *V. canina*, *V. lactea* and *V. pumila*. The lack of an extra CHS copy for *V. elatior* also suggests that it is an autoploid derivative of *V. stagnina*.

The analyses also confirmed that the closest relatives of *V. stagnina* were indeed the other arosulate violets *V. canina*, *V. elatior*, *V. pumila*, and *V. lactea*.

Viola stagnina var. lacteoides

The variety *V. stagnina* var. *lacteoides* was first described by Kloos and Becker in 1924. Since then it disappeared and resurfaced several times in Dutch flora's, because some botanists questioned the observed variation between var. *lacteoides* and the common variety, while others thought that the differences in morphology and geographic distribution justified the recognition of var. *lacteoides*. Some even thought it deserved a higher taxonomic rank as subspecies. In an attempt to settle this debate among Dutch botanist about the taxonomic status of *V. stagnina* var. *lacteoides*, the morphological and

genetic variation within *V. stagnina* were studied using a DNA fingerprinting technique called AFLP, morphometrics analyses, a common garden experiment, and a crossing experiment.

The AFLPs showed that there was only a very weak genetic differentiation between the two varieties of *V. stagnina*. The morphometrics study demonstrated that there are statistically significant differences between *V. stagnina* var. *stagnina* and *V. stagnina* var. *lacteoides*. The endemic variety was significantly smaller in size, had smaller leaves, shorter petioles, and the ventral and dorsal petals were less wide than those of the common variety. Also, the calycine appendages were significantly shorter, causing the spur to exceed more than is observed in *V. stagnina* var. *stagnina*, without actually being longer, as was assumed earlier.

The common garden experiment demonstrated that many characters of *V. stagnina* show a high degree of phenotypic plasticity. The different observations for 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. However, lamina size and petiole length are most probably genetically determined, because these characters remained significantly different over time between the two varieties. The crossing experiment demonstrated that manual crosses between plant of both varieties produced viable seeds and that the two varieties are probably not reproductively isolated.

Based on the genetic and morphological differences found, and the unique distribution, we recommend maintaining the infraspecific taxon *V. stagnina* var. *lacteoides*. Since genetic differentiation is low, and because of the small overlap in geographic distribution between both morphs of *V. stagnina*, we prefer to use the infraspecific rank of variety rather than subspecies.

Nomenclature

In many European floras, including the latest edition of the Heukels' Flora of The Netherlands, *V. stagnina* is mentioned under the name *V. persicifolia* Schreb. The nomenclatural study presented in this thesis, however, has pointed out that this name should be interpreted as referring to *V. elatior* Fries. In fact, the name *V. persicifolia* has also been applied to the closely related taxa *V. lactea* Sm. and *V. pumila* Chaix., because of the ambiguous description and the lack of a type specimen. Kitaibel, published the name *V. stagnina* for The European Fen Violet in 1814. This name still has an existing type specimen and was never used as scientific name for another species. We therefore propose to give priority to the latter name and to reject the older name *V. persicifolia*.

The Dutch variety of the Fen Violet was first published as var. *lacteaeoides* W. Becker & Kloos. The correct merge of the two elements "lactea"and "oides" from the epithet is "lacteoides" because it is a compound. 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", "lacteoïdes" and "lactaeoides" are incorrect.

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.

Conclusions

In this thesis, different speciation processes were studied that were involved in the origin of *V. stagnina* and its closest relatives. Phylogenetic analyses of the CHS intron showed that hybridization and polyploidisation played an important role during speciation and that *Viola stagnina* is one of the parental species of the alloploid species *V. canina*, *V. lactea* and *V. pumila* and the parental species of the autotetraploid *V. elatior*. The analyses also confirmed that the closest relatives of *V. stagnina* were the other arosulate violets *V. canina*, *V. elatior*, *V. pumila* and *V. lactea*.

In an attempt to settle a debate among Dutch botanist about the taxonomic status of *V. stagnina* var. *lacteoides*, the morphological and genetic variation within *V. stagnina* were studied using AFLP, morphometrics analyses, a common garden experiment, and a crossing experiment. The genetic and morphological differences found support for the recognition of the infraspecific taxon *V. stagnina* var. *lacteoides*.

The nomenclatural studies carried out resulted in a recommendation to formally reject the ambiguous name *V. persicifolia* for the European Fen Violet and use the name *V. stagnina*, instead. To bring the common name into line with the usage in Belgium, it is also recommended to change the Dutch common name from Melkviooltje into Vals melkviooltje.

Evolutie van het Melkviooltje en verwanten door hybridisatie en polyploïdie

De systematiek is de wetenschap binnen de biologie die zich bezighoudt met inventariseren, identificeren van de verwantschapsrelaties van alle levende organismen op aarde. Het leven op aarde is eenmalig ontstaan, circa 4 miljard jaar geleden. Sinds Charles Darwin de evolutietheorie heeft ontwikkeld, weten we dat dit eerste leven sindsdien door aanpassingen aan de omgeving (adaptatie) en natuurlijke selectie (survival of the fittest) is geëvolueerd tot de enorme diversiteit aan organismen die we tegenwoordig op aarde aantreffen. Systematici proberen deze diversiteit in natuurlijke groepen in te delen en van namen te voorzien. Deze natuurlijke groepen worden weergegeven in een afstammingsgeschiedenis, de zogenaamde fylogenie. Bovendien bestuderen systematici aan de hand van fylogenieën de processen die hebben geleid tot de grote vormenrijkdom die we waarnemen.

In dit proefschrift worden van een Nederlandse viooltjessoort en haar nauwste verwanten de evolutionaire geschiedenis en de achterliggende soortvormingsprocessen onderzocht. Het plantengeslacht *Viola* waar de viooltjes onder vallen behoort tot de familie van de Violaceae. Deze plantfamilie bestaat uit ongeveer 22 genera, waarvan het geslacht *Viola* het grootste is met ongeveer 500 soorten. *Viola* soorten zijn kruidachtig, overblijvend en makkelijk te herkennen aan de tweezijdig symmetrische bloemen. Door de korte bloeitijd van sommige *Viola* soorten kan het voorkomen dat bloemen niet op tijd bestoven worden. De normaal ontwikkelde bloemen verdorren dan zonder dat er zaadzetting plaatsvindt. Sommige *Viola* soorten hebben echter nog ander voortplantingsmechanisme; deze soorten ontwikkelen ook halfontwikkelde, zogenaamd cleistogame bloemen die zichzelf kunnen bevruchten en zonder hulp van bestuivers zaad kunnen produceren. Mede dankzij dit extra voortplantingsmechanisme heeft het geslacht *Viola* een wereldwijde verspreiding waarvan de oorsprong hoogstwaarschijnlijk in de Zuid-Amerikaanse Andes ligt. De vormenrijkdom binnen het geslacht is groot, zo bevat het geslacht niet alleen de viooltjes die we kennen uit de tuin en het bos, maar zijn er ook zeldzame soorten met vetachtige bladeren of houtige stengels die niet in Europa voorkomen.

De soort die in dit proefschrift centraal staat is *Viola stagnina* Kit. (Melkviooltje). Het Melkviooltje bloeit in het voorjaar en komt voor op zandige en kleiige veengrond. De soort is een pioniersplant die groeit op weinig bemeste terreinen die 's winters onder water staan en 's zomers oppervlakkig uitdrogen. De zaadvoorraad in de bodem kan tientallen jaren lang kiemkrachtig blijven. Om de soort niet kwijt te raken in een terrein is echter een periodieke verstoring nodig zoals uitbaggeren of afplaggen. Het verspreidingsgebied

van *V. stagnina* reikt van de Ierse westkust tot aan de Oeral en van centraal Zweden tot aan de Alpen en het Balkan gebergte (Fig 5.1). Ondanks haar grote verspreidingsgebied is *V. stagnina* een zeldzame soort, waarschijnlijk omdat periodieke verstoringen nodig zijn die bij de moderne beheersvorm van "niets doen" vaak niet meer worden uitgevoerd. In Nederland is *V. stagnina* op de rode lijst geplaatst in de categorie bedreigd. Dit betekent dat overheden en terreinbeherende organisaties maatregelen dienen te nemen om de soort weer van de rode lijst afgevoerd te krijgen.

In Nederland heeft deze soort bijzondere aandacht, omdat Becker en Kloos in 1924 binnen *V. stagnina* een nieuwe variëteit hebben beschreven: *V. stagnina* var. *lacteoides* (Heide-melkviooltje). Volgens sommige botanici verschilt deze variëteit substantieel in een aantal kenmerken van de algemene variëteit (Veen-melkviooltje). Bovendien komt het Heide-melkviooltje alleen in Nederland voor, een zogenaamd endeem voor Nederland. Andere botanici twijfelen hier echter aan, waardoor de variëteit meerdere malen opdook en weer verdween in de Nederlandse flora's die vanaf 1924 tot 2005 zijn gepubliceerd. In dit proefschrift proberen we 1) de evolutionaire verwantschappen van een aantal soorten rond *V. stagnina* in kaart te brengen, 2) de taxonomische realiteit van de infraspecifieke taxa binnen *V. stagnina* vast te stellen aan de hand van morfologische en genetische data, en 3) meer duidelijkheid te brengen in de verwarde nomenclatorische situatie rond de namen van een aantal soorten.

