Development and Characterization of Genic Microsatellites for the Ornamental Plant Green and Gold (*Chrysogonum virginianum*)

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Additional index words. subspecies delimitation, genic SSR, population genetics

Abstract. The genus *Chrysogonum* is native to the eastern United States. Three entities have been recognized—either as three varieties of *Chrysogonum virginianum* or as two species, one of them with two varieties. The current study suggests that a fourth entity should be recognized. Several forms of the complex are in commercial trade as ornamentals. As very limited molecular information on *Chrysogonum* is available, we developed a set of genic simple sequence repeat markers (eSSRs) from *de novo* transcriptome sequencing. We tested a set of 17 eSSRs on a collection of *C. virginianum* genomic DNA samples from the three botanical varieties, and a new putative type observed in Tennessee, dubbed “Ocoee-type” for its geographic origin. The polymerase chain reaction and capillary electrophoresis analyses with downstream population genetics tools verified the usefulness of the eSSRs. By applying this approach, we showed recognizable variation within *Chrysogonum*, although it did not correspond exactly to previous infraspecific classifications. Finally, as demonstrated for the commercial cultivar Pierre included in the study, the eSSRs can be used for enhancing the future breeding or hybridization efforts of this ornamental plant.

Received for publication 6 Nov. 2018. Accepted for publication 10 Dec. 2018.

This study was funded fully by an Agricultural Research Service–U.S. Department of Agriculture grant (58-6062-6) to the Trigiano laboratory for ornamental plants research. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Use of trade names is for identification purposes only and does not imply their endorsement by the authors or the study funding entities. The authors are grateful to the plant material collectors and herbaria for making them available to this study. The RNA sequencing raw read files are available as National Center for Biotechnology Information Sequence Read Archive accession SRR8181940.

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The monotypic genus *Chrysogonum* (Linnaeus, 1754; Asteraceae: Heliantheae) is native to the eastern United States. The single species currently recognized taxonomically is *Chrysogonum virginianum* L. (Green and Gold; Goldenstar), a perennial herb that produces small, bright yellow flower heads. The following three botanical varieties of *C. virginianum* are separated by habit and geographic location: var. *virginianum* has the tallest plant height, lacks stolons, has leafy flowering stems, and is found in the north-eastern portion of the generic range; var. *australe* is the shortest, has prominent, long stolons, leafless flowering stems, and occurs in the most southern and western part of the

range; var. *brevistolon* is of intermediate plant height, has stolons of medium length, has both leafy and leafless flowering stems, and occurs in the middle portion of the generic range (Nesom, 2001; Stuessy, 1973, 1977). The relationships and generic limits of *Chrysogonum* have been a source of controversy, as has the circumscription of taxa within the genus (Gray, 1882; Nesom, 2001; Stuessy, 1973, 1977). An alternative taxonomy of the genus was proposed (Weakley, 2015); it recognized the “*australe*” entity at specific rank, presumably because of its allopatry and distinct morphology, in contrast to previous categorization that overlooked that fact (Nesom, 2001). Issues with subtribal relationships were resolved with molecular phylogenetic data, starting with the studies based on enzymatic restriction of the chloroplast genome that explored the phylogenetic position of *Chrysogonum* in the Heliantheae tribe (Panero and Jansen, 1997; Panero et al., 1999). Sequence-based studies analyzing the nuclear ribosomal sequences of the internal transcribed spacer and the external transcribed spacer provided support for removal of all but one of the classically recognized species of *Chrysogonum* and placement of the genus *Chrysogonum* in subtribe Engelmanniinae, together with *Silphium* L. and four other small genera (Clevinger and Panero, 2000). The genome sequence of *C. virginianum* has not been reported, and before this study only 14 sequences were available in the National Center for Biotechnology Information GenBank for the species. The species ploidy is assumed to be diploid ($2N = 2 \times = 32$; Nesom, 2001; Solbrig et al., 1972), although some authors suggested it is tetraploid, based on the relatively high base chromosome number (Stuessy, 1973). Only scant phytopathological information is available on Green and Gold and includes a recent report on powdery mildew (*Golovinomyces spadi-ceus*; Trigiano et al., 2018a) and aerial blight (*Sclerotinia sclerotiorum*; Trigiano et al., 2018b). Green and Gold is used as a home garden ornamental plant in shady, moist areas with several cultivars of all three taxa available commercially, and some of them recognized by patents (Hawks, 2008; Hoffman et al., 2005). At the basal part of Green and Gold diaspores, there is an elaiosome, a fleshy, oil-bearing structure. Ants foraging on the elaiosomes contribute to the plant dispersal (Gaddy, 1986; Nesom, 1978). The plant flowering time was used as a marker of global climate change in the Washington, DC area and was confirmed to advance by 31 d over 30 years of studies, ranking it as the fifth most altered among the 89 plant species investigated (Abu-Asab et al., 2001).

