Conservation Genet Resour (2014) 6:527–529
DOI 10.1007/s12686-014-0170-3

TECHNICAL NOTE

Characterization of 15 novel microsatellite loci for *Cypripedium calceolus* (Orchidaceae) using MiSeq sequencing

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Received: 2 February 2014 / Accepted: 24 March 2014 / Published online: 2 April 2014

Abstract Lady's slipper orchid (*Cypripedium calceolus*) serves as a flagship species for plant conservation in many European countries. Its populations are threatened by over-collecting and loss of suitable habitat. Information on local and regional genetic structure can help to develop appropriate conservation strategies. A total of fifteen novel microsatellite markers were developed using MiSeq sequencing. All loci found to be polymorphic, with the number of alleles per locus ranging from 2 to 8. Observed heterozygosity ranged from 0.19 to 0.89. The developed microsatellite markers will be useful to analyze genetic diversity and genetic structure of *C. calceolus* populations.

Keywords Genetic diversity · Genetic structure · Microsatellites · Orchidaceae

Lady's slipper orchid (*Cypripedium calceolus* L.) is one of the largest and the most spectacular elements of European flora. This Euro-Asiatic species has suffered a marked decrease of localities and area occupied (Terschuren 1999). It is legally protected in all European countries, and listed in various national conventions and directives (Terschuren 1999). Studies of genetic diversity and fine-scale spatial genetic structure in relation to habitat management will improve management strategies of this vulnerable species. Two, out of four recently published microsatellite loci for *C. calceolus,* may pose scoring problems (Pedersen et al. 2012). Therefore a higher number of variable loci is needed to estimate genetic structure and distinguish close relatives. Here we report the isolation and characterization of polymorphic loci that will be useful in future population genetic studies.

Seeds of *C. calceolus* were collected from population Bukówki (Poland) and asymbiotically germinated in in vitro culture. Total genomic DNA was isolated from three seedlings using procedure described by Bekesiova et al. (1999). Extracted DNA was used for a library preparation with a NEBNext® DNA Library Prep Master Mix Set for Illumina. The sequencing was performed on the MiSeq Benchtop Sequencer (Illumina) using the 2 × 250 bp read mode. The obtained data (10,19 Mega reads) was assembled using CLCGenomicWorkbench (CLCBio) into 513,225 contigs and the microsatellites were then detected using QDD 2.1 Beta (Meglecz et al. 2010). A total of 22,162 contigs contained at last one microsatellite of which 53 loci were selected for initial screening. We screened 32 plants from two Polish populations (Bukówki and Prokowo) for polymorphisms at these loci. All forward primers were tagged with M13(-21) (5′-TGTAAAACGACGGCCAGT-3′) at the 5′ end. The 10 μL PCR volume contained: 4.5 μL MyTaq™ HS Mix (Bioline), 0.4 μM of both forward and reverse primers, 0.2 μM dye labelled primer, 0.3 % DMSO, 1.2 μL water and 1 μL DNA template (∼50 ng). The following PCR conditions were used: 2 min initial denaturation at 96 °C, followed by 35 cycles of 95 °C for 30 s/primer specific annealing temperature for 30 s/72 °C for 45 s. Next, the reaction was paused at 72 °C and fluorescently labelled (6-FAM, NED, PET or VIC) M13 primer was added. Amplification was then continued for the next 10 cycles

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Table 1 Characteristics of 15 loci for *Cypripedium calceolus*