De nauwste verwanten van Viola stagnina

Binnen het geslacht *Viola* is, net als in veel andere plantengeslachten, hybridisatie een algemeen voorkomend verschijnsel. Hybriden zijn vaak steriel. Soms kan bij hybriden het aantal chromosomen verdubbelen: dit proces noemen we polyploïdisatie. Polyploïden waarvan het genoom bestaat uit de chromosomensets van twee verschillende oudersoorten worden allopolyploïden genoemd. Door een dergelijke chromosoomverdubbeling kunnen steriele hybriden vruchtbaar worden omdat de meiose weer kan functioneren. Allopolyploïden zijn door hun afwijkend chromosoom aantal reproductief geïsoleerd van hun vooroudersoorten en kunnen zo een nieuwe soort vormen. Veel *Viola* soorten zijn allopolyploïden. Door middel van kruisingsexperimenten en het bestuderen van de chromosomen van de kruisingsproducten uit deze experimenten heeft men de relaties tussen een aantal polyploïde viooltjes en hun oudersoorten weten te bepalen (fig. 1). Veel recente *Viola* soorten zijn dus geen afsplitsing van één vooroudersoort (dichotome evolutie), maar zijn het resultaat van hybridisatie tussen twee of meer vooroudersoorten; dit wordt reticulate evolutie genoemd. Deze verwantschappen zijn goed in kaart te brengen door in moleculair onderzoek gebruik te maken van genen waarvan verscheidene kopieën aanwezig zijn in het genoom. Zo'n gen is bijvoorbeeld het *Chalcone Synthase* gen. Dit gen wordt in hoofdstuk twee gebruikt om de nauwst verwante soorten van *V. stagnina* te bepalen en om de reticulate verwantschappen in kaart te brengen. Analyses tonen aan dat *V. canina* (Hondsviooltje) en de niet in Nederland voorkomende *V. elatior* (Hoog viooltje), *V. lactea* (Echt melkviooltje) en *V. pumila* (Klein melkviooltje) de nauwste verwanten zijn van *V. stagnina* (Fig. 4). Bovendien wordt in onze analyses bevestigd dat *V. stagnina* één van de oudersoorten is van de allopolyploïden *V. canina*, *V. lactea* en *V. pumila*. *Viola elatior* is hoogstwaarschijnlijk een autopolyloid van *V. stagnina*. Dat wil zeggen dat deze soort is ontstaan nadat *V. stagnina* haar chromosomen heeft verdubbeld zonder eerst met

een ander soort te hybridiseren (Fig. 1). Daarnaast wordt de evolutionaire geschiedenis van het *Chalcone Synthase* gen zelf bestudeerd. Analyses tonen aan dat dit gen zich meerdere malen in de evolutionaire geschiedenis van de Violaceae en andere plantenfamilies heeft gedupliceerd (Fig. 5).

Latijnse naamgeving

In de Heukels' Flora van Nederland, net als in vele andere West-Europese flora's, staat het Melkviooltje nog vermeld onder de Latijnse naam *V. persicifolia* Schreb. (1771) (Perzikbladig viooltje). Andere flora's uit Centraal en Oost Europa gebruiken echter *V. stagnina* Kit. (1814). Uit nomenclatorisch onderzoek, beschreven in hoofdstuk drie, blijkt dat de beschrijving van *V. persicifolia* niet eenduidig is, temeer omdat een type exemplaar ontbreekt. Het is daarom zeer de vraag of de beschrijving van *V. persicifolia* ook echt op het Melkviooltje slaat. Van de locatie die beschreven wordt – de Funkenburg bij Leipzig – zijn ook de soorten *V. elatior* en *V. pumila* bekend, soorten die beide sterk lijken op het Melkviooltje. Behalve voor het Melkviooltje is de naam *V. persicifolia* door de eeuwen heen daarom ook gebruikt voor *V. elatior* en *V. pumila*. De naam *V. stagnina*, daarentegen, is altijd maar voor één soort gebruikt. In hetzelfde onderzoek wordt aangetoond dat de naam van de niet in Nederland voorkomende *V. montana* de oudste naam voor *V. elatior* is. Na de eerste publicatie echter is de naam *V. montana* vaak foutief gebruikt voor een variëteit van *V. canina*. In hoofdstuk vier wordt een voorstel ingediend bij de *International Association for Plant Taxonomy* ter besluitvorming op het eerstvolgende botanische congres, om de namen *V. persicifolia* en *V. montana* te verwerpen en voortaan respectievelijk de namen *V. stagnina* en *V. elatior* te gebruiken, zoals dat in dit proefschrift al gebeurt.

Variatie binnen V. stagnina

De taxonomische identiteit van *V. stagnina* var. *lacteoides* is bestudeerd door zowel de genetische als de morfologische variatie van verschillende populaties van het Melkviooltje te analyseren. De genetische variatie is bestudeerd met behulp van een DNA fingerprint techniek: Amplified Fragment Length Polymorphism (AFLPs). Met deze techniek worden er van iedere individuele plant DNA-fragmenten uit het gehele genoom gebruikt om een genetische blauwdruk te maken. Aan de hand van deze fingerprints kunnen evolutionaire verwantschapsanalyses gedaan worden. De analyse van de AFLPs in hoofdstuk vijf laat zien dat twee *V. stagnina* var. *lacteoides* takken met lage statistische ondersteuning zijn te onderscheiden van *V. stagnina* var. *stagnina* (Fig. 13). Uit de morfologische analyses blijkt verder een groot deel van de morfologische kenmerken significant verschillend te zijn voor de twee vormen van *V. stagnina*. Daarnaast laat een common garden experiment, beschreven in hoofdstuk zes, zien dat een aantal van deze significant verschillende kenmerken beïnvloed worden door abiotische factoren zoals licht en vochtigheid. Er zijn echter ook kenmerken die significant van elkaar blijven verschillen onder gelijke omstandigheden en dus veroorzaakt worden genetische differentiatie. Een kruisingsexperiment toont aan dat planten bestoven met pollen van de andere vorm nog steeds levensvatbare zaden opleveren, hetgeen ook mag worden verwacht bij kruising van eenheden die tot dezelfde soort behoren.

Conclusies

In dit proefschrift werd de rol van hybridisatie en polyploïdie als soortsvormingmechanismen bij *V. stagnina* onderzocht. We hebben aangetoond dat verschillende kopieën van het *Chalcone Synthase* gen eerdere hypothesen bevestigden over de afstamming van de nauwste verwanten van *V. stagnina* door reticulate evolutie. Na onderzoek naar de Latijnse naamgeving stellen we voor om de verwarrende naam *V. persicifolia* te laten vervallen en te vervangen door het meer eenduidige *V. stagnina*. Op basis van de gevonden genetische en morfologische verschillen en de uitkomsten van ons common garden experiment, vinden we dat de endemische vorm van *V. stagnina* als de aparte variëteit *lacteoides* erkend moet worden. Voor de taxonomische status van aparte (onder)soort verschillen de twee vormen te weinig en overlappen ze geografisch teveel. Het is mogelijk dat de endemische vorm van *V. stagnina* zich uiteindelijk toch tot een aparte soort zal ontwikkelen. Een goede bescherming van dit zeldzame viooltje en de terreinen waarin het voorkomt blijft dan ook van groot belang.

Abbott RJ, Ireland HE, Joseph L, Davies MS, Rogers HJ. 2005. Recent plant speciation in Britain and Ireland: origins, establishment and evolution of four new hybrid species. Biology and Environment: Proc. R. Ir. Acad. 105: 173–183.

Althoff DM, Gitzendanner MA, Segraves KA. 2007. The utility of amplified fragment length polymorphisms in phylogenetics: a comparison of homology within and between genomes. Syst. Biol. 56: 477–484.

Álvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phyl. Evol. 29: 417–437.

Ballard HE, Sytsma KJ, Kowal RR. 1999. Shrinking the violets: Phylogenetic relationships of infrageneric groups in *Viola* (*Violaceae*) based on internal transcribed spacer DNA sequences. Syst. Bot. 23: 439–458.

Ballard HE. 1993. Three new violet hybrids from Appalachia. Castanea 58: 1–9.

Beattie AJ. 1971. Pollination mechanisms in *Viola*. New Phytol. 70: 343–360.

Beattie AJ. 1974. Floral evolution in *Viola*. Ann. Missouri Bot. Gard. 61:781–793.

Becker W. 1910. *Violae Europaeae*. Verlag von C. Heinrich, Germany.

Becker W. 1917. Violae Asiaticae et Australenses. Beih. Bot. Centralbl. 34: 373–433.

Bennett MD. 2004. Perspectives on polyploidy in plants - ancient and neo. Biol. J. Linn. Soc. 82: 411–423.

Bergdolt E. 1932. Morphologische und physiologische Untersuchungen über *Viola* zugleich ein Betrag zur Lösung des Problems der Kleistogamie. Bot. Abh. 20: 1–120.

Boehmer GR. 1750. Flora Lipsiae indigena. Joh. Gothofred. Dyckium, Germany.

