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Authors: Moeller, Sina, Wöhrmann, Tina, Huettel, Bruno, and Weising, Kurt

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Primer Note

Development of 18 polymorphic microsatellite markers for *Vinca minor* (Apocynaceae) via 454 pyrosequencing[1]

Sina Moeller[2,4], Tina Wöhrmann[2], Bruno Huettel[3], and Kurt Weising[2]

[2]Systematics and Morphology of Plants, Institute of Biology, Universität Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany; and [3]Max Planck-Genome-centre Cologne, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany

- *Premise of the study:* Polymorphic microsatellite markers were developed in *Vinca minor* (Apocynaceae) to evaluate the level of clonality, population structure, and genetic diversity of the species within its native and introduced range.
- *Methods and Results:* A total of 1371 microsatellites were found in 43,565 reads from 454 pyrosequencing of genomic *V. minor* DNA. Additional microsatellite loci were mined from publicly available cDNA sequences. After several rounds of screening, 18 primer pairs flanking di-, tri-, or tetranucleotide repeats were identified that revealed high levels of genetic diversity in two native Italian populations, with two to 11 alleles per locus. Clonal growth predominated in two populations from the introduced range in Germany. Five loci successfully cross-amplified in three additional *Vinca* species.
- *Conclusions:* The novel polymorphic microsatellite markers are promising tools for studying clonality and population genetics of *V. minor* and for assessing the historical origin of Central European populations.

Key words: Apocynaceae; clonality; introduced species; relic of cultivation; simple sequence repeat (SSR) markers; *Vinca minor.*

The lesser periwinkle (*Vinca minor* L.; Apocynaceae) is an evergreen subshrub that is native to Southern Europe but has become naturalized in wider parts of Central Europe and North America (Meusel et al., 1978; Swearingen et al., 2010). In Germany, *V. minor* is nowadays mainly found in the surroundings of ancient Roman remains, medieval castle ruins, and abandoned settlements, but is also cultivated (and propagated asexually) in a number of horticultural varieties (Labhart, 2005). It is commonly assumed that *V. minor* had been introduced to Germany as an ornamental, symbolic, and/or medicinal plant with the expansion of the Roman Empire. The species is therefore considered as a so-called "relic of cultivation" (Prange, 1996; Celka, 2011). However, little is known about the origin of the Central European populations and their colonization history.

The ability of *V. minor* to form stolons often results in the formation of compact carpet-like mats (Hegi, 1966). Because this growth form is often an indicator for clonal growth, vegetative reproduction by the expansion of stolons is frequently considered to be the predominant means of propagation for *V. minor* (Prange, 1996), especially because mature fruits and seeds are rarely observed in populations north of the Alps (Hegi, 1966). However, the relative importance of asexual vs. sexual propagation in *V. minor* has never been assessed by molecular methods.

Microsatellite or simple sequence repeat (SSR) markers are among the most sensitive tools for the evaluation of intraspecific variation and population structure. Here, we present 18 polymorphic SSR loci developed for *V. minor* using 454 pyrosequencing technology. These markers are important tools for analyzing genetic diversity, population structure, and clonality of *V. minor* in its native and introduced ranges.

METHODS AND RESULTS

A standard cetyltrimethylammonium bromide (CTAB) procedure (Weising et al., 2005) was used for extracting genomic DNA from fresh leaf tissue of one individual *V. minor* plant of garden origin (VM_454_01; see Appendix 1). Library preparation and shotgun pyrosequencing of a 5-μg DNA aliquot on a 454 GS-FLX Titanium instrument (Roche Diagnostics, Rotkreuz, Switzerland) were performed as described in Wöhrmann et al. (2012). A total of 43,565 sequence reads with an average length of 431 bp were obtained, and assembled into unique sequences using Geneious 5.4 (Drummond et al., 2010). SciRoKo 3.4 software (Kofler et al., 2007) was applied to search for perfect SSRs, accepting minimum thresholds of seven repeat units for di-, six for tri-, five for tetra-, and four for penta- and hexanucleotide repeats, respectively. A total of 1371 nonredundant SSRs were present in 24,886 unique sequences, with di- and trinucleotide repeats being almost equally abundant (47.4% and 46.9%, respectively). In a complementary approach, we applied the same SSR search criteria to 723,230 publicly available cDNA sequences (average length = 536 bp) derived from 454 sequencing of the *V. minor* transcriptome (deposited in GenBank by January 2011; accession number SRX039641). After assembly, a total of 25,253 perfect SSRs were detected within 267,199 unigenes. Trinucleotide repeats were most abundant within

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[4]Author for correspondence: sina.moeller@yahoo.de

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the assembled cDNA collection (63.4%), with $(ACT)_n$ being the most common motif (22.0%).

