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DEVELOPMENT OF MICROSATELLITE PRIMERS IN THE PROTECTED SPECIES *VIOLA ELATIOR* (VIOLACEAE) USING NEXT-GENERATION SEQUENCING¹

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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.

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.

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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 R: AGCATATGTTGTTTCACCTTGC	(AC) ₁₀	NED	256–270	59	Pr032286348
Ve3	F: TCATAGGTTGAGTTTGGGTAC R: ACCCAGCTTCCATTTCATATG	(AAT) ₁₆	VIC	319	55	Pr032302549
Ve4	F: CTCACGCCAGATTCAACCC R: ATCTGGGCGTGATGGAGATC	(AAG) ₆	PET	330	59	Pr032302550
Ve5*	F: GGCTGTGCTTGCTTTGTCTC R: GTCCAAGCACAGATGAAGG	(AG) ₇	PET	224–228	59	Pr032286352
Ve9	F: TGGTGGCCACTCGTTAGAAC R: ATCTTGCTGCCATGGTTGAC	(AG) ₆	NED	229	59	Pr032302551
Ve10*	F: AGAAAGACGATGCAAGACGG R: TTTAGCTTCCAACGACACGC	(AG) ₆	FAM	223–259	58	Pr032286345
Ve11	F: ATGATGTAGCTGAGCCAAGG R: TGGGCATTGTAAGTGAAGCTG	(AAT) ₆	PET	228	59	Pr032302545
Ve12*	F: CAGCTAAGATTGAAGCAGCAG R: CATGATCCCAAGCATTTCTCC	(AAG) ₉	PET	127–139	59	Pr032286346
Ve13*	F: CAGAGAAAGCCACAAACAGTC R: GCCTCACTCTTCACTATTGTTTC	(AG) ₆	NED	314–322	59	Pr032286347
Ve14	F: TCATGCCCTTTATTTCCGAGTG R: AGAAGGGAATGTCATGGCTTG	(AG) ₆	VIC	206	55	Pr032302546
Ve21	F: CAACCGGAGACTACAAGCCC R: GCGTGTATAAATGCGTGG	(AAT) ₆	VIC	172	58	Pr032302547
Ve22	F: GGAGGAGGGAATAGGACAGC R: ACAGGAATGTTTGAGGTTGCC	(AAT) ₁₁	VIC	145	58	Pr032302548
Ve23*	F: AACCAAGACTAACGGCAGCAG R: TTTCGTGATTGTGCAGCTCC	(AG) ₆	FAM	216–234	58	Pr032286349
Ve24*	F: AGGGTTTAGGTTTAGAAGACG R: AGGCCCAAGACATAGAAGTG	(AAG) ₆	VIC	127–130	58	Pr032286350
Ve25*	F: CAATTCCACTCACCCGCTTC R: TCCAGCAATCTGAACACGC	(AGG) ₆	FAM	122–140	58	Pr032286351

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, Hoerd, 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 MgCl₂ (MP Biomedicals, Illkirch, France), 0.05 units *Taq* DNA polymerase (MP Biomedicals), and 2 mM MgCl₂. 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola elatior</i> 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
<i>Viola pumila</i> 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.