Development and Characterization of Microsatellite Loci for the Pseudometallophyte Commelina communis (Commelinaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR THE PSEUDOMETALLOPHYTE *COMMELINA COMMUNIS* (COMMELINACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed for the pseudometallophyte *Commelina communis* (Commelinaceae), an important pioneer plant for phytoremediation of copper-contaminated soil. Two wild populations collected from metalliferous and nonmetalliferous sites were used to assess the polymorphism at each locus.
- *Methods and Results:* Based on the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) method, a total of 34 pairs of simple sequence repeat (SSR) markers were designed. When 40 specimens from two populations were screened, 12 microsatellite loci were found to be highly polymorphic. The number of alleles per locus ranged from one to 11 and the observed and expected heterozygosity per locus ranged from 0.000 to 1.000 and from 0.195 to 0.941, respectively.
- *Conclusions:* These markers will be useful for examining genetic diversity, population structure, and gene flow in populations of *C. communis* under different edaphic conditions and guiding sustainable management plans for phytoremediation.

Key words: *Commelina communis*; Commelinaceae; genetic diversity; microsatellites; pseudometallophyte.

Commelina communis L. (Commelinaceae), commonly known as dayflower, is a pseudometallophyte (facultative metallophyte) that is distributed extensively on both cupriferous habitats and surrounding nonmetalliferous sites (Tang et al., 1999, 2001; Ye et al., 2012). It is an annual multibranched herb that exhibits sexual reproduction and clonal propagation (Tang et al., 1999; Ushimaru et al., 2007; Ye et al., 2012). This species can also accumulate extraordinarily high concentrations of copper, with foliar Cu concentration reaching as much as 1000 mg/kg (Tang et al., 1997; Shu et al., 2001). Because of its good reproductive capacity and high biomass production, *C. communis* has been considered an important pioneer plant for phytoremediation of copper-contaminated soil and restoration of mined land (Tang et al., 1997, 1999).

To effectively use wild metal-tolerant plants for phytoremediation and ecological restoration, an accurate knowledge of their life history traits and population genetics (notably gene flow, breeding system, and genetic diversity organization) is needed (Salt et al., 1998; Escarré et al., 2000). Compared with dominant markers such as random-amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR) markers are useful in studies of genetic diversity, population genetic structure, and genome mapping because of their high level of polymorphism and codominance (Jarne and Lagoda, 1996; Zhang and Hewitt, 2003). However, microsatellite loci have yet to be developed in *C. communis* or in congeneric species. In this study, we developed and characterized 12 polymorphic microsatellite loci for *C. communis* and tested the applicability of these SSR loci to estimate the genetic diversity of *C. communis* in metallicolous and nonmetallicolous populations.

METHODS AND RESULTS

Two populations of *C. communis* were sampled in Central China (nonmetalliferous population CS: Changsha, Hunan Province; metalliferous population YP: Jiangxi Province; Appendix 1). Voucher specimens (CS: WH06051793, YP: WH06051794) were deposited at the Wuhan University Herbarium (WH). Soil Cu concentrations at these sampling sites were measured following the method described by Ye et al. (2012) (Appendix 1). Total genomic DNA was extracted from silica gel–dried leaves of one individual of *C. communis* sampled from the CS population using the QIAGEN DNA Extraction Kit (QIAGEN, Hilden, Germany). Microsatellite loci from an enriched $(AG)_n$ library were isolated following the procedure of Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) (Zane et al., 2002). Approximately 250 ng of genomic DNA was completely digested by *Mse*I (Fermentas, Burlington, Ontario, Canada) and then ligated to an *Mse*I adapter pair (F: 5′-TACTCAGGACTCAT-3′; R: 5′-GACGATGAGTCCTGAG-3′) with T4 ligase (Promega Corporation, Madison, Wisconsin, USA). A total of 5 µL of a 10-fold diluted digestion-ligation mixture was directly amplified with 1 µL of the *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′; 25 µM), 1 unit of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, China), 2 µL of 10× PCR buffer, 1.6 µL of dNTPs (2.5 mM each), and 1.2 µL of $MgCl_2$ (25 mM) in a total volume of 20 µL using the following thermocycler conditions: 3 min of denaturation at 95°C; followed by 26 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 53°C, and 1 min of extension at 72°C; with a final extension of 72°C for 5 min.