Thirty-five SSR loci from the genomic 454 data (ngVm01–ngVm35) as well as 60 SSR loci from the cDNA collection (Vimi01–Vimi60), all specifying single, perfect di-, tri-, tetra-, penta-, or hexanucleotide repeats, were arbitrarily selected for primer design using the BatchPrimer3 interface (You et al., 2008). For primer construction, we used the following criteria: length ranging from 18 to 23 nucleotides (20 as the optimum), PCR product size ranging from 100 to 300 bp, annealing temperature from 50°C to 70°C (55°C as the optimum), and GC content between 30% and 70% (50% as the optimum). PCR amplifications were performed in 10-μL final volumes using a T-Gradient thermocycler (Biometra, Göttingen, Germany), following the indirect labeling procedure described by Schuelke (2000). Each assay contained approximately 20 ng of DNA in 1× PCR Mango*Taq* buffer (Bioline, Taunton, Massachusetts, USA), 5 μg bovine serum albumin (BSA), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.1 units of *Taq* DNA polymerase (Mango*Taq*, Bioline), 0.04 μM forward or reverse primer carrying a 5′-M13 tail, 0.16 μM of M13 forward or reverse primer labeled with fluorescent 5′-IRDye700 or 5′-IRDye800 (Metabion, Martinsried, Germany), and 0.16 μM unlabeled forward or reverse primer, respectively. The cycling conditions described by Shaw et al. (2007) were used for all PCRs.

All primer pairs were initially tested for successful PCR amplification in five *V. minor* individuals (including accession VM_454_01 as a positive control and one sample each from four different populations; Appendix 1) on 0.8% agarose gels. Thirty-two primer pairs yielded distinct bands on agarose, and PCR fragments from these loci were separated on denaturing 6% polyacrylamide gels in 1× TBE buffer, using an automated sequencer (Li-Cor 4300 DNA Analyzer; Li-Cor Biosciences, Lincoln, Nebraska, USA). Fragment sizes were scored manually as previously described (Wöhrmann et al., 2012). Eighteen primer pairs yielded distinct polymorphic single or double bands within the expected size range. Locus characteristics, primer sequences, and GenBank accession numbers are summarized in Table 1. They were used for genotyping 40 *V. minor* plants from four populations, each with *n* = 10 (Appendix 2). Total DNA was extracted from dried leaf material using the CTAB procedure described above. Two populations were from the native range in northern Italy, and two from the introduced range in Germany.

Allele numbers and observed and expected heterozygosity values were determined with Arlequin 3.5.1.2 (Excoffier et al., 2005). Results are summarized in Table 2. All 18 loci proved to be polymorphic, exhibiting two to 11 alleles per locus among the 40 *V. minor* plants. In the Italian samples, observed and expected heterozygosities ranged from 0.1 to 1 and from 0.189 to 0.868, respectively (Table 2). Extremely low levels of genotypic diversity and a pronounced heterozygote excess were found in the two populations from the introduced range, indicating a high degree of clonality (Table 2). Overall, 105 alleles were detected with a strongly uneven distribution between the native and the introduced range (Appendix 2): 62 alleles were only found in the Italian populations, whereas 17 alleles were restricted to Central Europe. Twenty-six alleles were shared between the two regions.

The potential for cross-species amplification of the 18 SSR primer pairs was determined with one accession each of *V. major* L., *V. herbacea* Waldst. & Kit., and *V. difformis* Pourr. (Appendix 1). Primer transferability was considered successful when either one or two distinct bands in the expected size range were detected after polyacrylamide gel electrophoresis. Following

Table 1. Characteristics of 18 microsatellite loci and primer pairs developed for *Vinca minor*.