Borbás V. 1892. Violarieen. In: Hallier, E. (ed), Koch's Synopsis der deutschen und Schweizer Flora: 161–226. O. R. Reisland, Germany.

Clausen J. 1927. Chromosome number and the relationship of species in the genus Viola. Ann. Bot. 61: 677–714.

Clausen J. 1929. Chromosome number and relationship of some North American species of *Viola*. Ann. Bot. 43: 741–764.

Clegg MT, Durbin ML. 2003. Tracing floral adaptations from ecology to molecules. Nat. Rev. Genet. 4: 206-215.

Corriveau JL, Coleman AW. 1988. Rapid screening method to detect potential biparental inheritance of plastid DNA and results from over 200 angiosperm species. Am. J. Bot. 75: 1443–1458.

Cracraft J. 1983. Species concepts and speciation analysis. Curr. Orn. 1: 159–187

Cronquist A. 1988. The evolution and classification of flowering plants, ed. 2. New York Botanical Garden, New York.

Dandy JE. 1970. Annotated list of the new names published in Allioni's Auctuarium ad synopsim stirpium horti reg. taurinensis. Taxon 19: 617–626.

Danihelka J, van den Hof K, Marcussen T, Jonsell B. 2009. *Viola montana* and *V. persicifolia* (Violaceae): two names to be rejected. Taxon: in review.

Darwin C. 1859. On the origin of species by means of natural selection or the preservation of favored races in the struggle for life. Murray, UK.

De Queiroz K. 2005. Ernst Mayr and the modern concept of species. Proc. Natl. Acad. Sci. USA. 102: 6600–6607.

Dengler NG. 1994. The influence of light on leaf development. In: Iqbal M, ed. Growth patterns in vascular plants 100–136. Dioscorides Press, USA.

Dobzhansky T. 1955. Evolution, genetics, and man. John Wiley and sons, USA.

Domac, R. 1994. Flora Hrvatske. Školska kniga, Croatia.

Doyle JJ, Davis JI. 1998. Homology in molecular phylogenetics: a parsimony perspective. In: Soltis DE, Soltis PS, Doyle JJ (eds), Molecular Systematics of Plants II: 101–131. Kluwer Academic Publishers, USA.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. Bot. Soc. Amer. 19:11–15.

Durbin ML, Learn GH, Huttley GA, Clegg MT. 1995. Evolution of the chalcone synthase gene family in the genus *Ipomoea*. Proc. Natl. Acad. Sci. USA. 92: 3338–3342.

Eckstein RL, Hölzel N, Danihelka J. 2006. Biological Flora of Central Europe: *V. elatior*, *V. pumila* and *V. stagnina*. Perspec. Plant. Ecol. 8: 45–66.

Eckstein RL, O'Neill RA, Danihelka J, Otte A, Köhler W. 2006. Genetic structure among and within peripheral and central populations of three endangered floodplain violets. Mol. Ecol. 15: 2367–2379.

Ellstrand NC, Schierenbeck KA. 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? Proc. Natl. Acad. Sci. USA. 97: 7043–7050.

Elven, R. 2005. Lid & Lid: Norsk flora. Det Norske Samlaget, Norway.

Erben M. 1996. The significance of hybridization on the forming of species in the genus *Viola*. Bocconea 5: 113–118.

Espeut M. 1999. Errata et addenda de l'article: "Approche du genre Viola dans le Midi Mediterranéen français". Monde Pl. 467: 7-9.

Evans JR, von Caemmerer S, Adams WW. 1988. Ecology of photosynthesis in sun and shade. CSIRO, Australia.

Farzad M, Griesbach R, Hammond J, Weiss MR, Elmendorf HG. 2003. Differential expression of three key anthocyanin biosynthetic genes in a color-changing flower, *Viola cornuta* cv. Yesterday, Today and Tomorrow. Plant Sci. 165: 1333–1342.

Farzad M, Soria-Hernanz DF, Altura M, Hamilton MB, Weiss MR, Elmendorf HG. 2005. Molecular evolution of the chalcone synthase gene family and identification of the expressed copy in flower petal tissue of *Viola cornuta*. Plant Sci. 168: 1127–1134.

Felsenstein J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. Mol. Evol. 17: 368–376.

Felsenstein J. 1985. Confidence-limits on phylogenies - An approach using the bootstrap. Evolution 39: 783–791.

Fries, EM. 1828. Novitiae florae suecicae. Edit. altera. Londini gothorum, ex officinal berlingiana. Gerold's Sohn, Austria.

Gerstlauer, L. 1943. Vorschläge zur Systematik der einheimischen Veilchen. Ber. Bayer. Bot. Ges. 26: 12–55.

Grant V. 1981. Plant speciation. Columbia University Press, USA.

Guinochet M, de Vilmorin, R. 1982. Flore de France, fasc. 4. Éditions du Centre National de la Recherche Scientifique, France.

Haeupler H, Wisskirchen R. 1998. Standardliste der Farn- und Blütenpflanzen Deutschlands mit Chromosomenatlas von Focke Albers. Bundesamt für Naturschutz, Germany.

Haller A. 1768. Historia stirpium indigenarum Helvetiae inchoata. sumptibus Societatis typographicae, Switzerland.

Hamilton CW, Reichard SH. 1992. Current practice in the use of subspecies, variety and forma in the classification of wild plants. Taxon 41: 485–498.

Harborne JB. 1994. The Flavonoids; Advances in Research since 1986. Chapman and Hall, UK.

Hardtke HJ. & Ihl A. 2000. Atlas der Farn- und Samenpflanzen Sachsens. Sächsisches Landesamt für Umwelt und Geologie, Germany.

Harmatta J. 1962. Die frühen Forschungsreisen Pál Kitaibels. Bot. Közlem. 49: 334–345.

Hartman, CJ. 1820. Handbok i Skandinaviens flora. Zacharias Haeggström, Sweden.

Harvey MJ. 1966. Cytotaxonomic relationships between the European and North American rostrate violets. New Phytol. 65 :469–476.

Hegarty MJ, Hiscock SJ. 2005. Hybrid speciation in plants: new insights from molecular studies. New Phytol. 165: 411–423.

Heimans E, Heinsius HW, Thijsse JP. 1924. Geïllustreerde Flora van Nederland. W. Versluys, The Netherlands.

Heimans E, Heinsius HW, Thijsse JP. 1965. Geïllustreerde Flora van Nederland. W. Versluys, The Netherlands.

Helariutta Y, Kotilainen M, Elomaa P, Kalkkinen N, Bremer K, Teeri TH, Albert VA. 1996. Duplication and functional divergence in the chalcone synthase gene family of Asteraceae: Evolution with substrate change and catalytic simplification. Proc. Natl. Acad. Sci. USA. 93: 9033-9038.

Heukels H. 1927. Schoolflora voor Nederland. P. Noordhoff, The Netherlands.

Hey J. 2001. The mind of the species problem. Trends Ecol. Evol. 16: 326–329.

Hey J. 2006. On the failure of the modern species concepts. Trends Ecol. Evol. 21: 447–450.

Hölzel N. 2003. Re-assessing the ecology of rare flood-meadow violets (*Viola elatior*, *V. pumila*, *V. persicifolia*) with large phytosciological data sets. Folia Geobot. 38: 281–298.

Huang J, Qu L, Yang J, Gu H. A preliminary study on the origin and evolution of chalcone synthase (CHS) Gene in Angiosperms. Acta Bot. Sin. 46: 10–19.

Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.

Hulten E, Fries M. 1986. Atlas of North European vascular plants north of the tropic of cancer II. Koeltz Scientific Books, Germany.

Hylander N. 1945. Nomenklatorische und systematische Studien über nordische Gefässpflanzen. Uppsala Univ. Årsskr. 7: 1–337.

Isaac NJB, Mallet J, Mace GM. 2004. Taxonomic inflation: its influence on macroecology and conservation. Trends Ecol. Evol. 19: 464–469.

Jaillon O, Aury J-M, Noel B, et al. (56 co-authors) 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449: 463–468.

Jávorka S. 1936. Kitaibel herbáriuma. Herbarium kitaibelianum. Ann. Mus. Natl. Hung., pars bot. 30: 7–118.

Jonsell B, Nordal I, Roberts FJ. 2000. *Viola rupestris* and its hybrids in Britain. Watsonia 23: 269–278.

Kadereit G, Kadereit JW. 2005. Phylogenetic relationships, evolutionary origin, taxonomic status, and genetic structure of the endangered local Lower Elbe river (Germany) endemic *Oenanthe conioides* (Nolte ex Rchb.f.) Lange (Apiaceae): ITS and AFLP evidence. Flora 200: 15–19.

Kirschner J, Skalický V. 1989. Notes on *Viola* in the new flora of the Czech lands. Preslia 61: 315–319.

Kirschner J, Skalický V. 1990. *Viola* L. – violka. In: Hejný S, Slavík B (eds.), Kvetena Ceské republiky vol. 2: 394–431. Academia, Czech Republic.

Kloos AW. 1924. Het geslacht *Viola* in Nederland. Nederl. Kruid. Arch. 33-34: 138–208.

Koch GDJ. 1836. Synopsis florae germanicae et helveticae. Sect. 1. sumptibus Friederici Wilmans, Germany.

