# Development of Microsatellite Primers in the Protected Species Viola elatior (violaceae) Using Next-Generation Sequencing

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Source: Applications in Plant Sciences, 3(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500011

Applications in Plant Sciences 2015 3(5): 1500011

PRIMER NOTE

# DEVELOPMENT OF MICROSATELLITE PRIMERS IN THE PROTECTED SPECIES *VIOLA ELATIOR* (VIOLACEAE) USING NEXT-GENERATION SEQUENCING[1]

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- *Premise of the study: Viola elatior* (Violaceae) is a Eurasian perennial plant species in which French populations are threatened by anthropogenic pressures. Microsatellite primers were developed to investigate its genetic structure and diversity.
- *Methods and Results:* Eight microsatellite markers were isolated using next-generation sequencing. Loci were amplified and screened for 138 individuals in 17 populations from France. Two of the eight polymorphic loci presented no variability across populations. The total number of alleles per locus varied from two to four. Observed heterozygosity ranged from 0.051 to 1.000. All primers amplified successfully in the closely related species *V. pumila*.
- *Conclusions:* This set of microsatellites offers a valuable tool for assessing population genetic diversity of the species to improve its conservation and base management efforts. High observed heterozygosity values probably reflect the particular mating system of the species and suggest an important tendency to clonality.

**Key words:** conservation; microsatellites; next-generation sequencing; population genetics; *Viola elatior*; Violaceae.

*Viola elatior* Fr. (Violaceae) is a perennial plant species that is found in large alkaline floodplains in continental climates in Eurasia (Eckstein et al., 2006). The species has both chasmogamous and cleistogamous flowers, with chasmogamous flowers opening first in April–May, allowing cross pollination. Later, in June–July, plants develop cleistogamous flowers, leading to self-pollination (Eckstein et al., 2006). In France, populations are found only in the eastern half of the country, mainly in floodplains subject to large groundwater variations.

French populations are threatened by anthropogenic pressures (agricultural practices, economic development of territories, regulation of river flows, and water regime modification of alluvial plains). Despite a wide geographic range, these populations are fragmented due to their specific ecology, hence *V. elatior* is considered in Ile-de-France to be a rare and vulnerable species (Auvert et al., 2011).

To characterize the genetic structure of the French populations of *V. elatior* and to quantify gene flow among them, we developed a set of variable microsatellite markers that are the first reported for *V. elatior*. These loci will be valuable as part of a conservation program aimed at identifying and strengthening connectivity between these populations. Their use can be extended to another closely related species, *V. pumila*, for which amplification was carried out successfully.

[1]Manuscript received 4 February 2015; revision accepted 9 March 2015.

The authors thank Olivier Bardet (Conservatoire Botanique National du Bassin Parisien [CBNBP]), Frédéric Hendoux (CBNBP), and Yorick Ferrez (Conservatoire Botanique National du Franche-Compté [CBNFC]) for their help during plant sampling in Saône, Marne, and Marais-de-Saône. This research was funded by GRTgaz Val-de-Seine and Conseil Régional d'Ile-de-France.

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doi:10.3732/apps.1500011

## METHODS AND RESULTS

Microsatellite markers were isolated by following a high-throughput genomic sequencing approach developed by Abdelkrim et al. (2009). Genomic DNA used to isolate the microsatellite loci was extracted from a single individual of *V. elatior* (V2-18; Appendix 1), utilizing the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to a protocol for herbarium specimens. Genomic shotgun sequencing was conducted using an Ion Torrent Personal Genome Machine (PGM) System with a Sequencing 400 Kit (Life Technologies, Saint Aubin, France). First, a single-stranded DNA library was constructed using physical fragmentation of gDNA with the Bioruptor Sonication System (Diagenode, Seraing, Belgium). Then, an emulsion PCR was performed to enrich the library and, finally, amplified fragments were sequenced. Shotgun sequencing generated 17,340 random sequences. These reads were converted into a FASTA format file and screened for the presence of microsatellites using MSATCOMMANDER version 1.0.8-beta (Faircloth, 2008). A search was performed for di-, tri-, and tetranucleotides with a minimum of six, six, and five repeats, respectively, and a minimum product size of 80 bp. Primers were designed using Primer3 (Rozen and Skaletsky, 1999) as implemented in MSATCOMMANDER. The minimum primer annealing temperature was set to 55°C, primer size was between 18–22 bp with an optimal size of 20 bp, and other settings were left at default values.