Among the neutral molecular markers, the microsatellites or simple sequence repeats (SSRs) are often used for population genetics and (sub)species delimitation (Duminil and Di Michele, 2009; Dupuis et al., 2012; Esfandani Bozchaloyi et al., 2017; Meudt et al., 2009). Compared with the genomic SSRs (gSSRs) developed on the nuclear DNA sequences, the eSSRs are developed

from the assembled transcriptomes (Ellis and Burke, 2007; Varshney et al., 2005). eSSRs have become an attractive and efficient high-throughput molecular tool owing to comparatively lower cost for their development from species lacking sequenced genomes. The eSSRs conserved in the transcriptomes usually display somewhat lower diversity indices and higher conservation across taxa than gSSRs (Ellis and Burke, 2007; Eujayl et al., 2001; Varshney et al., 2002).

To better substantiate the current taxonomic subspecies delineation in the complex *C. virginianum*, we developed eSSRs based on de novo RNA sequencing (RNA-seq). We selected 17 eSSRs and assessed their utility using a collection of 32 genomic DNA (gDNA) samples from the three currently recognized *C. virginianum* varieties from the eastern United States and a recently-discovered putative new form (“Ocoee-type”) first observed in eastern Tennessee. Our survey of *Chrysogonum* also included a sample of the commercial cultivar Pierre, whose varietal attribution was not described. The purpose of this report was to present the results of the *Chrysogonum* eSSR development and the initial results on variability within the genus.

Materials and Methods

Plant materials and nucleic acids extraction. Samples of *C. virginianum* were obtained to represent a variety of locations from throughout its range in the southeastern United States. Sources included herbarium specimens collected between 1967 and 2003 and living material collected 2016 through 2018. The samples (n = 31) belonged to the three currently recognized varieties assigned following Nesom (2001) and the newly observed putative “Ocoee-type” (Ocoee and Hiwassee River Valley samples, Tennessee). The “Ocoee-type” is distinct in having a tapering, cuneate leaf base similar to var. *australe* but lacking its distinctive long stolons. gDNA was isolated from dried leaves (herbarium specimens) or fresh leaf material (Table 1) using the DNA Plant Mini Kit (Qiagen, Germantown, MD), according to the manufacturer’s protocol. A single leaf sample from a home garden (a morphologically typical *C. virginianum* of the “Ocoee-type,” collected and transplanted from the Hiwassee River Valley, TN; ‘Chry_2’, Maryville, TN, collected 2018 and stored on dry ice) was also included. The gDNA of this sample was isolated from leaf tissue using the DNA Plant Mini Kit (Qiagen) according to the manufacturer’s protocol. A leaf sample of the same living plant was also processed for total RNA using the Ribospin II kit (GeneAll Biotechnology, Seoul, Korea) as per the manufacturer’s protocol.