Koch, GDJ. 1843. Synopsis florae germanicae et helveticae, ed 2. Sect. 1. sumptibus Gebhardt et Reisland, Germany.

Koes RE, Spelt CE, Mol J, Gerats A. 1987. The chalcone synthase multigene family of *Petunia hybrida* (V30): sequence homology, chromosomal localization and evolutionary aspects, Plant Mol. Biol. 10: 159–169.

Koopman WJM. 2005. Phylogenetic signal in AFLP data sets. Syst. Biol. 54: 197–217.

Koopman WJM, Vosman B, Sabatino GJH, Visser D, Van Huylenbroeck J, De Riek J, *et al.* 2008. AFLP markers as a tool to reconstruct complex relationships in the genus *Rosa* (Rosaceae). Am. J. Bot. 95: 353–366.

Kornet D, McAllister J. 1993. The composite species concept. Reconstructing species: Demarcations in genealogical networks. Unpublished phd dissertation, Institute for Theoretical Biology, Rijksherbarium, Leiden.

Lambinon J, de Langhe J, Delvosalle L, Duvigneaud J. 2004. Flora van België, het Groothertogdom Luxemburg, Noord-Frankrijk en de aangrenzende gebieden. Nationale Plantentuin van België, Belgium.

Levin DA. 2002. The role of chromosomal change in plant evolution. Oxford University Press, UK.

Lindberg H. 1958. Växter kända från Norden i Linnés herbarium. Acta Bot. Fenn. 60: 1–133.

Link, HF. 1821. Enumeratio plantarum horti regii botanici berolinensis altera. Pars I. G. Reimer, Switzerland.

Linnaeus, C. 1755. Flora suecica, ed. 2. Sumtu & literis Laurentii Salvii, Sweden.

Luo R, Hipp L, Larget B. 2007. A Bayesian model of AFLP marker evolution and phylogenetic inference. SAGMB. 6: article 11.

Maddison DR, Maddison WP. 2003. Macclade 4.06. Sunderland MA, Sinauer Associates.

Malécot V, Marcussen T, Munzinger J, Yockteng R, Henry M. 2007. On the origin of the sweet-smelling Parma Violet cultivars (Violaceae): wide intraspecific hybridization, sterility, and sexual reproduction. Am. J. Bot. 94: 29–41.

Mansfeld, R. 1939. Zur Nomenklatur der Farn- und Blütenpflanzen Deutschlands. VII. Zweiter Nachtrag zu den Gattungsnamen. Repert. Spec. Nov. Regni Veg. 47: 263–287.

Marcussen T. 2003. A new Violet species from the south-west Alps. Bot. J. Linn. Soc. 142: 119–123.

Marcussen T, Borgen L. 2000. Allozynic variation and relationships within *Viola* (Violaceae). Plant Syst. Evol. 223: 29–57.

Marcussen T, Nordal I. 1998. *Viola suavis*, a new species in the Nordic flora, with analyses of the relation to other species in the subsection *Viola* (Violaceae). Nord. J. Bot. 18: 221–237.

Marcussen T, Borgen L, Nordal I. 2001. *Viola hirta* and its relatives in Norway. Nord. J. Bot. 21: 5–18.

Marcussen T, Borgen L, Nordal I. 2005. New distributional and molecular information call into question the systematic position of the West Asian Viola sintenisii (Violaceae). Bot. J. Linn. Soc. 147: 91–98.

Marcussen T, Wind P, Jonsell B, Karlsson T. 2009. Violaceae. In: Jonsell B, (ed), Flora Nordica, vol. 6. In review, scheduled for publication 2009. Bergius Foundation, Sweden.

Mayr E. 1969. The biological meaning of species. Bot. J. Linn. Soc. 1: 311–320.

Mayden RL. 1997. A hierarchy of species concepts: the denouement in the saga of the species problem. In Claridge MF, Dawah, HA, Wilson, MR (eds), Species: the units of biodiversity: 381–424. Chapman & Hall, UK.

McDade LA. 1995. Hybridisation and phylogenetics. In: Hoch PC, Stephenson AG (eds), Experimental and molecular approaches to plant biosystematics: 305–331. Missouri Botanical gardens, USA.

McNeill J, Barrie FR, Burdet HM, Demoulin V, Hawksworth DL, Marhold, K, Nicolson DH, Prado J, Silva PC, Skog JE, Wiersema JH, Turland NJ. 2006. International Code of Botanical Nomenclature (Vienna Code) adopted by the Seventeen International Botanical Congress Vienna, Austria, July 2005. Gantner Verlag, Ruggel, Liechtenstein.

Mertens FC, Koch WDJ. 1826. J.C. Röhlings Deutschlands Flora, vol. 2. Friedrich Wilmans, Germany.

Miyaji Y. 1913. Untersuchungen über die chromosomezahlen bei einigen Viola-Arten. Bot. Mag. Tokyo. 27: 443–460.

Moore DM, Harvey MJ. 1961. Cytogenetic relationships of *Viola lactea* SM. and other West European

Arosulate violets. New Phytol. 60: 85–95.

Neilreich A. 1859. Flora von Nieder-Oesterreich, vol. 2. Druck und Verlag von Carl, Austria.

Neuffer B, Auge H, Mesch H, Amarell U, Brandl R. 1999. Spread of violets in polluted pine forests: moprhological and molecular evidence for the ecological importance of interspecific hybridization. Mol. Ecol. 8: 365–377.

Nikitin VV. 1988. On the typification of *Viola montana*. Bot. Žurn. 73: 1536–1542.

Nikitin, VV. 1995. Critical notes on taxonomy and nomenclature of some European species of the section *Trignocarpea* of the genus *Viola*. Bot. Žurn. 80: 84–96.

Nixon KC, Wheeler, QD. 1990. An amplification of the phylogenetic species concept. Cladistics 6: 211–223.

Nordal I, Jonsell B. 1998. A phylogeographic analysis of *Viola rupestris*: three post-glacial migration routes into the Nordic area? Bot. J. Linn. Soc. 128: 105–122.

Nylander JAA. 2004. MrModeltest v2. Evolutionary Biology centre, Uppsala University.

Page RDM, Charleston MA. 1997. From gene to organismal phylogeny: reconciled trees and the gene tree/species tree problem. Mol. Phyl. Evol. 7: 231–240.

Pelser PB, Gravendeel B, van der Meijden R. 2003. Phylogeny reconstruction in the gap between too little and too much divergence: the closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. Mol. Phyl. Evol. 29: 613–628.

Petermann, GL. 1838. Flora Lipsiensis excursoria. sumptibus Joannis Ambrosii Barth, Germany.

Pillon Y, Chase MW. 2007. Taxonomic exaggeration and its effects on Orchid conservation. Conserv. Biol. 21: 263–65.

Popp M, Oxelman B. 2001. Inferring the history of the polyploid *Silene aegaea* (Caryophyllaceae) using plastid and homoeologous nuclear DNA sequences. Mol. Phyl. Evol. 20: 474–481.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Rambaut A, Drummond AJ. 2004. Tracer. Version 1.3. University of Oxford, UK.

Rauschert S. 1973. Zur Nomenklatur der Farn- und Blütenpflanzen Deutschlands (III). Feddes Repert. 83: 645–662.

Redbo-Torstensson P, Berg T. 1995. Seasonal cleistogamy: a conditional strategy to provide reproductive assurance. Acta Bot. Neerl. 44: 247–256.

Reichenbach HGL. 1823. Iconographia botanica seu Plantae criticae. Centuria tabularum prima. Friedrich Hofmeister, Germany.

Reichenbach HGL. 1838–1839. Icones florae germanicae et helveticae, vol. 3. Friedrich Hofmeister, Germany.

Reichenbach HGL. 1839–1840. Deutschlands Flora. Familie der Mohne, Kappern,Veilchen, Cisten und Ranunkeln: Papaveraceae, Capparideae, Violaceae, Cistinae, Ranunculaceae. Mit 300 Abbildungen. Friedrich Hofmeister, Germany.

Reichenbach HGL. 1832. Flora germanica excursoria, vol. 2(2). Carolum Cnobloch, Germany.

Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzback AE, Donovan LA, Lexer C. 2003. Major ecological transitions in wild sunflowers facilitated by hybridization. Science 301: 1211–1216.

Rieseberg LH. 1997. Hybrid origins of plant species. Annu. Rev. Ecol. and Syst. 28: 359–389.

Rohlf FJ. 1997. NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.02k. Exeter Software, USA.

Ronquist F, Huelsenbeck JP, van der Mark P. 2005. MrBayes 3.1 Manual Draft 5/26/2005. School of computational Science Florida State University, USA.

Röpert D. (ed). 2000. Digital specimen images at the Herbarium Berolinense. http://ww2.bgbm.org/herbarium/default.cfm (continuously updated).

Røren V, Stabbetorp O, Borgen L. 1994. Hybridization between *Viola canina* and *V. persicifolia* in Norway. Nord. J. Bot. 14: 165–172.

Roth AW. 1789. Tentamen florae germanicae, vol. 2. I.G. Mülleriano, Germany.

Ruppius, HB. 1726. Flora jenensis, ed. 2. Ernestum Claud, Germany.