Locus[a]	Primer sequences (5′–3′)	T_a (°C)	Repeat motif	Expected allele size (bp)[b]	GenBank accession no.
ngVm05	F: TTTTGCCGACTTCTTATGTT	56	$(CA)_{14}$	249	KP644241
	R: CTTTATGTTCCTTGCTTCCA	56			
ngVm07	F: GCATAATTGGTGCAGTTTAG	54	$(TTA)_{16}$	138	KP666033
	R: GGGCAATAAAAATTCTCCTC	56			
ngVm11	F: CTCAAGGCTAAATTGATAGC	52	$(ATA)_{14}$	195	KP666034
	R: TGACATCTCTGTTCAAGTACAC	53			
ngVm15	F: CATGTCCTTTATTCTAGCTG	50	$(AAT)_{12}$	173	KP666035
	R: TCTCAAGTGTGCTACTCATAG	51			
ngVm21	F: ATAATCAATGCCACCCACT	55	$(CT)_{11}$	148	KP666036
	R: CTAATGAGGATTTGGAAGACTC	55			
ngVm24	F: TTCAAGCCCTTCTATTCC	53	$(CT)_{11}$	160	KP666037
	R: TATATTCTGGACGGTGGAG	53			
ngVm26	F: ACGGCTATGCTACAGACAATA	55	$(GA)_{11}$	130	KP666038
	R: GAAGATAGAAATGGAGTGAGGT	54			
ngVm33	F: ACACTCGCAATCAACTCTATG	56	$(AGTG)_6$	181	KP666039
	R: CTCTGTTGCATCGACATATTAG	55			
ngVm34	F: GCGCTCGATCAACATATTA	55	$(TCTT)_5$	199	KP666040
	R: TCCTAGTCCAAGAACTCACAA	55			
Vimi25	F: CCGTTTTCCTATTCATTTTCT	55	$(TGT)_{14}$	133	KP666041
	R: CCTGAACCTGGAATTAGAACT	55			
Vimi26	F: GTGGTTGTTGTAACAGAGGAA	55	$(TTA)_{14}$	162	KP666042
	R: GGAAACTCAAATCCTTCTGA	54			
Vimi27	F: ACGTAGTATGGCTACTCGACA	55	$(GTT)_{16}$	162	KP666043
	R: AGCAGTGTCCTCCTCAGAT	55			
Vimi33	F: AACGGATACTTTCTCAATCG	55	$(GCT)_7$	156	KP666044
	R: CCTCATAAATCAATCAGACTCC	55			
Vimi34	F: TCTCATTTACTCCCAACCTTC	55	$(TAT)_{14}$	163	KP666045
	R: TTTGTGTCTGTAGCTTCTCG	56			
Vimi39	F: CTAGTGAAGCAAGATCAGCTC	55	$(ACC)_{10}$	155	KP666046
	R: TCCATCCCTTTTACAGTTTC	54			
Vimi43	F: GCTGCTTAGACTTCTGATTTC	54	$(ATT)_{13}$	144	KP666047
	R: GAGTCCCTGTTTCTGTTGAT	54			
Vimi47	F: CACCAATCCAAATGACCTAA	56	$(TAT)_{11}$	162	KP666048
	R: TCCGAAAACACCTCTCTTTA	55			
Vimi53	F: ACACCTGAGAATAGAGGTTCC	55	$(TC)_{19}$	162	KP666049
	R: CCAAACCATTTCCATCTAAG	55			

Note: T_a = optimal annealing temperature.

[a] The acronyms ngVm (next-generation *V. minor*) and Vimi (*V. minor*) define primer pairs derived from either 454 genomic sequences or transcriptomic data, respectively.

[b] Expected allele sizes were deduced from the original 454 sequencing data.

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Table 2. Results of screening of 18 polymorphic SSR markers in four populations of *Vinca minor* (two from the native range in Italy, two from the introduced range in Germany).[a]