De novo sequencing and development of eSSR markers. The RNA of *C. virginianum* specimen ‘Chry_2’ was submitted for Illumina MiSeq [pair-end 2 × 250 base pairs (bp)] sequencing (GeneWiz, South Plainfield, NJ). RNAseq data for *C. virginianum*

Table 1. *Chrysogonum virginianum* collections included in the study.^z

DNA#	<i>C. virginianum</i> var.	Collector and voucher details	Location	Yr collected
3511	<i>brevistolon</i>	Bailey & McCoy	Scott Co., TN	2003
4027	<i>australe</i>	Rogers 2058-A	Forrest Co., MS	1969
4028	<i>brevistolon</i>	McNeilus 00-489	Shenandoah Co., VA	2000
4029	<i>australe</i>	Rogers 9437-A	Wayne Co., MS	1974
4033	<i>virginianum</i>	Filipczak 161	Alamance Co., NC	1980
4036	<i>brevistolon</i>	Panero 695	Scott Co., TN	1987
4038	<i>brevistolon</i>	Schell s.n.	Greene Co., TN	1994
4039	<i>brevistolon</i>	Gaylon & Schnabel 40873	Claiborne Co., TN	1967
4500	Ocoee-type	Floden et al., 2879	Polk Co., TN	2016
4501	Ocoee-type	Floden et al., 2880	Polk Co., TN	2016
4502	Ocoee-type	Floden et al., 2881	Polk Co., TN	2016
4580	<i>brevistolon</i>	Schilling 95-1	*	2016
4581	Ocoee-type	Schilling 16-05	Modoc Co., SC	2016
4582	<i>virginianum</i>	Schilling DS1	Chatham Co., NC	2016
4583	<i>virginianum</i>	Schilling DS2	Wake Co., NC	2016
4586	Ocoee-type	Diggs s.n.	Oakwood Co., GA	2016
4605	<i>brevistolon</i>	Floden & Schilling 3046	Hancock Co., TN	2016
4608	<i>brevistolon</i>	Floden & Schilling 3046	Hancock Co., TN	2016
4609	<i>brevistolon</i>	Floden & Schilling 3181	Clairborne Co., TN	2016
4624	<i>australe</i>	Garden plant	*	2016
4631	<i>virginianum</i>	Schilling A1	Montgomery Co., NC	2016
4632	<i>virginianum</i>	Schilling B1	Montgomery Co., NC	2016
4633	<i>virginianum</i>	Schilling A2	Montgomery Co., NC	2016
4634	<i>virginianum</i>	Schilling B2	Montgomery Co., NC	2016
4650	Ocoee-type	Datillo s.n.	Polk Co., TN	2016
4768	<i>australe</i>	McDonald 5864 (MO)	Houston Co., AL	1967
4769	<i>australe</i>	Kral 54955 (MO)	Antaga Co., AL	1975
4770	Ocoee-type	Kral 64940 (MO)	McDuffie Co., GA	1980
4771	Ocoee-type	Kral 64940	Dawson Co., GA	1976
4772	Ocoee-type	Kral 57573	Lumpkin Co., GA	1976
Chry_2	Ocoee-type	Garden plant	Blount Co., TN	2018
Pierre	<i>virginianum</i>	Commercial source	*	2018

^zDNA# column marks the laboratory genomic DNA sample code. Variety was established following Nesom (2001). Herbarium voucher details, sample location information (*; garden plant of unknown origin), and year of collection are listed. Individuals ‘Chry_2’ (serving for RNA extraction and RNA sequencing) and commercial cultivar Pierre are in bold.

produced were assessed for read quality using the quality control program FastQC, which identifies base content, sequence quality, and sequence duplication in fastq files of interest (Andrews, 2014). Adapter content was removed using the trimming program Skewer (Jiang et al., 2014) (minimum length: 30 bp, defaults used for all other parameters). To enable microsatellite identification, reads were assembled using Assembly By Short Sequences (ABYSS), specifically its paired-end option, abyss-pe, using a k-mer size of 81 and default settings for all other options (Simpson et al., 2009). Low-complexity regions of the transcriptome were masked out with Dustmasker, with a level of 1, before finding SSRs within the assembly (Morgulis et al., 2006). This study used a custom perl script to identify SSRs and call primers with Primer3 (Staton and Ficklin, 2018; Untergasser et al., 2012). This script searched for di-, tri-, and tetra-repeating motifs with an amplified fragment range of 100 to 250 bases.