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TABLE 1. Characteristics of 34 microsatellite loci developed in *Commelina communis.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye	GenBank accession no.
YP5	F: CACGGCTGATGGGAAGTTG R: CTATGCATGCTTGGTTGAT	$(CT)_7$	164–204	54	6-FAM	KJ647361
YP6	F: AAGCATTCTCCATTGTAAA R: AGAAATGTGGGGGATAAA	$(CT)_{10}$	102–138	55	6-FAM	KJ647362
YP7	F: AAGCACTCTCCACTGTAAT R: AGAAATGTAGGGGATAAAC	$(TC)_{17}$	140	55	6-FAM	KJ647363
YP8	F: TTAAGGCTGGCATAACCCC R: AAACTTGCTGGACAGAAGA	$(CT)_{22}$	173	57	ROX	KJ647364
YP9	F: ATTCAACGAAAAAGGGTAG R: CTTGGGATTGTTAGAGAGAT	$(TCC)_{10}$	162	53	ROX	KJ647365
YP10	F: AAGCACTCTCAACTGAAAAT R: CAGTACAAGGAGACATAGA	$(TC)_{13}$	133–157	55	ROX	KJ647366
YP11	F: GAATTCACAAATAACCACCA R: GACGCTCAAGTCAATAAAG	$(CT)_9$	129	57	6-FAM	KJ647367
YP12	F: CAACCAGAGAAGAGGATAA R: CTCGGTGTGAAAATGAAGA	$(AG)_{34}$	260	56	HEX	KJ647368
YP13	F: CATGTTATGGTTGCAAGCTC R: ACCGATATATGATCCCGTCC	$(TC)_{12}$	199	58	HEX	KJ647369
YP14	F: GCTTTTAGGATAACTTCAAC R: GAGGACATTTAGGGCACAT	$(TC)_{10}$	202–218	55	HEX	KJ647370
YP15	F: TTAAACTGAGCATAGGTCCG R: TGTGGAGAAGGAAGGAGAG	$(TC)_{19}$	201	57	ROX	KJ647371
YP16	F: GGTTGCAAGTTCTCTTTTTA R: GTGCTTGTTCACTTCATCAT	$(TC)_6…(CT)_8$	317	59	HEX	KJ647372
YP17	F: TAGTATGGTTGCAAGCTCT R: CCAATAATGTATCTCTAGTC	$(TC)_{19}$	238	56	ROX	KJ647373
YP18	F: CCTTTTCAACGCATATACA R: TCTTCCAGGATCAAGTCAA	$(CT)_{13}$	99–127	55	HEX	KJ647374
YP19	F: AGGTTGAGTGGATAAAATG R: GAAGGAGAAAGGTGATGAG	$(CT)_{10}$	192	60	ROX	KJ647375
YP20	F: CTCCAACCTTGCCATCCTAC R: TCTGGTGCTCCAAATCCTGC	$(TC)_{11}$	149	55	ROX	KJ647376