Ruppius, HB. 1745. Flora jenensis, ed. 3. Christ. Henr. Cunonis, Germany.

Schlosser JC, Vukotinovic L. 1869. Flora croatica. Fr. Župan (Albrecht et Fiedler), Croatia.

Schenk FM, Thienpont CN, Koopman WJM, Gilissen LJWJ, Smulders MJM. 2008. Phylogenetic relationships in Betula (Betulaceae) base don AFLP markers. Tree Genet. Genomes 4: 911–924.

Schkuhr, Ch. 1803. Botanisches Handbuch, vol. 3. auf Kosten des Verfassers, Germany.

Schreber JCD. 1771. Spicilegium florae lipsicae. Dykiano, Germany.

Schultes JA. 1814. Österreichs Flora, ed. 2(1). C. Schaumburg und Compagnie, Austria.

Schweigger AF. 1804. Specimen florae Erlangensis. Typis Hilpertianis, Germany.

Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, Siripun KC, Winder CT, Schilling EE, Small RL. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J. Bot. 92: 142–166.

Simmons MP, Pickett KM, Miya M. 2004. How meaningful are Bayesian support values? Mol. Biol. Evol. 21: 188–199.

Smedmark JEE, Eriksson T, Bremer B. 2005. Allopolyploid evolution in Geinae (Colurieae: Rosaceae)

– building reticulate species trees from bifurcating gene trees. Org. Divers. Evol.. 5: 275–283.

Smith, JE. 1798. English Botany, vol. 7. J. Davis, UK.

Soltis DE, Albert VA, Leebens-Mack J, Bell CD, Paterson AH, Zheng C, Sankoff D, dePamphilis CW, Wall PK, Soltis PS. 2009. Polyploidy and angiosperm diversification. Am. J. Bot. 96: 336–348.

Soltis PD, Soltis DE. 2000. The role of genetic and genomic attributes in the success of polyploids. Proc. Natl. Acad. Sci. USA. 97: 7051–7057.

Sommer H, Saedler H. 1986. Structure of the chalcone synthase gene of *Antirrhinum majus*. Mol. Gen. Genet. 202: 429–434.

Song K, Lu P, Tank K, Osborn TC. 1995. Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. Proc. Natl. Acad. Sci. USA. 92:7719–7723.

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C. 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol. Biol. 24: 743–755.

Stace C. 1997. New flora of the British Isles, ed. 2. Cambridge University Press, UK.

Stafleu FA, Cowan RS. 1976. Taxonomic literature, ed. 2(1): A-G. Bohn, Scheltema & Holkema, The Netherlands.

Stafleu FA, Cowan RS. 1983. *Taxonomic literature,* ed. 2(4): *P-Sak.* Bohn, Scheltema & Holkema, The Netherlands.

Stebbins GL. 1971. Chromosomal evolution in higher plants. Edward Arnold, UK.

Strand AE, Leebens-mack J, Milligan BG. 1997. Nuclear DNA-based markers for plant evolutionary biology. Mol Ecol. 6: 113–118.

Stuessy TF. 1990. Plant Taxonomy: The Systematic Evaluation of Comparative Data. Columbia University Press, USA.

Swofford DL. 2003. PAUP*: Phylogenetic analysis using parsimony (* and other methods). Version 4.0b10. Sunderland, MA: Sinauer Associates.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann C, Willerslev E. 2007. Power and limitations of chloroplast *trn*L (UUA) intron for plant DNA barcoding. Nucleid Acids Res. 35: e1-e8.

Templeton AR. 1980. The theory of speciation via the founder principle. Genetics 94: 1011–1038.

Tokuoka T. 2008. Molecular phylogenetic analysis of Violaceae (Malpighiales) based on plastid and nuclear DNA sequences. J. Plant Res. 121: 253–260.

Tuskan GA, DiFazio S, Jansson S, et al. (104 co-authors). 2006. The Genome of Black Cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313: 1596–1604.

Valentine DH, Merxmuller H, Schmidt A. 1968. Viola L. In: Tutin TG, Heywood VH, Burges NA,

Moore DM, Valentine DH, Walters SM, Webb DA (eds), Flora Europaea 2: 270–282. Cambridge Press, UK.

Valentine DH. 1950. The experimental taxonomy of two species of *Viola*. New Phytol. 49: 193–212.

Valentine DH. 1958. Cytotaxonomy of the Rostrate violets. Proc. Linn. Soc. London 169: 132–134.

Valentine DH. 1962. Variation and evolution in the genus *Viola*. Preslia 34: 190–206.

van den Hof K, van den Berg RG, Gravendeel B. 2009. Combined analyses of AFLP markers and morphology confirm the taxonomic status of *Viola stagnina* var. *lacteoides*. Mol. Phyl. Evol. submitted.

van den Hof K, Danihelka J, Marcussen T, Jonsell B, van den Berg RG, Gravendeel B. 2009. Proposal to reject the names *Viola montana* and *V. persicifolia* (Violaceae). Taxon: in review.

van den Hof K, van den Berg RG, Gravendeel B. 2008. Chalcone synthase gene lineage diversification confirms allopolyploid evolutionary relationships of European rostrate violets. Mol. Biol. Evol. 25: 2099–2108.

Van der Meijden R. 1983. Heukels' flora van Nederland. Wolters-Noordhoff, The Netherlands.

Van der Meijden R. 1990. Heukels' flora van Nederland. Wolters-Noordhoff, The Netherlands.

Van der Meijden, R. 2005. Heukels' Flora van Nederland. Wolters-Noordhoff, The Netherlands.

Van Oostroom SJ. 1977. Heukels-van Oostroom Flora van Nederland. Wolters-Noordhoff, The Netherlands.

Van Valen L. 1976. Ecological species, multispecies, and oakes, Taxon 25: 233–239

Verlaque R, Espeut M. 2007. IAPT/IOPB chromosome data 3. Taxon 56: 209.

Von Uechtritz R. 1871. Zur Flora Ungarns. Oesterr. Bot. Z. 21: 306–310.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, et al. 1995. AFLP a new technique for DNA fingerprinting. Nucl. Acids Res. 23: 4407–4414.

Wang WK, Schaal BA, Chiou YM, Murakami N, Ge XJ, Huang CC, Chiang TY. 2007. Diverse selective modes among orthologs/paralogs of the chalcone synthase (Chs) gene family of *Arabidopsis thaliana* and its relative *A. halleri* ssp. *gemmifera*. Mol. Phyl. Evol. 44: 503–520.

Weeda EJ. 2001. Melkviooltje (*Viola persicifolia* Schreber) in Nederland in verleden en heden. 1. Variëteiten, voorkomen, standplaats en plantensociologische positie. Stratiotes 23: 73–103.

Weeda EJ. 2002. Melkviooltje (*Viola persicifolia* Schreber) in Nederland in verleden en heden. 2. Vergelijking met gegevens van elders en een historische interpretatie. Stratiotes 24: 3–12.

Wendel JF, Schnabel A, Seelanan T. 1995. Bi-directional interlocus concerted evolution following allopolyploid speciation in cotton (Gossypium). Proc. Natl. Acad. Sci., USA. 92: 280–284.

Wiley EO. 1978. The evolutionary species concept reconsidered. Syst. Zool. 27: 17–26.

Willdenow CL. 1798. Species plantarum, vol. 1(2). impensis G.C. Nauk, Switzerland.

Wilmott AJ. 1916. What is *Viola montana* L.? J. Bot. 54: 257–262.

Wood TE, Takebayashic N, Barker MS, Mayrosee I, Greenspoond PB, Rieseberg LH. 2009. The frequency of polyploid speciation in vascular plants. Proc. Natl. Acad. Sci., USA. 106: 13875–13879.

Yamazaki Y, Suh D-Y, Sitthithaworn W, Ishiguro K, Kobayashi Y, Shibuya M, Ebizuka Y, Sankawa U. 2001. Diverse Chalcone synthase superfamily enzymes from the most primitive vascular plant, *Psilotum nudum*. Planta 214: 75–84.

Yang J, Gu H. 2006. Duplication and divergent evolution of the CHS and CHS like genes in the Chalcone synthase (CHS) superfamily. Chinese Sci. Bull. 51: 505–509.

Yoo KO, Jang SK, Lee WT. 2005. Phylogeny of Korean *Viola* based on ITS sequences. Korean J. Plant Taxon 35: 7–23.

Yousheng C, Qiner Z, Ohba H, Nikitin VV. 2007. *Viola* Linnaeus, Sp. Pl. 2: 933. 1753. In: Zhengyi W, Raven, PH (eds.), Flora of China vol. 13: 74–111. Science Press, Beijing & Missouri Botanical Garden Press, USA.

Zwaenepoel A, Vanallemeersch L. 2007. *Viola persicifolia* na 127 jaar opnieuw opgedoken in Belgie. Dumortiera 91: 1–6.