eSSR screening and plant gDNA collection genotyping. From the developed eSSRs, we initially screened a pool of 50 primer pairs (20 of di-, 20 of tri-, and 10 of tetra-motif repeats) on the gDNA sample whose leaf was also used for RNA isolation and sequencing (‘Chry_2’). The polymerase chain reaction (PCR) reaction volume was 10 µL and included 5 µL of 2 × AccuStart II PCR SuperMix (Quanta BioSciences, Inc., Beverly, MA), 0.25 µM of each primer, and 4 ng of

gDNA. The PCR thermal profile used was as follows: initial denaturation at 94 °C for 4 min, 10 touch-down cycles (94 °C for 20 s, 65 °C –1.0 °C per cycle for 20 s, 72 °C for 30 s), 30 cycles (94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s), and final extension at 72 °C for 5 min. The PCR products were visualized using capillary electrophoresis (QIAxcel Advanced Electrophoresis System; Qiagen) and analyzed using a 25 to 500 bp DNA size marker and an internal 15/600 bp alignment marker. The 17 best-performing eSSRs (four di-, eight tri-, and five tetra-motif repeats) based on agreement with the predicted size and one or two unambiguous alleles, were selected and used for genotyping of the *C. virginianum* gDNA collection. The resultant dataset was binned into allelic classes using FlexiBin (an MSExcel macro; Amos et al., 2007).

Estimation of the hierarchical fixation indices and population structure. The binned dataset was analyzed for an array of population genetics parameters. To estimate the basic indices, including number of alleles per locus, observed and expected heterozygosity, evenness, Shannon’s diversity index, and allelic richness (defined as the number of alleles divided by the number of samples without missing data at that locus), we used the following packages: *poppr* (Kamvar et al., 2014, 2015), *hierfstat* (Goudet, 2005; Goudet et al., 1996), and *strataG* (Archer et al., 2017) in R version 3.4.3 (The R Core

Team, 2014). Gene flow (Nm) was estimated using GenAEx version 6.503 (Peakall and Smouse, 2012). The package *poppr* in R was used for calculations of the (pairwise) linkage disequilibrium. Discriminant Analysis of Principal Components (DAPC) was performed in R using the package *ade4* version 2.1.1 (Jombart et al., 2010, 2018) and included cross-check and optimization by 1000 permutations over principal components number ranging from 1 to 32.

Results and Discussion

C. virginianum transcriptome and eSSR development and screening. After pre-processing the RNAseq data through Skewer, 7,054,370 of the initial 7,145,419 read pairs were available for further analysis; of these, 991,875 were trimmed using Skewer, and 6,062,495 did not require trimming. The assembly generated by running ABySS on these remaining reads contained 841,375 scaffolds. A total of 4940 SSRs with primers were identified from the *C. virginianum* assembled transcriptome; of these, 3162 were dinucleotide repeats, 995 were trinucleotide repeats, and 783 were tetranucleotide repeats. All unique di-repeat SSR types were discovered. Similarly, of 60 unique tri-repeat SSRs, only [AGT]_n-types (and their redundant iterations) were missing in the assembled transcriptome of *C. virginianum*. From among the total of 252 unique tetra-SSRs, only 118 were discovered in the assembled transcriptome of *C. virginianum* with at least one SSR present. The number of SSRs that yielded primers as per our algorithm corresponded with their respective counts of SSRs discovered in the assembled transcriptome, regarded both in the repeat-length fashion, or repeat-motif (Fig. 1). Similar to other studies (Arias et al., 2011), the most frequent SSRs were di-repeats with [GC]_n ranking the lowest among them. In contrast to other Asteraceae plants (Peng et al., 2014), the tetra-repeat SSRs occurred almost as frequently as the tri-repeat SSRs and were among the most frequently discovered in the unique motif manner.