Under these conditions, a total of 75 microsatellite loci were found (53 dinucleotides, 17 trinucleotides, and five tetranucleotides), and primers were designed successfully for 32 of them (22 dinucleotides and 10 trinucleotides). Among them, loci that contained repeats of (AT) bases were discarded, while loci larger than 100 bp were preferentially selected. At the end of selection, 17 loci were retained for the following analyses of polymorphism.

 

TABLE 1. Characteristics of 15 microsatellite primers isolated from *Viola elatior* used for amplification of seven monomorphic and eight polymorphic loci. Loci are available in GenBank in the Sequence Read Archive database under accession number SRP055804.

| Locus | Primer sequences (5′–3′) | Repeat motif | Fluorescent dye[a] | Allele size range (bp) | $T_a$ (°C) | GenBank accession no. |
|---|---|---|---|---|---|---|
| Ve2* | F: AGTTAAGTCCTCCCTGCTGG | $(AC)_{10}$ | NED | 256–270 | 59 | Pr032286348 |
| | R: AGCATATGTTGTTTCACCTTGC | | | | | |
| Ve3 | F: TCATAGGTTGAGTTTGGGTAC | $(AAT)_{16}$ | VIC | 319 | 55 | Pr032302549 |
| | R: ACCCAGCTTCCATTCATATG | | | | | |
| Ve4 | F: CTCACGCCCAGATTCAAACC | $(AAG)_6$ | PET | 330 | 59 | Pr032302550 |
| | R: ATCTGGGCGTGATGGAGATC | | | | | |
| Ve5* | F: GGCTGTGCTTGCTTTGTCTC | $(AG)_7$ | PET | 224–228 | 59 | Pr032286352 |
| | R: GTCCAAGCACACGATGAAGG | | | | | |
| Ve9 | F: TGGTGGCCACTCGTTAGAAC | $(AG)_6$ | NED | 229 | 59 | Pr032302551 |
| | R: ATCTTGCTGCCATGGTTGAC | | | | | |
| Ve10* | F: AGAAAGACGATGCAAGACGG | $(AG)_6$ | FAM | 223–259 | 58 | Pr032286345 |
| | R: TTTAGCTTCCAACGACACGC | | | | | |
| Ve11 | F: ATGATGTAGCTGAGCCAAGG | $(AAT)_6$ | PET | 228 | 59 | Pr032302545 |
| | R: TGGGCATTGTAACTGAAGCTG | | | | | |
| Ve12* | F: CAGCTAAAGATTGAAGCAGCAG | $(AAG)_9$ | PET | 127–139 | 59 | Pr032286346 |
| | R: CATGATCCCAAGCATTCTCC | | | | | |
| Ve13* | F: CAGAGAAGCCACAAACCAGTC | $(AG)_6$ | NED | 314–322 | 59 | Pr032286347 |
| | R: GCCTCACTCTTCACTATTGTTC | | | | | |
| Ve14 | F: TCATGCCCTTTATTTCCGAGTG | $(AG)_6$ | VIC | 206 | 55 | Pr032302546 |
| | R: AGAAGGGAATGTCATGGCTTG | | | | | |
| Ve21 | F: CAACCGGAGACTACAAGCCC | $(AAT)_6$ | VIC | 172 | 58 | Pr032302547 |
| | R: GCGTGTATAAATGCGTGG | | | | | |
| Ve22 | F: GGAGGAGGGAATAGGACAGC | $(AAT)_{11}$ | VIC | 145 | 58 | Pr032302548 |
| | R: ACAGGAATGTTTGAGGTTGCC | | | | | |
| Ve23* | F: AACAAGACTAACGGCAGCAG | $(AG)_6$ | FAM | 216–234 | 58 | Pr032286349 |
| | R: TTTCGTGATTGTGCAGCTCC | | | | | |
| Ve24* | F: AGGGTTTAGGTTTAGAAGACG | $(AAG)_6$ | VIC | 127–130 | 58 | Pr032286350 |
| | R: AGGCCCAAGACATAGAAGTG | | | | | |
| Ve25* | F: CAATTCCACTCACCCGCTTC | $(AGG)_6$ | FAM | 122–140 | 58 | Pr032286351 |
| | R: TCCAGCAATCTTGAACACGC | | | | | |

*Note*: $T_a$ = annealing temperature.
[a]Added to the 5′ end of the forward primers.
*Polymorphic microsatellite loci.