YP21	F: AGCACGCTATTACATTCCAG R: CACACACAAAAAAGTCAAG	$(GA)_{11}$	121	53	6-FAM	KJ647377
YP22	F: AGTTGGCAAAAGAAAGGGA R: GCAGTATGTGACGGAGGGT	$(TC)_{23}$	260	56	ROX	KJ647378
YP23	F: GGGATTTTAGATTCTAATTG R: GCATACTGCTTTTCTTGTT	$(TC)_{15}$	192	54	HEX	KJ647379
YP24	F: CACATACTTGTAAGGCTAC R: TTAATATAGTGGAATTGGC	$(CT)_{26}$	107	55	6-FAM	KJ647380
YP25	F: AAATACGTCAAATGTTCTG R: ACCTGAGTCAACGACAAAC	$(CT)_{17}$	118	55	HEX	KJ647381
YP26	F: ACAAAGAGGGGAGGCAACA R: GGAGAAAGGGGAAAGGTGA	$(CT)_{25}$	234	58	6-FAM	KJ647382
YP27	F: TCTTCGATCTTCGTTCTTC R: CCAACTCACACTCTCATTTT	$(TC)_{14}$	121	58	6-FAM	KJ647383
YP28	F: TTAACGTTCACCTCACATG R: GGCTTATATCAAATCCTACT	$(CT)_{15}$	208–239	55	6-FAM	KJ647384
YP29	F: TGAGGAAAGATGACTGTTG R: GTTATGTGATGACGTACCA	$(TC)_{12}$	125	59	ROX	KJ647385
YP30	F: AGTATTGCGACACGAGACA R: GAAAACTAAGCCGAGATGA	$(TC)_{18}$	238	60	ROX	KJ647386
YP31	F: AAATTCAAATTCGATAGCA R: AGAGTTTATATTTTGTGGA	$(TC)_7…(CT)_6$	84–120	56	ROX	KJ647387
YP32	F: CATCAACAAGAACTTGCCA R: CAATCAATGGTTGAGGCTA	$(TC)_8$	178	59	HEX	KJ647388
YP33	F: ATTATCTCTATGCCATCTCC R: TTCTCTCCCTACACTTTTCT	$(AG)_{18}$	295–319	55	HEX	KJ647389
YP34	F: GCGTATTCAACCTGACAAAC R: ACAAGAAGGGACATCAAAA	$(TC)_{16}$	161–191	56	ROX	KJ647390
YP35	F: GTTAGTCGAGTTACCAGCCT R: GAAATGTTCTACGCCAATAG	$(CT)_{19}$	256	55	ROX	KJ647391
YP36	F: CTCCCTTGTCCTCCCTCTTC R: GGACTTTTTGGTTATTTGGT	$(CT)_{14}$	194–206	57	ROX	KJ647392
YP37	F: CAACAAATTGTTTTGAGAAT R: AAGTGGTGTCTAACGATTGC	$(CA)_7…(AG)_{16}$	205–231	54	ROX	KJ647393
YP38	F: ATATTTATTCCTTTTTCCCC R: TATCATCACAAGCTCGCTAC	$(TC)_{14}$	126–178	58	6-FAM	KJ647394