C. virginianum eSSR characteristics and varietal distances. Based on the genotyping dataset accrued, the used eSSRs are well dispersed over the *C. virginianum* genome (Fig. 2A). The small range of pairwise values of Index of Association $\bar{r}_d$ (from -0.2 to 0.3) corroborates this result. Furthermore, the overall value of $\bar{r}_d = 0.014$ ($P = 0.083$) (Fig. 2B) indicative of the outcrossing mode of reproduction, is in agreement with the species biology (Nesom, 2001; Stuessy, 1973, 1977).

With regard to the diversity indices calculated, the selected 17 eSSRs detected from 4 to 15 alleles per locus with a mean of ≈ 8 (Table 2). The allelic richness ranged from 0.09 to 0.52, with a high cumulative mean of 0.32. The observed heterozygosity (0.26) was much lower than the expected heterozygosity (0.70). This was likely derived from the low number of accessions present in this

collection, or, given the growth habit, the plants may be relatively inbred. The very low value for Shannon's diversity index (1.17) indicative of generally low allele species richness/evenness and far from saturating the expected species diversity, may have stemmed from the same relatively low sample number in this study. The same limitation also applied to the relatively high values of the eSSR evenness indices, ranging from 0.56 to 0.96, with a mean of 0.74. Very high values of the Wright's fixation index (F) implied an excess of homozygosity relative to that expected under the Hardy-Weinberg equilibrium of the varieties of *C. virginianum*, despite rather strong support for the outcrossing mode of this plant reproduction

(Fig. 2B). High gene flow calculated for our *C. virginianum* collection ($Nm = 1.68$) likely had a smoothing effect on the allele frequencies, rendering high values of the evenness index. All these characteristics, however, must be taken with caution owing to the limited sampling and, although promising, certainly needs to be confirmed in a large-scale study.

Despite the low number of samples included in this study, we achieved reliable separation of some of the *C. virginianum* varieties with DAPC (Fig. 3). Specifically, we observed distant placement of the var. *australe* from the other varieties, and a possible emergence of an additional new botanical variety, tentatively dubbed 'Ocoee-type'

Fig. 1. Genic simple sequence repeats (eSSRs) discovered in the transcriptome of *Chrysogonum virginianum* 'Chry_2'. Assembled RNA sequencing reads were screened for presence of di-, tri-, and tetra-motif repeat SSRs. Frequency (counts) of top 20 eSSR nonredundant motifs found in the assembled transcriptome are depicted in white; SSRs with primers constructed as per our algorithm are shown in gray. Insert: Frequency (counts) of di-, tri-, and tetra-motif repeat eSSRs. Note: Frequencies of nonredundant tetra-repeat eSSRs were not inspected before devising the primers for them. bp = base pair.

Fig. 2. Linkage disequilibrium of the tested genic simple sequence repeats (eSSRs) of *Chrysogonum virginianum*. (A) Pairwise Index of Association ($\bar{r}_d$) was tested to infer the eSSR distribution over *C. virginianum* genome by the feature of the probability of their cosegregation. The $\bar{r}_d$ values are color-coded, according to the legend depicted on the right side. (B) Collection-wise $\bar{r}_d$ was inferred over 1000 permutations of the entire dataset ($n = 32$). The overall $\bar{r}_d$ (black dashed line) and the corresponding P value are denoted.

