DEVELOPMENT OF 18 POLYMORPHIC MICROSATELLITE MARKERS FOR VINCA MINOR (APOCYNACEAE) VIA 454 PYROSEQUENCING¹

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

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• 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 populations 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 (Kofler et al., 2007). 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). Among a total of 25,253 perfect SSRs were detected within 267,199 unigenes. Trinucleotide repeats were most abundant within...
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 MangoTaq 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 (MangoTaq, Bioline), 0.04 μM forward or reverse primer carrying a 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 0.8% agarose 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 heterozygosity values 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

| Locus | Primer sequences (5′→3′) | \( T_a \) (°C) | Repeat motif | Expected allele size (bp) | GenBank accession |
|-------|--------------------------|--------------|-------------|--------------------------|------------------|
| ngVm05 | F: TTTGCGCAGCTTCTTATGTT  
R: CTTATATGCTTCCTTCCCA | 56 | (CA)\( _{14} \) | 249 | KP644241 |
| ngVm07 | F: GCATAATTGGCTCGTATTAG  
R: GCGACACAAATATCTCTCTCC | 54 | (TTA)\( _{16} \) | 138 | KP666033 |
| ngVm11 | F: CTCAAGGCCTAATTGATAGCC  
R: TGACATCTCTGTATGAGCTAC | 52 | (ATA)\( _{14} \) | 195 | KP666034 |
| ngVm15 | F: CATGGCTCCTATTCGAGCTG  
R: TCTCAAGTGGCTCATTCTAG | 50 | (AAT)\( _{12} \) | 173 | KP666035 |
| ngVm21 | F: ATATCAATTGCCCACCCACT  
R: CTAATTGAGGGTTTGGAGACTC | 55 | (CT)\( _{11} \) | 148 | KP666036 |
| ngVm24 | F: TCCAGGCCTCTTCTATCC  
R: TATATGCTTGGACGCGTTGAG | 53 | (CT)\( _{11} \) | 160 | KP666037 |
| ngVm26 | F: ACGGGCTATGTCAAGCAATA  
R: GAGATTAGAAGGTTAGGCTG | 55 | (GA)\( _{11} \) | 130 | KP666038 |
| ngVm33 | F: ACACCTGCAATCCTCCTATG  
R: CTCCTGGCAGCTCATTTAG | 56 | (AGTG)\( _{6} \) | 181 | KP666039 |
| ngVm34 | F: GCCGTCTGACAACATATA  
R: TCTCAAGTGCTGCTCTCATC | 55 | (TCTT)\( _{5} \) | 199 | KP666040 |
| Vimi25 | F: CGGTCTTCTATTCTATCTCT  
R: CTCGACTCTGGATAGACTC | 55 | (TGT)\( _{14} \) | 133 | KP666041 |
| Vimi26 | F: GTGGTGTCTGGATAGAGGGA  
R: GGAAGCTCAAGCTCTCTCCT | 55 | (TTA)\( _{14} \) | 162 | KP666042 |
| Vimi27 | F: AGCATGATGGCTACCTAGCA  
R: AGCACTCTTCTTCTGATAT | 55 | (GTT)\( _{16} \) | 162 | KP666043 |
| Vimi33 | F: ATCTCTACTCTTCTCTCAG  
R: AAGGCTAATCTCTTCTCAG | 55 | (GCT)\( _{7} \) | 156 | KP666044 |
| Vimi34 | F: TCTCTATCCTATCTCCTCCT  
R: TTTTGCTGTCAGGCTTCTTG | 56 | (TAT)\( _{14} \) | 163 | KP666045 |
| Vimi39 | F: CTAAGTGAAGAGCAGCTCCT  
R: TCCATCCCTTATCAGTTC | 55 | (ACC)\( _{10} \) | 155 | KP666046 |
| Vimi43 | F: GCTGCTGCTGCTGTATCCT  
R: GATGCTCTGGTCTGGAGTG | 54 | (ATT)\( _{13} \) | 144 | KP666047 |
| Vimi47 | F: CACCAATCTCAAGAGCTTCA  
R: TCCGAAAAACTCCTCTCTTA | 55 | (TAT)\( _{11} \) | 162 | KP666048 |
| Vimi53 | F: ACAAATTGCGAAAAGCTTCC  
R: CCAAATTCATTTTAGCTAAC | 55 | (TC)\( _{10} \) | 162 | KP666049 |

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.

http://www.bioone.org/loi/apps
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.

**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.

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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* | n    | Geographic coordinates                  |
|-----------------------------|----------------------------------------------------------------|-------------------|------|-----------------------------------------|
| *V. minor* L.              | Lagoi 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 Schlesen, 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.

* 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)* | Germany (n = 20) |
|-----------|-----------------------|----------------|-----------------|
| 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   | 185             |
| ngVm21    | 148 150               | 158            | 156             |
| ngVm24    | 172 182               | 174 180        | 147 149         |
| ngVm26    | 179 180               | 140 142 145 158 | 192 198 |
| ngVm33    | 181 190               | 199            | 192 198         |
| ngVm34    | 136 154               | 133 169        | 139             |
| Vimi25    | 181 190               | 155 158 161 170 | 181 190 |
| Vimi26    | 181 190               | 178 184 187 193 196 199 | 188 190 |
| Vimi27    | 181 190               | 192 195 216 222 228 231 | 198 |
| Vimi33    | 189 210               | 161 167        | 180 195         |
| Vimi34    | 168 171               | 162 174 183 186 192 204 | 198 |
| Vimi47    | 159                   | 153 162 165 171 | 185 |
| Vimi53    | 157 158 162           | 172 178 184    | 171             |

Note: *n* = number of individuals.

* Alleles private to Italy.

* Alleles private to Germany.