Development of simple sequence repeat markers for *Chamerion angustifolium* (Onagraceae)

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**PREMISE:** Rosebay willow herb, or fireweed (*Chamerion angustifolium* (L.) Holub: Onagraceae), is a protandrous, insect-pollinated perennial herb. It has three different cytotypes: diploids, tetraploids, and hexaploids (Mosquin, 1966, 1967). Ploidy level is known to facilitate outcrossing in this species (Routley and Husband, 2003). Hardy and drought tolerant, *C. angustifolium* is common and widely distributed across temperate Europe, Asia, and North America (Mosquin, 1966, 1967; Chen et al., 1988, 2007). Different *C. angustifolium* ploidy levels are associated with environmental factors; for example, diploid populations occur in higher latitudes and at higher elevations, whereas tetraploids occur in generally more temperate environments, and hexaploids favor warmer climates (Mosquin, 1966, 1967; Chen et al., 1988, 2007; Husband and Schemske, 1998). In *C. angustifolium,* certain morphological traits such as plant height, pollen size, and the number of pollen pores are also known to be associated with different ploidy levels (Mosquin, 1967). Due to its cytotype polymorphism, *C. angustifolium* has been used as a model organism to study divergence among cytotypes, including adaptation to variation in elevation (Martin and Husband, 2013), microclimate (Thompson et al., 2014), ecophysiological responses to drought (Guo et al., 2016), and the effects of ploidy on reproductive isolation (Husband and Schemske, 2000).

We have developed simple sequence repeat (SSR) markers for *C. angustifolium* to explore genetic differentiation among its three cytotypes. However, several genotypes for each ploidy that share the same electrophoresis band type are indistinguishable from each other. For example, when two alleles (*A* and *B*) are present at a locus in an autotetraploid individual, there are three types of heterozygotes (i.e., *AAAB*, *AABB*, and *ABBB*) that can produce the same band type (i.e., *AB*). This is referred to as “genotyping ambiguity” (Huang et al., 2014). Although there are some methods that can be used to determine the genotype of heterozygous polyploids (Gidskehaug et al., 2011; Serang et al., 2012; Uitdewilligen et al., 2013), this requires additional equipment or next-generation sequencing. Here, we report a newly developed population genetic method to enable accurate analysis for polyploids. This is directly based on the estimation of genetic diversity indices from the allelic phenotypes (i.e., electrophoresis band types).

**METHODS AND RESULTS**

Genomic DNA was extracted from 10 dried leaf samples of *C. angustifolium*. We used double restriction endonuclease EcoRI and MseI digestions to break the genomic DNA into numerous short DNA fragments. From these DNA fragments, DNA segments with simple short repeats were captured by the magnetic bead enrichment protocol (MagneSphere Magnetic Separation products; Promega...
Of the 63 primers, eight pairs were unamplifiable, 33 had amplified alleles were segregated using polyacrylamide gel electrophoresis. We conducted a preliminary test using 10 individuals to exclude unamplifiable, monomorphic, or incorrectly amplified primer pairs. The remaining parameters were manually determined from the electropherogram peaks and then selected the sequences containing SSR motifs. We used DNAMAN version 5.2.2 (Lynnon Biosoft, San Ramon, California, USA) to perform sequence alignment analysis. After removing the sequences of the adapters, and CodonCode Aligner version 5.2.2 (Lynnon Biosoft, San Ramon, California, USA) to perform sequence alignment analysis. After removing the repetitive sequences, SSR primer pairs were designed using Primer Premier 5 (Lalitha, 2000) with the following settings: primer length 18–22 bp, product size 100–300 bp, and the remaining parameters set as default. In total, 63 pairs of primers were designed. We carried out a preliminary test using 10 individuals to exclude unamplifiable, monomorphic, or incorrectly amplified primer pairs. The alleles were segregated using polyacrylamide gel electrophoresis. Of the 63 pairs, eight primers were unamplifiable, 33 had amplified polymorphic bands, and 22 exhibited nonspecific amplification. We further selected 16 primer pairs with high numbers of alleles, high intensity of electrophoresis bands, and low stutter.