Appendix I

Voucher information of taxa sampled in Chapter 2

Species	Voucher	Locality	GenBank accession numbers		
			CHS intron	CHS exon II	*trnS-trnG* intron + spacer
Allexis batangae Melch.	Bos 4241	Cameroon			EU356072
Cubelium concolor Raf.	Plack & Bodin s.n.	Missouri, Jasper Co.			EU358071
V. acuminata Ledeb.	Marcussen 101	Cultivated, Russia	EU311428 (1) EU311487 (2)		EU311508
V. alba Bess.	van den Hof 210	Montserrat, Spain	EU311433 (1) EU311460 (2)		EU311522
V. banksii K.R.Thiele & Prober	Marcussen 603	Sydney, Australia	EU311453 (2a) EU311454 (2b)		EU311502
V. biflora L.	Wieringa 5713	Trentino, Italy	EU311455 (2)	EU295681	EU311528
V. canina L.	van den Hof 031	Svensrud, Norway	EU311439 (1) EU311472 (2a) EU311478 (2b) EU311493 (3)	EU295667 EU295674 EU295678 EU295685	EU311515
V. caspia (rupr.) Freyn	Marcussen 222	Quba, Azerbaijan	EU311446 (1) EU311466 (2a) EU311490 (2b) EU311495 (3)		EU311523
V. cornuta L.	Gravendeel 3066	Cipieres, France			EU311531
V. elatior Fr.	Hepper 8989	Alpes-Maritimes, France	EU311441 (1) EU311476 (2)		EU311516
V. grayii Franch. & Sav.	Marcussen 785	St.-Dalmas-de-Tende, France	EU311429 (1) EU311484 (2) EU311494 (3)	EU295676 EU295677 EU295690	EU311520
V. jordanii Hanry	Marcussen 303	Alpes-Maritimes, France	EU311436 (1) EU311462 (2a) EU311467 (2b)		EU311512
V. lactea Sm.	van den Hof	Cultivated, Netherlands	EU 311440 (1) EU311473 (2a) EU311475 (2b) EU311477 (2c) EU311492 (3a)		EU311530
V. mirabilis L.	van den Hof 145	Cultivated, Netherlands	EU311469 (2) EU311497 (3)	EU295672	EU311529
V. odorata L.	van den Hof 155	Cultivated, Netherlands	EU311432 (1) EU311458 (2)		EU311519
V. oligyrtia Tiniakou	Marcussen 337	Argolída, Greece	EU311430 (1) EU311485 (2)		EU311509
V. ovato-oblonga makino	Marcussen 602	Origin unknown	EU311431 (1) EU311486 (2)		EU311521
V. pedatifida G. Don	van den Hof 158	Cultivated, Netherlands			EU311505
V. pseudo-mirabilis Coste	van den Hof 209	Cavalier, France	EU311448 (1) EU311482 (2) EU311498 (3a) EU311500 (3b)	EU295670 EU295673 EU295689 EU295693	EU311507

Species	Voucher	Locality	GenBank accession numbers		
			CHS intron	CHS exon II	*trnS-trnG* intron + spacer
V. pubescens Aiton	Pelser	Oxford, Ohio, USA	EU311456 (2)	EU295680 EU295682	EU311503
V. pumila Chaix	van den Hof 136	Öland, Sweden	EU311444 (1) EU311474 (2a) EU311480 (2b)		EU311514
V. reichenbachiana Jord. Ex Bor	Chase 16402	Origin unknown	EU311435 (1) EU311488 (2)	EU295694	EU311526
V. riviniana Rchb.	van den Hof 198	Polder d'Erstein, France	EU311449 (1) EU311470 (2a) EU311491 (2b)	EU295687	EU311527
V. riviniana Rchb. f. *purpurea*	van den Hof 157	Cultivated, Netherlands	EU311445 (1) EU311471 (2) EU311496 (3)	EU295688 EU295691 EU295692	EU311525
V. rosulata Poepp. & Endl.	Marcussen 643	Origin unknown			EU311501
V. rupestris F.W. Schmidt	van den Hof 156	Oostvoorne, Netherlands	EU311437 (1) EU311465 (2a) EU311483 (2b)		EU311517
V. sieheana W. Becker	Marcussen 326	Messinía, Greece	EU311447 (1) EU311463 (2a) EU311464 (2b)		EU311513
V. sororia Willd.	Pelser	Oxford, Ohio, USA	EU311459 (2)		EU311504
V. stagnina Kit.	van den Hof 129	Brændemose, Denmark	EU311442 (1) EU311481 (2)	EU295665 EU295683	EU311510
V. stagnina var. lacteaeoides W. Becker & Kloos	van den Hof 005	Boetelerveld, Netherlands	EU311443 (1) EU311479 (2)		EU311511
V. suavis M. Bieb.	van den Hof 207	Mende, France	EU311434 (1) EU311457 (2)		EU311518
V. uliginosa Bess.	Marcussen 662	Gästrikland, Sweden	EU311450 (1) EU311461 (2)	EU295671 EU295686	EU311524
V. willkommii De Roem ex Willk.	van den Hof 212	Montserrat, Spain	EU311438 (1) EU311468 (2a) EU311489 (2b) EU311499 (3)	EU295668 EU295669 EU295675 EU295679 EU295684	EU311506

List of species and accessions used for AFLP analyses in Chapter 5

species	Accession nr.	Voucher nr.	Locality
Viola canina[1]	32	van den Hof 32	Bönsnes, Norway
Viola canina[1]	142	van den Hof 142	Lisjöberg, Sweden
Viola canina[1]	143	van den Hof 143	Lisjöberg, Sweden
Viola canina	179	van den Hof 179	Polder de Dulf, The Netherlands
Viola canina[1]	232	van den Hof 232	Frankfurt, Germany
Viola canina	255	van den Hof 255	Moerputten, The Netherlands
Viola canina[2]	256	van den Hof 256	Moerputten, The Netherlands
Viola canina	260	van den Hof 260	Moerputten, The Netherlands
Viola canina[2]	266	van den Hof 266	Emsterbroek, The Netherlands
Viola canina[2]	269	van den Hof 269	Emsterbroek, The Netherlands
Viola canina[2]	274	van den Hof 274	Kienveen, The Netherlands
Viola canina[2]	286	van den Hof 286	Polder de Dulf, The Netherlands
Viola canina[2]	289	van den Hof 289	Polder de Dulf, The Netherlands
Viola elatior[1]	240	van den Hof 240	Frankfurt, Germany
Viola elatior[2]	298	van den Hof 298	Moravia, Czech Republic
Viola elatior[1]	306	Danihelka DA07/004 BRNU	Moravia, Czech Republic
Viola elatior[1]	307	Danihelka DA07/0325 BRNU	Russia
Viola elatior[2]	310	J. Barth. s.n. (1874)	Transylvania
Viola elatior[2]	311	H. Calliér s.n. (1890)	Breslau, Poland
Viola elatior[2]	312	Haynald 2855	Szent-Benedek, Hungary
Viola pumila[1]	135	van den Hof 135	Öland, Sweden
Viola pumila	136	van den Hof 136	Öland, Sweden
Viola pumila[1]	137	van den Hof 137	Öland, Sweden
Viola pumila	139	van den Hof 139	Öland, Sweden
Viola pumila[1]	238	van den Hof 238	Frankfurt, Germany
Viola pumila[1]	242	van den Hof 242	Frankfurt, Germany
Viola pumila[1]	243	van den Hof 243	Frankfurt, Germany
Viola pumila[1]	300	van den Hof 300	Moravia, Czech Republic
Viola pumila	303	Danihelka DA07/005 BRNU	Moravia, Czech Republic
Viola pumila	304	Danihelka DA07/007 BRNU	Moravia, Czech Republic
Viola pumila[1]	309	Danihelka DA07/0326 BRNU	Russia
Viola pumila[2]	313	Würschmidt s.n. (1849)	Spire, France
Viola pumila[2]	314	Wilh. Becker s.n. (1921)	Magdeburg, Germany
Viola pumila[2]	315	A. Nicolsen s.n.	Unknown
Viola pumila[2]	316	Velenovsky s.n. (1887)	Unknown
Viola pumila[2]	317	9082681149	Speyer, Germany
Viola riviniana	198	van den Hof 198	Polder d'Erstein, France
Viola riviniana[1]	201	van den Hof 201	Meroz, France
Viola riviniana[1]	202	van den Hof 202	Meroz, France
Viola riviniana[1]	203	van den Hof 203	Meroz, France
Viola stagnina var. *lacteoides*[1]	89	van den Hof 089	Boetelerveld, The Netherlands
Viola stagnina var. *Lacteoides*[1]	94	van den Hof 094	Stelkampsveld, The Netherlands
Viola stagnina var. *Lacteoides*[2]	263	van den Hof 263	Emsterbroek, The Netherlands
Viola stagnina var. *Lacteoides*	264	van den Hof 264	Emsterbroek, The Netherlands
Viola stagnina var. *Lacteoides*	265	van den Hof 265	Emsterbroek, The Netherlands
Viola stagnina var. *Lacteoides*	267	van den Hof 267	Emsterbroek, The Netherlands
Viola stagnina var. *Lacteoides*	268	van den Hof 268	Emsterbroek, The Netherlands
Viola stagnina var. *Lacteoides*	270	van den Hof 270	Kienveen, The Netherlands