Note: T_a = annealing temperature.

TABLE 2. Results of initial primer screening in two populations of *Commelina communis*.[a]

	YP (N = 20)			CS (N = 20)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]
YP5	3	0.200	0.195	5	0.500	0.442
YP6	4	0.200	0.284**	3	0.500	0.532
YP10	11	1.000	0.941	9	0.600	0.879**
YP14	6	0.400	0.621**	9	0.500	0.905**
YP18	2	0.000	0.667*	6	0.429	0.791*
YP28	1	0.000	0.241*	2	0.000	0.533*
YP31	4	0.300	0.363	5	0.000	0.758**
YP33	2	0.250	0.250	6	0.625	0.767
YP34	8	0.300	0.863**	9	0.400	0.879**
YP36	3	0.300	0.532	3	0.500	0.426
YP37	3	0.000	0.800**	5	0.000	0.80**
YP38	7	0.500	0.853**	3	0.000	0.653**
Average	4.5	0.288	0.551	5.4	0.338	0.697

Note: A = total number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population.

[a] See Appendix 1 for population locality information.

[b] Deviations from Hardy–Weinberg equilibrium: $*P < 0.05$, $**P < 0.01$.

Amplified DNA fragments with a range of 200–800 bp were enriched for microsatellite repeats by magnetic bead selection with 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$ probes. Nonspecific DNA fragments were removed by three nonstringency washes with TEN1000 (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl [pH 7.5]) and then three stringency washes using 0.2× saline sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS). After stringent washing, the enriched DNA fragments were eluted in 50 µL of 1× TE and then amplified with *Mse*I-N primers for 26 cycles as described above. The PCR products were purified using a Gel Extraction Kit (TaKaRa Biotechnology Co.) according to the manufacturer's instructions. The purified PCR products with enriched microsatellite repeats were ligated into pGEM-T vector (Promega Corporation) and transformed into DH5α competent cells (TransGen Biotech, Beijing, China). Recombinant clones were screened by blue/white selection, and positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$ and T7/Sp6 as primers.

The 125 clones with positive inserts were sequenced with an ABI PRISM 3730xl DNA sequencer (Applied Biosystems, Foster City, California, USA). These sequences were analyzed for microsatellite repeat motif regions using the software SSR Hunter (Li and Wang, 2005). Of the 125 sequences analyzed, 90 had microsatellite motifs. After exclusion of redundant sequences, 42 high-quality sequences were selected for microsatellite primer design using OLIGO 7.0 software (Rychlik, 2010) and evaluated in 20 individuals from the CS population. Thirty-four pairs of primers (Table 1) that showed single and clear bands were chosen and labeled with the fluorescent dyes 6-FAM, ROX, or HEX (Invitrogen, Carlsbad, California, USA). Polymorphisms at the 34 SSR loci were assessed using 40 individuals from two populations of *C. communis* (Appendix 1), each with 20 individuals. Amplifications were performed in a total volume of 20 µL containing 30–50 ng genomic DNA, 0.6 µM of each primer, 7.5 µL of 2× *Taq* PCR MasterMix (0.1 unit *Taq* polymerase/µL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, 3 mM $MgCl_2$; Tiangen, Beijing, China). The thermocycling conditions were: 95°C for 3 min, 30 cycles of 94°C for 30 s, with the annealing temperature optimized for each specific primer for 30 s (Table 1), 72°C for 60 s, and a final extension step at 72°C for 7 min. The amplified products were separated using an ABI PRISM 3730xl DNA sequencer with GeneScan 600 LIZ (Applied Biosystems) as an internal size standard, and the sizes were determined using GeneMapper version 4.0 (Applied Biosystems).

Out of the 34 primer pairs, 12 primer pairs displayed polymorphism among individuals of the two populations of *C. communis* (Table 1). For each population, the number of alleles per locus (A), observed and expected heterozygosity (H_o and H_e), deviation from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between all pairs of polymorphic loci were analyzed using GENEPOP version 4.2 (Rousset, 2008). Across the two populations of *C. communis*, A ranged from one to 11, with a mean of 4.5 in the YP population and 5.4 in the CS population. The H_o and H_e per locus ranged from 0.000 to 1.000 and from 0.195 to 0.941, respectively. A relatively high level of genetic diversity was found in the CS population (H_o = 0.338, A = 5.4) compared with the YP population (H_o = 0.288, A = 4.5). Some loci showed significant deviation from HWE (Table 2).

CONCLUSIONS

The SSR markers developed here will enable the estimation of genetic diversity in populations of *C. communis* under different edaphic conditions, and will be helpful for exploring the origin and evolutionary history of metallicolous populations under heavy metal stress in this pseudometallophyte. Their use at larger scales will provide detailed information on the genetic consequences of heavy metal concentration on *C. communis* that may guide the sustainable management plans for phytoremediation and ecological restoration.

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Appendix 1. Voucher and location information for populations of *Commelina communis* used in the study and the total Cu concentrations in the substrates associated with plants sampled. The voucher specimens are deposited in the Wuhan University Herbarium (WH).

Edaphic type	Population code	Population locality	Geographic coordinates	Concentration of Cu in substrates (mg/Kg, mean ± SD)	Voucher no.
Metallicolous	YP	Yongping, Jiangxi Province, China	28°19′N, 117°78′E	2500 ± 128	WH06051794
Nonmetallicolous	CS	Changsha, Hunan Province, China	28°17′N, 112°94′E	80 ± 15	WH06051793