To confirm the polymorphism of these 16 primer pairs, 50 individuals of *C. angustifolium* were collected from three populations in Shaanxi Province, China (Taibai County *n* = 20, Langao County *n* = 19), and Chang’an County *n* = 11; Appendix 1). The sample leaf tissues were cut in 1-cm² pieces, immersed in a 500-μL nuclei extraction buffer (CyStain UV Precise P; Sysmex Partec, Münster, Germany), and quickly chopped to release nuclei using a double-edged blade. The isolated nuclei were stained with stain buffer (CyStain UV Precise P; Sysmex Partec), whose absorbance was measured using a flow cytometer (CyFlow; Sysmex Partec). By comparing the absorbance of *C. angustifolium* to that of a related diploid species (*Epilobium hirsutum* L.), the cytotypes of all individuals were calculated.

These 16 selected fluorescent primer pairs (Table 1) were amplified for capillary electrophoresis to genotype. The allelic phenotypes were manually determined from the electropherogram peaks. For reference purposes, these same 16 primer pairs were also amplified in five samples each of *E. palustre* L. and *C. conspersum* (Hausskn.) Holub (Appendix 1).

Due to genotype ambiguity (Huang et al., 2014), the true genotypes of polyploids cannot be revealed by electrophoresis band types from PCR-based codominant markers. We developed a method to

**TABLE 1.** Characteristics of 16 polymorphic simple sequence repeat markers developed for *Chamerion angustifolium*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Expected allele size (bp) | *T*ₚ (°C) | Fluorescent label* | GenBank accession no. |
|-------|-------------------------|--------------|--------------------------|-----------|------------------|---------------------|
| WK-P01 | F: ACCAGGATTAGCAGAAGGCAA<br>R: GAAATCCGGAATAAGAGGAGGA | (CTAT)₂₀ | 171 | 50.6 | TAMRA | MK153166 |
| WK-P02 | F: ACCTACAATAGCAGTGGACA<br>R: GCTAAAAATGGCCAGTACTCT | (CTCA)₁₁ | 251 | 46.1 | HEX | MK153167 |
| WK-P03 | F: ATCTCTTGAGCAGCCTTGTA<br>R: TTGACCGACAACCCGGACAT | (GAGT)₄ | 107 | 60.7 | FAM | MK153168 |
| WK-P10 | F: GAAATCACACTAGCAGGATC<br>R: CCGTGAGTGAAGAAGAGTGG | (AG)₁₀ | 218 | 50.4 | HEX | MK153169 |
| WK-P11 | F: CTGAGAAATGATGAGGTTGG<br>R: GAGCATGACAACTGACAAGGAC | (AG)₆ | 232 | 47.8 | HEX | MK153170 |
| WK-P20 | F: TGAGCAACTTTGAGATCAGT<br>R: CACCCCTCCCTCTGATAGGG | (GA)₁₃ | 134 | 53.8 | FAM | MK153171 |
| WK-P25 | F: CACCATTGTCATTCAAGTAA<br>R: GATATTTCCAACAGTACATCA | (GA)₁₂ | 137 | 52.0 | FAM | MK153172 |
| WK-P27 | F: AGTCCTCGGACCTGGAGG<br>R: GGTCATGTCCTCTGAGGG | (AG)₁₂ | 164 | 58.8 | TAMRA | MK153173 |
| WK-P32 | F: CGGCGGACAAGCTGTGCTTTAC<br>R: CCGATCCTGCTGGTCGAAA | (AG)₁₂ | 126 | 56.9 | FAM | MK153174 |
| WK-P34 | F: ATCTGATCTGACGGACCTGGA<br>R: CGCTACCCGAGCACACATTACA | (GT)₁₆ | 158 | 54.8 | FAM | MK153175 |
| WK-P35 | F: GCTTTCCGAGTGGCGAACC<br>R: TCTCCCTCAGTGTACCTCCCAT | (CT)₁₂ | 108 | 57.2 | FAM | MK153176 |
| WK-P38 | F: GATCAAGATACAAAGGGCA<br>R: GGTGAGTAAGAAATCAAGAAA | (CT)₁₁ | 213 | 48.2 | HEX | MK153177 |
| WK-P41 | F: AGCAGGAGTTCGACAGTG<br>R: AGCGAGGTTGAGTAAGG | (CT)₁₄ | 141 | 54.2 | TAMRA | MK153178 |
| WK-P43 | F: AGTCCCTCCTGCCCTTCTGG<br>R: ATGGGAAACCTTGGCTTG<br> | (GA)₁₂ | 171 | 53.7 | HEX | MK153179 |
| WK-P44 | F: GAATTTCCCTGATTGCCGG<br>R: TAGACGCTTGTACTGAGG<br> | (CT)₁₂ | 106 | 53.8 | HEX | MK153180 |
| WK-P58 | F: ATAGACTCATTATTTTAGGTT<br>R: CCACTACTGCTGTGTCGAC | (AC)₁₁ | 143 | 51.2 | TAMRA | MK153181 |

Note: *T*ₚ = annealing temperature.