species	Accession nr.	Voucher nr.	Locality
Viola stagnina var. *Lacteoides*	271	van den Hof 271	Kienveen, The Netherlands
Viola stagnina var. *Lacteoides*	272	van den Hof 272	Kienveen, The Netherlands
Viola stagnina var. *Lacteoides*	273	van den Hof 273	Kienveen, The Netherlands
Viola stagnina var. *Lacteoides*[2]	275	van den Hof 275	Kienveen, The Netherlands
Viola stagnina var. *Lacteoides*[2]	276	van den Hof 276	Achterveld, The Netherlands
Viola stagnina var. *Lacteoides*	277	van den Hof 277	Achterveld, The Netherlands
Viola stagnina var. *Lacteoides*	278	van den Hof 278	Achterveld, The Netherlands
Viola stagnina var. *Lacteoides*	279	van den Hof 279	Achterveld, The Netherlands
Viola stagnina var. *Lacteoides*[2]	280	van den Hof 280	Achterveld, The Netherlands
Viola stagnina var. *Lacteoides*	283	van den Hof 283	Koolmansdijk, The Netherlands
Viola stagnina var. *stagnina*[1]	34	van den Hof 34	Bönsnes, Norway
Viola stagnina var. *stagnina*[1]	35	van den Hof 35	Bönsnes, Norway
Viola stagnina var. *stagnina*[1]	43	van den Hof 43	Bönsnes, Norway
Viola stagnina var. *stagnina*[1]	47	van den Hof 47	Åsa, Norway
Viola stagnina var. *stagnina*[1]	51	van den Hof 51	Åsa, Norway
Viola stagnina var. *stagnina*[1]	63	van den Hof 63	Ringstad, Norway
Viola stagnina var. *stagnina*[1]	64	van den Hof 64	Ringstad, Norway
Viola stagnina var. *stagnina*[1]	90	van den Hof 90	Bennekomse Hooilanden, The Netherlands
Viola stagnina var. *stagnina*[1]	92	van den Hof 92	Bennekomse Hooilanden, The Netherlands
Viola stagnina var. *stagnina*[1]	93	van den Hof 93	Bennekomse Hooilanden, The Netherlands
Viola stagnina var. *stagnina*[1]	96	van den Hof 96	Polder de Dulf, The Netherlands
Viola stagnina var. *stagnina*[1]	103	van den Hof 103	Sluis, The Netherlands
Viola stagnina var. *stagnina*[1]	104	van den Hof 104	Sluis, The Netherlands
Viola stagnina var. *stagnina*[1]	107	van den Hof 107	Zijdebrug, The Netherlands
Viola stagnina var. *Stagnina*	108	van den Hof 108	Zijdebrug, The Netherlands
Viola stagnina var. *stagnina*[1]	117	van den Hof 117	Bremen, Germany
Viola stagnina var. *Stagnina*[2]	123	van den Hof 123	Bremen, Germany
Viola stagnina var. *Stagnina*	127	van den Hof 127	Brændemose, Denmark
Viola stagnina var. *Stagnina*	128	van den Hof 128	Brændemose, Denmark
Viola stagnina var. *Stagnina*	129	van den Hof 129	Brændemose, Denmark
Viola stagnina var. *stagnina*[1]	146	van den Hof 146	Sweden
Viola stagnina var. *stagnina*[1]	147	van den Hof 147	Sweden
Viola stagnina var. *Stagnina*	149	van den Hof 149	Aetsa, Finland
Viola stagnina var. *Stagnina*	150	van den Hof 150	Aetsa, Finland
Viola stagnina var. *Stagnina*	178	van den Hof 178	Polder de Dulf, The Netherlands
Viola stagnina var. *stagnina*[2]	182	van den Hof 182	Polder de Dulf, The Netherlands
Viola stagnina var. *Stagnina*	183	van den Hof 183	Polder de Dulf, The Netherlands
Viola stagnina var. *Stagnina*	184	van den Hof 184	Bennekomse Hooilanden, The Netherlands
Viola stagnina var. *Stagnina*	185	van den Hof 185	Bennekomse Hooilanden, The Netherlands
Viola stagnina var. *Stagnina*	186	van den Hof 186	Bennekomse Hooilanden, The Netherlands
Viola stagnina var. *stagnina*[1]	188	van den Hof 188	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	192	van den Hof 192	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	193	van den Hof 193	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	214	van den Hof 214	Etangs de la Puisaye, France

species	Accession nr.	Voucher nr.	Locality
Viola stagnina var. *Stagnina*	216	van den Hof 216	Etangs de la Puisaye, France
Viola stagnina var. *Stagnina*	217	van den Hof 217	Etangs de la Puisaye, France
Viola stagnina var. *Stagnina*[1]	222	van den Hof 222	Frankfurt, Germany
Viola stagnina var. *Stagnina*[1]	227	van den Hof 227	Frankfurt,Germany
Viola stagnina var. *Stagnina*[1]	229	van den Hof 229	Frankfurt,Germany
Viola stagnina var. *Stagnina*[1]	234	van den Hof 234	Frankfurt,Germany
Viola stagnina var. *Stagnina*[1]	235	van den Hof 235	Frankfurt,Germany
Viola stagnina var. *Stagnina*[1]	244	van den Hof 244	Frankfurt,Germany
Viola stagnina var. *Stagnina*[1]	245	van den Hof 245	Frankfurt,Germany
Viola stagnina var. *Stagnina*[1]	246	van den Hof 246	Frankfurt,Germany
Viola stagnina var. *stagnina*[2]	257	van den Hof 257	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	258	van den Hof 258	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	259	van den Hof 259	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	261	van den Hof 261	Moerputten, The Netherlands
Viola stagnina var. *Stagnina*	262	van den Hof 262	Moerputten, The Netherlands
Viola stagnina var. *stagnina*[2]	282	van den Hof 282	Koolmansdijk, The Netherlands
Viola stagnina var. *stagnina*[2]	285	van den Hof 285	Polder de Dulf, The Netherlands
Viola stagnina var. *stagnina*	290	van den Hof 290	Polder de Dulf, The Netherlands
Viola stagnina var. *stagnina*[2]	291	van den Hof 291	Polder de Dulf, The Netherlands
Viola stagnina var. *stagnina*[2]	299	van den Hof 299	Czech Republic
Viola stagnina var. *stagnina*[2]	301	Danihelka DA07/003 BRNU	Czech Republic
Viola stagnina var. *stagnina*	302	Danihelka DA07/003 BRNU	Czech Republic
Viola stagnina var. *stagnina*[2]	318	J. Kern 5552	Gorkum, The Netherlands
Viola stagnina var. *stagnina*[2]	319	M. Boinski & W. Gugnacka 100	Lake Wójcinskie, Poland
Viola stagnina var. *stagnina*[2]	320	Bachmann s.n.	Breslau, Poland
Viola stagnina var. *stagnina*[2]	321	B. Jonsell 8170 UPS	Lake Ukzhezero, Russia
Viola stagnina var. *stagnina*[2]	322	N. Lunqvist 1102 UPS	Lake Vänern, Sweden
Viola stagnina var. *stagnina*[2]	323	O.J. Hasslow 1178	Kristianstad, Sweden
Viola stagnina var. *stagnina*[2]	324	H. Calliér 782	Bunzlau, Poland
Viola stagnina var. *stagnina*[2]	325	N.Y. Sandwith 182	Woodwalton fen, Great Britain
Viola x *ritschliana*	194	van den Hof 194	Moerputten, The Netherlands
Viola x *ritschliana*	195	van den Hof 195	Moerputten, The Netherlands
Viola x *ritschliana*[2]	287	van den Hof 287	Polder de Dulf, The Netherlands
Viola x *ritschliana*	288	van den Hof 288	Polder de Dulf, The Netherlands

All specimens are deposited at L, unless stated otherwise.

[1]Only used for AFLP dataset
[2]Only used for morphological dataset

Appendix III

List of characters used for morphometric analyses in Chapter 5

Reproductive characters	*Character type*
Flower color	0: White 1: Pale blue 2: Blue
Spur length/ventral petal length ratio	Morphometric
Dorsal petal ratio length width	Morphometric
Lateral petal ratio length width	Morphometric
Ventral petal ratio length width	Morphometric
Sepal length	Morphometric
Sepal length/width ratio	Morphometric
Sepal length /sepal appendage length ratio	Morphometric
Upper bract length	Morphometric

Vegetative characters	*Character type*
Plant height	Morphometric
Lamina length	Morphometric
Lamina length/width ratio	Morphometric
Lamina length/petiole length ratio	Morphometric
Petiole/stipule ratio	Morphometric
Leafbase shape	0: Atenuate 1: Truncate 2: Cordate

Kevin van den Hof werd geboren op 15 mei 1980 in Geleen. Na het behalen van zijn Atheneum diploma in juni 1998 aan het Bisschoppelijk College Sittard, ging hij Biologie studeren aan de Universiteit Leiden. Op 25 augustus 2000 behaalde hij zijn propedeuse. Tijdens de doctoraal fase van zijn studie raakte hij steeds meer geïnteresseerd in de systematiek en evolutiebiologie. Een stage aan de Leidse vestiging van het Nationaal Herbarium Nederland (NHN-Leiden) was dan ook een logische stap. Onder begeleiding van dr. Pieter Pelser en dr. Ruud van der Meijden deed hij onderzoek naar de morfologie van Jakobskruiskruid en aanverwante soorten. Na het afronden van zijn stage aan het NHN-Leiden bleef Kevin werken aan Jakobskruiskruid. In Zwitserland bij het Institut für Umweltwissenschaften deed hij onder begeleiding van dr. Jasmin Joshi en dr. Klaas Vrieling onderzoek naar de genetische variatie in Jakobskruiskruid populaties van zowel inheemse als exotische origine. Dit onderzoek werd gefinancierd door het Funke Fonds voor Experimentele Plantwetenschappen en een Erasmus beurs. Op 28 oktober 2003 behaalde hij het doctoraal examen in de Biologie.