Total genomic DNA was extracted from sampled specimens using the NucleoSpin 96 Plant Kit (Macherey-Nagel, Hoerdt, France) (Appendix 1). To detect polymorphic markers, initial analyses were conducted on two specimens of each population using an M13 protocol as described in Schuelke (2000). Thus, an M13(−21) tail was added on the 5′ end of the forward primers. PCRs were carried out in a 12-µL final volume containing 0.2 mM dNTPs, 0.167 µM of M13 modified forward primer, 0.667 µM of each reverse primer and M13 primer fluorescently labeled with 6-FAM, VIC, NED, or PET (Eurofins Genomics, Courtaboeuf, France), 10× incubation mix without $MgCl_2$ (MP Biomedicals, Illkirch, France), 0.05 units *Taq* DNA polymerase (MP Biomedicals), and 2 mM $MgCl_2$. Between 5 and 80 ng of genomic DNA was used as template. Cycling was performed on a C1000 Touch Thermal Cycler (Bio-Rad, Marnes-la-Coquette, France). Conditions of PCR amplification were as follows: 94°C (5 min); 30 cycles at 94°C (30 s), 58–59°C (45 s), 72°C (45 s); then eight cycles at 94°C (30 s), 53°C (45 s), 72°C (45 s); and a final elongation at 72°C for 30 min. Thereafter, 1 µL of the PCR product containing the fluorescent dye-labeled fragments was added to 8.8 µL of formamide and 0.2 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems, Life Technologies) and subsequently run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Subsequent analyses were conducted with the polymorphic markers for all specimens of each population.

Genotypes were called using GeneMapper software (version 5; Applied Biosystems). Two loci did not amplify, and seven loci were monomorphic across individuals tested for all 17 populations (Table 1; Appendix 1). Eight polymorphic loci were characterized. Two of them (Ve10 and Ve24) possessed the same two fixed heterozygous alleles for all populations. Locus Ve24 showed inconsistent peaks, whereas Ve10 presented a clear signal without variability across populations and was therefore discarded from analysis. For the remaining six loci, allelic variability was calculated for the 138 individuals collected in 17 different populations in French floodplains of the Seine (Ile-de-France and Champagne-Ardenne), the Marne (Champagne-Ardenne), the Saône (Bourgogne), and in the Marais de Saône (Franche-Comté) (Table 2). Allele frequencies at each locus and observed and expected heterozygosities were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012). Tests for deviation from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were performed using GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008). The number of alleles observed per locus ranged from two to four, and the observed heterozygosity ranged from 0.051 to 1.000 (mean = 0.593). After Bonferroni correction, seven loci deviated significantly from HWE expectations and no linkage disequilibrium was detected for any loci. High observed heterozygosity values probably reflect the particular mating system of the species

TABLE 2. Genetic properties of eight polymorphic microsatellite loci isolated from *Viola elatior*.

| Locus | M13 label | *A* (*N*) | $H_o$ | $H_e$ |
|---|---|---|---|---|
| Ve2 | NED | 3 (138) | 1.000* | 0.537 |
| Ve5 | PET | 2 (138) | 0.080 | 0.077 |
| Ve10 | FAM | 2 (23) | 1.000* | 0.500 |
| Ve12 | PET | 4 (138) | 0.920* | 0.595 |
| Ve13 | NED | 3 (138) | 0.109* | 0.342 |
| Ve23 | FAM | 2 (138) | 0.051* | 0.188 |
| Ve24 | VIC | 2 (138) | 1.000* | 0.500 |
| Ve25 | FAM | 4 (138) | 0.993* | 0.557 |

*Note*: *A* = number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; *N* = number of individuals genotyped.
*Designates significant deviation from Hardy–Weinberg equilibrium genotypic proportions after sequential Bonferroni correction for multiple tests ($P < 0.0001$).

and suggest an important tendency to clonality (Eckstein et al., 2006). The significant deviation from HWE for almost all loci could be explained by a better ability of the heterozygous to reproduce asexually. This mode of reproduction might have been underestimated in this species and deserves further study. Amplifications for the seven polymorphic loci were carried out successfully on the closely related species *V. pumila* Chaix.

## CONCLUSIONS

These eight newly developed microsatellite markers should be useful to compare genetic diversity, structure, and connectivity across the landscape within *V. elatior*. They should offer a valuable tool for understanding the consequences of habitat fragmentation on this species' population genetic structure and will help to inform management practices.