* Fluorescent label placed at the 5′ end of each primer.
TABLE 2. Polymorphism of the 16 simple sequence repeat sites in three populations of *Chamerion angustifolium*.a

| Locus       | Chang’an County population (n = 11) | Taibai County population (n = 20) |
|-------------|-------------------------------------|-----------------------------------|
|             | A | H_e | H_o | PIC | A_s | A | H_e | H_o | PIC | A_s |
| WK-P01      | 8 | 0.746 | 0.742 | 0.699 | 3.881 | 9 | 0.737 | 0.723 | 0.682 | 3.612 |
| WK-P02      | 10 | 0.809 | 0.838 | 0.819 | 6.169 | 13 | 0.754 | 0.733 | 0.756 | 4.397 |
| WK-P03      | 4 | 0.585 | 0.562 | 0.465 | 2.282 | 5 | 0.768 | 0.735 | 0.688 | 3.772 |
| WK-P10      | 11 | 0.729 | 0.750 | 0.715 | 4.006 | 9 | 0.662 | 0.809 | 0.784 | 5.239 |
| WK-P11      | 5 | 0.771 | 0.746 | 0.709 | 3.931 | 9 | 0.709 | 0.805 | 0.784 | 5.135 |
| WK-P20      | 6 | 0.646 | 0.623 | 0.575 | 2.655 | 8 | 0.674 | 0.729 | 0.701 | 3.686 |
| WK-P25      | 12 | 0.843 | 0.801 | 0.774 | 5.013 | 13 | 0.815 | 0.871 | 0.858 | 7.737 |
| WK-P27      | 9 | 0.810 | 0.794 | 0.768 | 4.861 | 7 | 0.685 | 0.708 | 0.681 | 3.429 |
| WK-P32      | 12 | 0.822 | 0.839 | 0.820 | 6.229 | 9 | 0.743 | 0.843 | 0.825 | 6.352 |
| WK-P34      | 12 | 0.899 | 0.840 | 0.820 | 6.269 | 5 | 0.732 | 0.793 | 0.760 | 4.837 |
| WK-P35      | 9 | 0.286 | 0.372 | 0.360 | 1.593 | 8 | 0.429 | 0.473 | 0.458 | 1.897 |
| WK-P38      | 13 | 0.813 | 0.792 | 0.767 | 4.800 | 11 | 0.763 | 0.815 | 0.792 | 5.410 |
| WK-P41      | 7 | 0.806 | 0.756 | 0.718 | 4.106 | 8 | 0.833 | 0.802 | 0.772 | 5.045 |
| WK-P43      | 7 | 0.869 | 0.680 | 0.628 | 3.129 | 8 | 0.719 | 0.711 | 0.673 | 3.465 |
| WK-P44      | 5 | 0.762 | 0.733 | 0.686 | 3.750 | 5 | 0.644 | 0.629 | 0.561 | 2.695 |
| WK-P58      | 5 | 0.639 | 0.676 | 0.636 | 3.087 | 6 | 0.829 | 0.764 | 0.724 | 4.232 |

Note: A = number of alleles per locus per population; A_s = allelic richness per locus within a population (I/∑i=1^p;i); H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled; PIC = polymorphic information content.

*Voucher and location information are provided in Appendix 1.

estimate the genetic diversity indices directly from the allelic phenotypes (Appendix 2). This method extracts the possible genotypes from the phenotype, then estimates the allele frequencies by an iterative algorithm developed by Kalinowski and Taper (2006); the genetic diversities are calculated from the allele frequencies or from the extracted genotypes by using their posterior probabilities as the weight.