Table 2. Characteristics of 17 microsatellite loci (eSSRs) developed for *Chrysogonum virginianum*.^z

eSSR locus	GenBank#	Primer sequences	Repeat motif	%	Allele size range [bp]	N	A _R	H _o	H _e	F	H'	N _m	E
eChry01	MK124548	CCCTTCTGCAATGGTTGAATGGG TGACTTACCAGCTCAAAATGCG	[TG] ₆	97	172–191	7	0.23	0.71	0.71	-0.11	1.13	1.24	0.69
eChry08	MK124549	GGTACGCCCATTTAGATTGG CAATGCTGTACAAGACCGTGC	[AT] ₆	100	191–214	8	0.25	0.38	0.75	0.47	1.32	1.49	0.70
eChry09	MK124550	TTCCCTGTTCTCACATGATCC GAGATGCTGAGACTCCCTGC	[TA] ₉	88	157–200	13	0.46	0.14	0.90	0.85	1.54	1.24	0.88
eChry18	MK124551	GTAGACCGACTTGAGAACCG CGTATCAGTCGCTACCTGTCC	[TA] ₆	91	257–295	7	0.24	0.17	0.62	0.48	0.86	0.59	0.56
eChry22	MK124552	TCGTGTATGGCATCTCTTGG TCAGCTTCACTTGGTCTCTCC	[GTG] ₆	100	348–361	6	0.19	0.06	0.80	0.88	1.42	2.69	0.90
eChry24	MK124553	TCGTGTATGGCATCTCTTGG CGCGCACACAACTTGAAACG	[GTT] ₆	100	318–330	5	0.16	0.03	0.68	0.92	1.02	1.42	0.80
eChry26	MK124554	GACGGAAAGAGTGAATGGG GCTCATAAACCTAGTCGGTAGG	[TTA] ₇	72	207–263	12	0.52	0.26	0.89	0.78	1.45	1.06	0.87
eChry28	MK124555	TCGTGAATGCTGAAGGTGGG GGTGCTCCTGTTGTCTCC	[TAA] ₆	97	202–221	7	0.23	0.13	0.80	0.76	1.11	0.93	0.85
eChry33	MK124556	CATCACTCCCTCAGAGAAGGC ACCATCAAGCTCATCTGACCC	[TTC] ₉	100	130–156	10	0.31	0.66	0.85	0.29	1.61	2.21	0.87
eChry34	MK124557	TGAGGCTTCTGAAGGACACG GTTGCAGGGAAGTTTGAAGCC	[ACT] ₆	100	108–117	4	0.13	0.12	0.48	0.80	0.72	1.45	0.70
eChry35	MK124558	AGAGCATATGAACACATTCCTCG GCAGTCAATGTTACAGTGAGGG	[AAT] ₉	100	228–289	15	0.47	0.72	0.91	0.16	1.96	3.09	0.86
eChry37	MK124559	AGGACCGTGACATAAAGTTTGC AGGAGAAATGTTTGACAGGC	[ATA] ₆	97	121–136	6	0.19	0.48	0.82	0.47	1.36	1.57	0.96
eChry41	MK124560	GGCTATCCAATACACATTCCTCG AGGAGGCTCTGCCCTAAAGG	[AATA] ₄	100	143–181	9	0.28	0.62	0.80	0.18	1.54	2.52	0.76
eChry42	MK124561	GGTGGGTCAAACTATAGGCC ATCGAATGAGGTTTGACTGCG	[GAT] ₄	100	137–144	3	0.09	0.00	0.12	1.00	0.18	2.76	0.42
eChry43	MK124562	GGAACTCAAAGATGCAGCTGC ACGTACACCAATAGATTCTGGG	[AAT] ₅	100	206–245	10	0.31	0.34	0.67	0.32	1.11	0.60	0.56
eChry44	MK124563	AAGACGGTAACCTCAGGG AAATGGTCTGTAGTTCTCACC	[TTTA] ₄	91	137–156	6	0.21	0.34	0.48	0.25	0.73	3.04	0.52
eChry45	MK124564	TGATTCCATGATGCGAGGATCC TACACATCACGTATGCGAGGC	[TATT] ₄	88	295–340	5	0.18	0.25	0.65	0.70	0.86	0.73	0.75
Mean				95		8	0.32	0.26	0.70	0.53	1.17	1.68	0.74