	Lagoni di Mercurago (I) ($n = 10$, $G = 8$)			Castel Boymont (I) ($n = 10$, $G = 2$)			Weidelsburg (GER) ($n = 10$, $G = 2$)			Wüstung Schleesen (GER) ($n = 10$, $G = 1$)			Total ($n = 40$, $G = 13$)		Cross-amplification in other *Vinca* species[b]			
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	Allele size range (bp)	*Vma*	*Vdiff*	*Vher*	Sr (%)
ngVm05	3	0.500	0.511	3	1.000	0.658	2	1.000	0.526	1	NA	NA	7	244–261	+	—	+	66.7
ngVm07	2	0.200	0.189	2	0.500	0.395	2	1.000	0.526	2	1.000	0.526	5	120–144	—	—	—	0
ngVm11	7	0.900	0.863	4	1.000	0.789	2	0.900	0.521	2	1.000	0.526	11	181–225	—	—	—	0
ngVm15	3	0.900	0.616	2	0.000	0.526	2	1.000	0.526	2	1.000	0.526	6	164–215	—	—	—	0
ngVm21	3	0.500	0.532	2	1.000	0.526	2	1.000	0.526	2	1.000	0.526	4	148–158	—	+	—	33.3
ngVm24	4	0.600	0.611	2	0.500	0.395	1	NA	NA	1	NA	NA	4	172–182	+	+	+	100
ngVm26	4	0.100	0.647	1	NA	NA	1	NA	NA	1	NA	NA	6	140–158	—	—	—	0
ngVm33	3	0.700	0.532	2	0.000	0.526	2	0.900	0.521	2	1.000	0.526	4	179–190	—	—	—	0
ngVm34	1	NA	NA	1	NA	NA	2	0.900	0.521	2	1.000	0.526	3	192–199	—	—	—	0
Vimi25	2	0.300	0.268	4	1.000	0.789	2	0.900	0.521	2	1.000	0.526	5	133–169	+	+	+	100
Vimi26	4	0.800	0.689	2	1.000	0.526	2	1.000	0.526	2	1.000	0.526	6	155–190	—	—	—	0
Vimi27	4	0.700	0.611	3	1.000	0.658	2	0.900	0.521	1	NA	NA	8	178–199	—	—	—	0
Vimi33	2	0.300	0.268	1	NA	NA	2	0.900	0.521	2	1.000	0.526	2	244–250	+	—	+	33.3
Vimi34	7	1.000	0.868	3	0.500	0.658	2	0.900	0.521	2	1.000	0.526	9	189–231	+	+	+	100
Vimi39	3	0.700	0.532	2	0.500	0.395	2	0.900	0.521	2	1.000	0.526	4	155–167	+	+	+	100
Vimi43	6	0.900	0.811	2	0.500	0.395	2	0.900	0.521	1	NA	NA	10	162–204	+	+	+	100
Vimi47	4	0.400	0.647	1	NA	NA	1	NA	NA	2	1.000	0.526	5	153–171	—	—	—	0
Vimi53	5	0.900	0.679	2	0.500	0.395	2	1.000	0.526	1	NA	NA	6	157–184	—	—	—	0
Mean	3.88	0.612	0.581	2.50	0.643	0.545	2.00	0.940	0.523	2.00	1.000	0.526	5.83	120–261	38.9%	33.3%	38.9%	

Note: A = number of alleles; G = number of genotypes; GER = Germany; H_e = expected heterozygosity; H_o = observed heterozygosity; I = Italy; n = number of individuals; NA = not available; Sr = success rate of cross-amplification; *Vdiff = Vinca difformis*; *Vher* = *V. herbacea*; *Vma* = *V. major*.

[a] See Appendix 1 for locality and voucher information.

[b] Single PCR product in the expected size range, success rates of cross-amplification per species and per locus are given in percent. + = successful amplification, with numbers of bands being in accordance with the ploidy level each species; — = no amplification.

these criteria, success rates ranged from zero to 100% with a mean of 35.2%. Eight loci (ngVm05, ngVm21, ngVm24, Vimi25, Vimi33, Vimi34, Vimi39, and Vimi43) amplified in one to three species included in the sample set (Table 2).

CONCLUSIONS

We developed a first set of 18 nuclear SSR markers for the lesser periwinkle, *V. minor*, a presumed "relic of cultivation." The markers displayed high levels of polymorphism across *V. minor* individuals and populations from the native range of the species in Italy and revealed a high extent of clonality in the introduced range in Germany. The markers are promising tools for population genetic analyses of *V. minor*. They will not only enable us to assess the relative importance of vegetative vs. sexual propagation in its native and introduced ranges, but will also help us to trace the species' phylogeographic history.

LITERATURE CITED

Celka, Z. 2011. Relics of cultivation in the vascular flora of medieval West Slavic settlements and castles. *Biodiversity: Research and Conservation* 22: 1–110.

Drummond, A. J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas, et al. 2010. Geneious v5.0. Website http://www.geneious.com [accessed 2 December 2010].

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47–50.

Hegi, G. 1966. Illustrierte Flora von Mitteleuropa (Vol. 5, No. 3). Paul Parey Verlag, Berlin, Germany.

Kofler, R., C. Schlötterer, and T. Lelley. 2007. SciRoKo: A new tool for whole genome microsatellite search and investigation. *Bioinformatics* 23: 1683–1685.

Labhart, G. 2005. Neues von *Vinca*, dem Bodendecker. *g'plus die Gärtner-Fachzeitschrift* 21: 26–29.