Flow cytometry showed that all individuals in the three populations of *C. angustifolium* are hexaploids. The results of microsatellite genotyping show that the number of alleles is between four and 13 in all three sampled populations of *C. angustifolium* (Table 2), and the levels of observed and expected heterozygosity range from 0.286 to 0.899 and from 0.372 to 0.871, respectively. The polymorphism information content values of the 16 loci exceed 0.3, and allelic richness is also high in all populations, denoting high polymorphism of the developed SSR markers. These parameters also suggest high levels of polymorphism among the 16 loci. Table 3 lists the 14 primers that successfully amplified in *E. palustre* and the 12 that successfully amplified in *C. conspersum*.

CONCLUSIONS

We report on the development of 16 new polymorphic SSR markers for *C. angustifolium*. These markers will be useful for future population genetic studies of this species, especially those investigating the effects of both cytotype and geographical distance on genetic differentiation among populations.

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AUTHOR CONTRIBUTIONS

K.H. and B.G.L. designed the project; R.M., K.H., D.W.D., and T.C.W. collected the samples; R.M. and T.C.W. performed the experiments; and R.M., K.H., and D.W.D. wrote the manuscript.
DATA ACCESSIBILITY

Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1.

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APPENDIX 1. Geographic locations and voucher information for species used in this study.

| Species | Location       | Population code | Voucher specimen accession no.* | Geographic coordinates | n   | Ploidy |
|---------|----------------|-----------------|---------------------------------|------------------------|-----|--------|
| C. conspersum (Hausskn.) Holub | Urumqi County, China | WNU-WLMQ-2018-HK-02 | 43.108578°N, 87.060690°E | 5 Diploid |       |
| | Langao County, China | WNU-CA-2017-HK-11 | 33.842662°N, 108.785287°E | 11 Hexaploid |       |
| Chamerion angustifolium (L.) Holub | Taibai County, China | WNU-TB-2017-HK-20 | 34.387486°N, 107.209336°E | 20 Hexaploid |       |
| | Chang'an County, China | WNU-CA-2017-HK-11 | 33.108578°N, 107.060690°E | 11 Hexaploid |       |
| | L. Taibai County, China | WNU-TB-2017-HK-01 | 34.387486°N, 107.209336°E | 11 Hexaploid |       |
| Epilobium palustre L. | Taiyuan County, China | WNU-TB-2018-HK-02 | 43.108578°N, 87.060690°E | 5 Diploid |       |

Note: n = sample size.

*Vouchers deposited at the Herbarium of the College of Life Sciences (WNU), Northwest University, Xi’an, China. HK = Kang Huang, collector.

APPENDIX 2. Analysis of genetic diversity based on allelic phenotypes in polysomic inheritance.

The possible genotypes hidden behind each allelic phenotype are weighted by their genotypic frequencies. The posterior probability of the ith candidate genotype (Gi) can be derived by the Bayes equation:

\[
Pr(G_i | P) = \frac{Pr(P | G_i) Pr(G_i)}{Pr(P)} = \frac{Pr(G_i)}{\sum Pr(G_i)},
\]

where Pr(P | G_i) and Pr(P) are the frequencies of G_i and P, respectively. Pr(P | G_i) is the probability that P is the allelic phenotype of the individual with a genotype of G_i, then Pr(P | G_i) is equal to one. We ignore any double-reduction (Butruille and Boiteux, 2000) and assume the alleles randomly appear within the genotype. Therefore:

\[
Pr(G) = v! \prod_{i=1}^{k} \frac{P_{ik}^{n_i}}{n_i!},
\]

where ki is the number of ploidy, K is the number of alleles, ni is the number of copies of the ith allele in G_i, and pi is the frequency of the ith allele.

The allele frequencies are estimated with an iterative algorithm modified from Kalinowski and Taper (2006): the frequency of each allele is assigned with an initial value of 1/K, then iteratively updated until it is converged. The updated frequency \( \hat{P}_k \) is calculated by:

\[
\hat{P}_k = \frac{\sum Pr(G_i | P) Pr(A_k | G_i)}{\sum Pr(G_i | P)}=
\]

where Pr(A_k | G) is the frequency of the kth alleles in G. Based on the estimated allele frequency, the genetic diversity indices can be calculated, including allelic richness, polymorphic information content, and both observed and expected heterozygosities. The heterozygosity in polysomic inheritance is defined as the probability of sampling two distinct alleles from a genotype without replacement. Our method has been implemented in Polygene version 1.0 (Huang et al., 2019; https://github.com/huangkang1987/polygene/).