^zShown for each locus are the GenBank accession number, the forward and reverse primer sequences, repeat motif, percent of the 32 samples amplified (%), allele size range [bp], number of alleles (N), allelic richness (A_R = number of alleles for a given locus divided by the number of samples without missing data at that locus), observed (H_o = frequency of heterozygous individuals per locus averaged over number of sampled loci) and expected heterozygosities (H_e = a measure of genetic diversity less sensitive to allele species richness), Wright's fixation index (F = a measure of inbreeding), Shannon's diversity index (H' = increases together with both richness and evenness of the species present), gene flow (Nm), and evenness (E); relative abundance of allele species), bp = base pair; eSSR = genic simple sequence repeat.

Fig. 3. Discriminant Analysis of Principal Components of the *Chrysogonum virginianum* genic simple sequence repeat (eSSR) dataset. All samples of *C. virginianum* used in this study are presented, with varieties labeled. The analysis was cross-checked by optimizing the number of principal components used from 1 to 32 (1000 permutations). *C. virginianum* 'Pierre' was added as a proof of concept for the subspecies placement by eSSR. The specimen 'Chry_2' that was used for the RNA sequencing is marked. (Insert) *C. virginianum* variety-wise unrooted tree of genetic distances. Samples used in the study were grouped by variety to infer the genetic distances [F_{ST}] among them (here, ranging from 0.02 to 0.11). The analysis was permuted 1000 times, and the bootstrap support [%] of each split is presented. PCA = principal components analysis.

for its place of origin. In contrast, vars. *virginianum* and *brevistolon* were not clearly delineated from each other. The morphological difference between typical var. *virginianum* and var. *brevistolon* is small, but both herbarium and field observations showed that the geographic boundary is sharply defined (as mapped), thus some kind of genic differentiation should be expected (G.L. Nesom, personal communication). Notably, our observations are tentative in character and need to be tested on a large-scale study, possibly involving auxiliary molecular evidence (e.g., sequencing of chosen informative or "bar-coding" loci; CBOL Plant Working Group, 2009), as well as descriptive taxonomy using

the geographical and ecological methods (Duminil and Di Michele, 2009; Reed and Frankham, 2001). Several reports successfully used the Amplified Fragment Length Polymorphism as molecular markers neutral in character and of nuclear origin, for plant species delimitation (Chen et al., 2013; Meudt et al., 2009; Segatto et al., 2017) or SSR alike (Chen et al., 2013; Dupuis et al., 2012; Meudt et al., 2009). In particular, the eSSR markers may be of interest due to their conservation and informativeness among the closely related species (Ellis and Burke, 2007). SSRs also help increase the power of low taxonomic level distinction in agreement with the "one marker is not enough" approach (Dupuis et al., 2012;

Segatto et al., 2017). The tentative assignment of the commercial cultivar Pierre to the large cluster with samples of vars. *virginianum* and *brevistolon* (which are not distinguished here) serves as proof of concept for such analyses in the *C. virginianum* subspecies complex. Although our collection did not allow for reliable distinction between those two varieties, we postulate that the eSSRs developed in this study can serve such a general purpose. Thus, an investigation using broader sampling of *C. virginianum* specimens may clarify the taxonomic classification of the genus at the lowest ranks (Nesom, 2001; Stuessy, 1973, 1977). In addition, this might lead to changes of varietal circumscriptions (Clausen et al., 1939; Duminil and Di Michele, 2009; Lowry, 2012).

In conclusion, our de novo eSSR markers provide a material progress in the analyses of *C. virginianum* complex in several aspects. As exemplified with the analyzed collection, the eSSRs can be used for species diversity studies, including conservation research. By applying the eSSR genotyping and downstream analyses, we achieved reliable separation of the *Chrysogonum* varieties, confirmed the molecular distinction of a tentative new variety, and postulated an enhanced separation for the vars. *virginianum* and *brevistolon*. Finally, as demonstrated for the commercial cultivar Pierre, the eSSRs can be used for enhancing the future breeding or hybridization efforts of this ornamental plant.

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