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Appendix 1. Voucher and locality information for specimens of *Viola elatior* and *V. pumila* used in this study. Vouchers of representative specimens are stored at the Muséum National d'Histoire Naturelle (MNHN/FABR) (*V. elatior*: FABR06834, Villefranche, France; *V. pumila*: FABR07471, Gap, France).

| Species | Geographic coordinates | Collector | Specimen ID no.[a] |
|---|---|---|---|
| *Viola elatior* Fr. | 48°26′11.43″N, 3°17′37.63″E | Jérôme Wegnez, Chantal Griveau | V1-1, V1-11, V1-12, V1-13, V1-16, V1-19, V1-20, V1-21 |
| *Viola elatior* Fr. | 48°27′6.17″N, 3°16′48.82″E | Jérôme Wegnez, Chantal Griveau | V2-3, V2-30, V2-34, V2-35, V2-36, V2-5, V2-6, V2-8, V2-18 |
| *Viola elatior* Fr. | 48°25′17.04″N, 3°15′11.89″E | Jérôme Wegnez, Chantal Griveau | V3-11, V3-12, V3-13, V3-14, V3-15, V3-17, V3-20 |
| *Viola elatior* Fr. | 48°26′27.04″N, 3°15′0.79″E | Jérôme Wegnez, Chantal Griveau | V4-3, V4-24, V4-25, V4-26, V4-27, V4-28, V4-29, V4-33, V4-34 |
| *Viola elatior* Fr. | 48°30′26.97″N, 3°29′36.02″E | Jérôme Wegnez, Chantal Griveau | V5-1, V5-10, V5-16, V5-21, V5-25, V5-28, V5-3, V5-5 |
| *Viola elatior* Fr. | 48°26′41.37″N, 3°16′41.99″E | Jérôme Wegnez, Mélina Celik | V7-27, V7-28, V7-29, V7-3, V7-30, V7-31, V7-32, V7-9 |
| *Viola elatior* Fr. | 48°26′40.05″N, 3°16′49.96″E | Jérôme Wegnez, Mélina Celik | V8-2, V8-20, V8-21, V8-22, V8-23, V8-24, V8-28, V8-29, V8-3, V8-31 |
| *Viola elatior* Fr. | 48°29′1.2″N, 3°24′39.11″E | Jérôme Wegnez, Mélina Celik | V9-1, V9-10, V9-11, V9-12, V9-13, V9-14, V9-15 |
| *Viola elatior* Fr. | 48°29′39.64″N, 3°24′58.02″E | Jérôme Wegnez, Mélina Celik | V10-23, V10-24, V10-25,V10-26, V10-27, V10-28, V10-29, V10-30 |
| *Viola elatior* Fr. | 48°28′28.3″N, 3°21′26.74″E | Jérôme Wegnez, Mélina Celik | V11-1,V11- 10, V11-11, V11-12, V11-13, V11-14, V11-15, V11-16 |
| *Viola elatior* Fr. | 48°27′51.33″N, 3°21′20.58″E | Jérôme Wegnez, Mélina Celik | V12-31, V12-32, V12-4, V12-5, V12-6, V12-7, V12-8, V12-9 |
| *Viola elatior* Fr. | 48°32′16.10″N, 3°38′23.51″E | Jérôme Wegnez, Mélina Celik | V15-1, V15-10, V15-11, V15-12, V15-13, V15-14, V15-15, V15-16 |
| *Viola elatior* Fr. | 48°31′18.49″N, 4°15′16.3″E | Jérôme Wegnez, Mélina Celik | V16-31, V16-32, V16-4, V16-5, V16-6, V16-7, V16-8, V16-9 |
| *Viola elatior* Fr. | 48°9′58.7″N, 4°3′58.82″E | Jérôme Wegnez, Mélina Celik | V17-1, V17-10, V17-11, V17-12, V17-13, V17-14, V17-15, V17-16 |
| *Viola elatior* Fr. | 49°1′16.11″N, 4°13′37.54″E | Frédéric Hendoux | V18-31, V18-32, V18-4, V18-5, V18-6, V18-7, V18-8, V18-9 |
| *Viola elatior* Fr. | 46°37′41.09″N, 4°57′7.40″E | Olivier Bardet | V19-1, V19-10, V19-11, V19-12, V19-13, V19-14, V19-15, V19-16 |
| *Viola elatior* Fr. | 47°12′57.9″N, 6°6′22.94″E | Yorick Ferrez | V20-31, V20-32, V20-4, V20-5, V20-6, V20-7, V20-8, V20-9 |
| *Viola pumila* Chaix | 48°26′44.0″N, 3°17′14.0″E | Jérôme Wegnez | Vp1–Vp12 |

[a] Stored in the Conservatoire Botanique National du Bassin Parisien, Paris, France.