Meusel, H., E. Jäger, S. Rauschert, and E. Weinert. 1978. Vergleichende Chorologie der zentraleuropäischen Flora, Vol. 2. Gustav Fischer Verlag, Jena, Germany.

Prange, W. 1996. Das Kleine Immergrün (*Vinca minor* L.) in Westdeutschland, eine Kulturreliktpflanze aus römischer Zeit. *Schriften des Naturwissenschaftlichen Vereins Schleswig-Holstein* 66: 71–96.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Shaw, J., E. B. Lickey, E. E. Schilling, and R. L. Small. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany* 94: 275–288.

Swearingen, J., B. Slattery, K. Reshetiloff, and S. Zwicker. 2010. Plant invaders of Mid-Atlantic natural areas, 4th ed. National Park Service and U.S. Fish and Wildlife Service, Washington, D.C., USA.

Weising, K., H. Nybom, K. Wolff, and G. Kahl. 2005. DNA fingerprinting in plants: Principles, methods and applications, 2nd ed. Taylor & Francis, Boca Raton, Florida, USA.

Wöhrmann, T., N. Wagner, F. Krapp, B. Huettel, and K. Weising. 2012. Development of microsatellite markers in *Fosterella rusbyi* (Bromeliaceae) using 454 pyrosequencing. *American Journal of Botany* 99: e160–e163.

You, F. M., N. Huo, Y. Q. Gu, M. C. Luo, Y. Ma, D. Hane, G. R. Lazo, et al. 2008. BatchPrimer3: A high throughput web application for PCR and sequencing primer design. *BMC Bioinformatics* 9: 253.

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Appendix 1. Locality and voucher information of *Vinca minor* and related species analyzed for this study.

Species	Locality/source	Plant ID/voucher[a]	*n*	Geographic coordinates
V. minor L.	Lagoni di Mercurago, Piedmont, Italy	ID015–ID024	10	45°44′34″N, 8°32′40″E
V. minor L.	Ruin of Castel Boymont, Trentino, Italy	IT001–IT010	10	46°29′41″N, 11°15′9″E
V. minor L.	Ruin of Weidelsburg, Hesse, Germany	ID113–ID136	10	51°16′23″N, 9°8′44″E
V. minor L.	Wüstung Schleesen, Saxony Anhalt, Germany	ID253–ID262	10	52°1′50″N, 12°22′17″E
V. minor L.	Universität Kassel, Hesse, Germany	VM_454_01	1	51°16′55″N, 9°26′58″E
V. major L.	Botanische Gärten der Friedrich-Wilhelms-Universität Bonn, Germany	BONN-6026	1	NA
V. herbacea Waldst. & Kit.	Botanischer Garten der Justus-Liebig-Universität Gießen, Germany	GIESS-0-U-3893	1	NA
V. difformis Pourr.	Staatliches Museum für Naturkunde Stuttgart, Germany	STUT (Kull M3914)	1	NA

Note: *n* = number of individuals; NA = data not available.

[a] Vouchers for each population (accession numbers ID015, IT001, ID113, ID253) have been deposited in the Herbarium of the Universität Kassel (KAS).

Appendix 2. Survey of allele sizes (in bp) detected at 18 polymorphic SSR loci and their distribution among 20 *Vinca minor* plants from the native range in Italy and 20 plants from the introduced range in Germany.

Locus	Both regions (*n* = 40)			Italy (*n* = 20)[a]									Germany (*n* = 20)[b]	
ngVm05	249			245	247	251	261						244	255
ngVm07	120	135		123	138								144	
ngVm11				181	183	201	204	210	213	216	219	225	192	207
ngVm15	170			164	173	197	215						185	
ngVm21	148	150		158									156	
ngVm24	172	182		174	180									
ngVm26				140	142	145	158						147	149
ngVm33	181	190		179	180									
ngVm34				199									192	198
Vimi25	136	154		133	169								139	
Vimi26	181	190		155	158	161	170							
Vimi27				178	184	187	193	196	199				181	190
Vimi33	244	150												
Vimi34	189	210		192	195	216	222	228	231				198	
Vimi39	155	158		161	167									
Vimi43	168	171		162	174	183	186	192	204				180	195
Vimi47	159			153	162	165	171							
Vimi53	157	158	162	172	178	184								

Note: *n* = number of individuals.

[a] Alleles private to Italy.

[b] Alleles private to Germany.

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