Op 2 augustus 2004 begon hij als promovendus bij het NHN-Leiden aan zijn promotieonderzoek naar de taxonomie en fylogenie van het Melkviooltje en verwante soorten. Prof. dr. Pieter Baas was destijds zijn promotor en dr. Barbara Gravendeel en dr. Ruud van der Meijden waren zijn co-promotoren. Door de pensionering van prof. dr. Pieter Baas en het overlijden van dr. Ruud van der Meijden zijn prof. dr. Erik Smets en dr. Ronald van den Berg als respectievelijk promotor en co-promotor in het onderzoek gestapt.

Tijdens zijn aanstelling heeft Kevin aan diverse internationale symposia deelgenomen. In 2005 heeft hij het International Botanical Congres in Wenen bijgewoond en in 2007 heeft hij zijn onderzoeksresultaten gepresenteerd te Edinburgh op de Biennal Meeting van de Systematics Association.

Tijdens zijn aanstelling is hij ook diverse malen betrokken geweest bij het onderwijs dat door het NHN-Leiden verzorgd wordt voor biologiestudenten van de Universiteit Leiden. Hij heeft tweemaal de cursus Biodiversiteit en Patroonanalyse geassisteerd in 2004 en 2007 en driemaal heeft hij de tweedejaars flora excursie in Limburg mogen assisteren. Daarnaast was hij verantwoordelijk voor de begeleiding van masterstudente Živa Fišer, tijdens haar stage aan mogelijk invasieve *Aronia* soorten. In januari 2009 heeft Kevin de Professor Lam prijs toegekend gekregen voor het artikel "CHS Gene Lineage Diversification confirms Allopolyploid Evolutionary Relationships of European Rostrate Violets" (hoofdstuk 2 van dit proefschrift).

Sinds mei 2009 is Kevin werkzaam als octrooideskundige voor het plantenveredelingsbedrijf Rijk Zwaan B.V.

Publicaties

Danihelka J, van den Hof K, Marcussen T, Jonsell B. *Viola montana* and *V. persicifolia* (Violaceae): two names to be rejected. Taxon (In review).

Doorduin LJ, van den Hof K, Vrieling K, Joshi J. 2010. The lack of genetic bottleneck in invasive Tansy ragwort populations suggests multiple source populations. Basic Appl. Ecol. 11/3: 244-250.

Pelser PB, van den Hof K, Gravendeel B, van der Meijden R. 2004. The systematic value of morphological characters in Senecio sect. Jacobaea (Asteraceae) as compared to DNA sequences. Syst. Bot. 29: 790-805.

van den Hof K, van den Berg RG, Gravendeel B. 2008. Chalcone Synthase Gene Lineage Diversification confirms Allopolyploid Evolutionary Relationships of European Rostrate Violets. Mol. Biol. Evol. 25: 2099-2108.

van den Hof K. 2008. Polyploïdie in Viooltje (*Viola*, L.). Gorteria 33: 149-155.

van den Hof K, Danihelka J, Marcussen T, Jonsell B, van den Berg RG, Gravendeel B. Proposal to reject the names *Viola montana* and *V. persicifolia* (Violaceae). Taxon (In review).

van den Hof K, van den Berg RG, Gravendeel B. Combined analyses of AFLP markers and morphology confirm the taxonomic status of *Viola stagnina* var. *lacteoides*. Conserv. Genet. (Submitted).

van den Hof K, Marcussen T, van den Berg RG, Gravendeel B. 2009. Phenotypic plasticity of *Viola stagnina*. Plant Ecol. Evol. (Submitted).

De resultaten van mijn onderzoek, beschreven in dit proefschrift, zijn natuurlijk niet het resultaat van alleen mijn inspanningen. Gedurende de afgelopen vierenhalf jaar heb ik bijzonder veel kunnen leren van de ervaring en expertise van mijn collega onderzoekers in binnen- en buitenland. Zonder de bijzondere hulp van deze mensen was dit boekje er niet gekomen en daarom wil ik hier mijn dank uitspreken aan een ieder die heeft bijgedragen aan de totstandkoming van dit proefschrift.

In 2004 ben ik begonnen aan dit promotie onderzoek met Ruud van der Meijden en Barbara Gravendeel als mijn directe begeleiders. Helaas werd Ruud halverwege het project ziek en overleed hij in 2007. Ik vind het bijzonder spijtig dat Ruud niet kan meemaken dat ook zijn laatste promovendus zijn project tot een goed einde weet te brengen. Toen ik in 2001 aan mijn doctoraal stage begon op het NHN-Leiden bij Pieter Pelser was ik vooral geïnteresseerd in de evolutionaire processen die leiden tot de vormenrijkdom om ons heen. Voor de vormenrijkdom zelf had ik eigenlijk geen oog. Plantjes, beestjes, schimmels, bacteriën, het maakte me niet uit, als ik maar de evolutionaire processen kon bestuderen en beschrijven die verantwoordelijk waren voor de diversiteit binnen een groep organismen. Ruud wist altijd enthousiast zijn kennis van planten over te brengen op anderen, ook op mij. Samen met Pieter heeft hij in mij de interesse voor de botanie weten aan te wakkeren. Tijdens het schrijven van mijn proefschrift heb ik Ruud dan ook vaak gemist. Vooral tijdens het schrijven van de AFLP en nomenclatuur hoofdstukken had ik graag nog naar zijn mening en advies willen vragen. Maar ik heb Ruud niet alleen gemist om zijn kennis van planten. Ook zijn aanstekelijke, drukke borrelpraat en blufpoker kunsten tijdens flora excursies in Limburg heb ik de tweede helft van mijn promotie gemist. Ruud is uiteindelijk van geen van de artikelen in dit proefschrift mede-auteur. Zonder hem was er echter weinig van deze artikelen terecht gekomen. Zijn kennis, ervaring en connecties waren van onschatbare waarde voor de totstandkoming van dit proefschrift.

Na het overlijden van Ruud heb ik vooral met de hulp van Barbara het project

weer op de rails weten te krijgen. Je vastberadenheid en eindeloze geduld hebben ervoor gezorgd dat de resultaten uit het lab hun weg hebben weten te vinden richting het papier. Bovendien wist je een zeer geschikte plaatsvervanger voor Ruud te regelen: Ronald van den Berg. Ronald, je kritische blik als buitenstaander en je ervaring met AFLPs kwamen zeer goed van pas.

De Viooltjes in Nederland, met name het melkviooltje, heb ik ook leren kennen met de hulp van Hanneke den Held en Eddy Weeda. Jullie kennis en ervaring waren zeer waardevol voor dit proefschrift.

In het lab heb ik altijd kunnen rekenen op de voortreffelijk hulp van Bertie Joan van Heuven, Marcel Eurlings en René Glas wanneer PCRs het weer eens niet wilden doen, of wanneer ik SEM foto's nodig had van stampers en stijlen. De AFLPs zijn er gekomen met de hulp van Ria Vrielink-van Ginkel en Nynke Groendijk-Wilders. Heel erg bedankt voor het samen turen naar al die 'vage' bandjes.

De illustraties zijn niet van eigen hand. Esmee Winkels verzorgde de prachtige tekeningen in dit proefschrift.

Met veel geduld hebben Leni Duistermaat, Wout Holverda, René van Moorsel en Hans Kruijer me verder wegwijs gemaakt in de Nederlandse flora tijdens excursies maar ook tijdens reuze gezellige 'werkbesprekingen' die ik zeker zal gaan missen!

I would also like to thank my collaborators from abroad, Thomas Marcussen and Jiří Danihelka. You both contributed a lot to this thesis. The manuscripts benefited a lot from your expertise on nomenclature and *Viola*, and would not have been the same without it. Also the sendings of *Viola* material during the past four and half years are much appreciated!

Besides all the people who were directly involved during my research, I have of course learnt a lot from my fellow PhD students and colleagues at the lab, and shared and enjoyed many experiences with them. Christian, Delia, Kanchana, Kristo, Niels, Natasha, Pieter, and Živa thank you for the wonderful times!

Tot slot natuurlijk een dankwoord aan mijn vrienden en familie voor hun steun de afgelopen jaren. Gerjo, bedankt voor het lenen van je auto. Dit heeft het veldwerk een stuk gemakkelijker gemaakt. Katja en Jan, heel erg bedankt dat jullie bereid zijn om als paranimf op te treden. De afgelopen vierenhalf jaar zijn uiteraard ook de steun en goede zorgen van mijn ouders Richard en Annie, mijn broer Amir, en mijn vriendin Sachar, van onschatbare waarde geweest. Zonder jullie had ik dit proefschrift nooit kunnen schrijven.