Locus	Primer pair sequence (5′–3′)	Repeat motif	T_m	Size range (bp)[a]	Accession no	All N_A	Prokowo				Bukówki			
							N	N_A	H_O	H_E	N	N_A	H_O	H_E
Ccal _5	CCACAAAGCCACACTACATAACA TGTAAGGGTGATCTTGGAAAGC	$(AG)_9$	62	141–143	KJ130946	2	16	2	0.44	0.34	16	1	0.00	0.00
Ccal _7	TAAGCACTTCTTGGGAGGCA GAGGTTGAGCACAAGAAAGAAA	$(AGT)_{12}$	60	195–207	KJ130947	4	15	4	0.53	0.60	16	3	0.81	0.58
Ccal _9	AGAAGAGATGTGGGAAGCCC CTTCCAAGCTCCAGCTACCA	$(AT)_{10}$	59	122–124	KJ130948	2	14	2	0.43	0.46	16	2	0.44	0.50
Ccal_19	CGAGCCTCTCGACAACATCT TGGTGGTTGTTTGGTGTGAA	$(AT)_{10}$	60	291–293	KJ130949	2	11	1	0.45	0.43	16	2	0.50	0.52
Ccal_24	GGCTTATAGAGAGAGAGGCTATGG CATGGGAGCTGACTCATCAT	$(ATC)_{13}$	59	118–123	KJ130950	5	16	4	0.87	0.70	16	5	0.87	0.72
Ccal_25	CAGCATTTTGCTCAATGTCTTT CAGATAATGGCCCTTTGGTC	$(ATC)_{16}$	60	162–180	KJ130951	8	16	5	0.87	0.76	16	7	0.69	0.78
Ccal_31	GGCAATGTCATTAGGGGAAG GGGGTTCAAGTAGCATAAGACAA	$(AT)_{10}$	62	115–119	KJ130952	3	16	2	0.19	0.27	16	2	0.12	0.12
Ccal_34	CATGGAAGGGAATAACATCCT TGCAATTCCATGTACTTGTTCATTA	$(AT)_{10}$	58	128–136	KJ130953	4	15	3	0.47	0.37	16	3	0.50	0.43
Ccal_39	TTCTCCTCAAAGAATGATTCCA CCATTGGGCAATTCACTCAT	$(AC)_{15}$	59	154–164	KJ130954	6	8	5	0.89	0.67	8	5	0.75	0.70
Ccal_47	AAGGCTCAAGATCCCAAGGA ATCATTATGGTTGTCTCTTTATCGTT	$(AAC)_{11}$	60	127–136	KJ130955	4	15	3	0.33	0.34	15	3	0.33	0.34
Ccal_48	CAATAAGCTAAGTGAGTAGCAGGTTG AGGTTCTTCCTTTCACTTCACTACC	$(AAC)_{12}$	61	141–150	KJ130956	4	15	4	0.27	0.24	15	4	0.27	0.24
Ccal_49	TGGAAGGGTCATGTTACTAGCAG TGGTGATGACACAACTAACTCCA	$(AAG)_{13}$	58	115–143	KJ130957	6	16	4	0.69	0.66	15	6	0.93	0.76
Ccal_50	GAGAAGGGATTCAATAGGTTTGG AAGTTCCTTCTCATTTCTAGCTCTC	$(AAG)_{10}$	60	122–134	KJ130958	6	16	3	0.56	0.47	16	5	0.53	0.67
Ccal_51	CCCTCCACCCATTCTCTAGC ATCTGTTGAAGGTGTTCGGC	$(AAG)_8$	60	174–177	KJ130959	2	15	2	0.33	0.28	15	2	0.33	0.28
Ccal_53	CCTACCTCCACCCTGACACA TGAGGCCTAGGCTAGCAAGT	$(AAG)_{13}$	60	164–185	KJ130960	6	8	4	0.50	0.61	9	4	0.33	0.57

N number of individuals analyzed, N_A number of alleles per locus, H_O observed heterozygosity, H_E expected heterozygosity

[a] Product size including M13 tag

according to Arruda et al. (2010) with 15 min of final extension at 72 °C. Samples were run on an ABI 3730 DNA Analyzer and analyzed with GeneMarker 2.2.0 (SoftGenetics) using GS-500 (LIZ) as a size standard.

The number of alleles per locus, departures from Hardy–Weinberg equilibrium (HWE), and heterozygosity for two Polish populations were calculated in GenAlEx (Peakall and Smouse 2012). The presence of linkage disequilibrium (LD) was tested in Arlequin (Excoffier and Lischer 2010). Presence of null alleles and scoring errors were checked using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). The statistical significances in multiple statistical tests were adjusted by the Bonferroni corrections (Rice 1989). A set of 18 SSR was polymorphic. Three of them showed evidence for presence of null alleles and were excluded. Remaining loci showed no evidence of LD after sequential Bonferroni correction. Number of alleles per locus for remaining 15 SSR ranged from 2 to 8 (Table 1) with mean of 4.3. In the studied populations, the mean N_A ranged from 3.27 to 3.6. Observed (H_o) and expected (H_e) heterozygosity ranged from 0.19 to 0.89 and 0.12 to 0.71 respectively. None of the loci showed deviation from Hardy–Weinberg equilibrium. The 15 polymorphic SSR loci reported in this study will be useful for assessing genetic diversity, population structure and parentage analysis.

Acknowledgments This work was supported by the Polish National Science Centre grant No. 3984/B/PO1/2010/39.

References

Arruda MP, Gonçalves EC, Schneider MPC, da Costa da Silva AL, Morielle-Versute E (2010) An alternative genotyping method using dye-labeled universal primer to reduce unspecific amplifications. Mol Biol Rep 37:2031–2036. doi:10.1007/s11033-009-9655-7

Bekesiova I, Nap JP, Mlynarova L (1999) Isolation of high quality DNA and RNA from leaves of the carnivorous plant *Drosera rotundifolia*. Plant Mol Biol Rep 17:269–277. doi:10.1023/A:1007627509824

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10:564–567. doi:10.1111/j.1755-0998.2010.02847.x

Meglecz E, Dubut V, Gilles A, Malausa T, Pech N, Martin JF (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinformatics 26(3):403–404. doi:10.1093/bioinformatics/btp670

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28:2537–2539. doi:10.1093/bioinformatics/bts460

Pedersen HA, Rasmussen HN, Kahandawala IM, Fay MF (2012) Genetic diversity, compatibility patterns and seed quality in isolated populations of *Cypripedium calceolus* (Orchidaceae). Conserv Genet 13:89–98. doi:10.1007//s10592-011-0267-0

Rice WR (1989) Analyzing tables of statistical test. Evolution 43:223–225. doi:10.2307/2409177

Terschuren J (1999) Action plan for Cypripedium calceolus in Europe. Nature and Environment no. 100. Council of Europe Publishing, Strasbourg

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotypig errors in microsatellite data. Mol Ecol Notes 4:535–553. doi:10.1111/j.1471-8286.2004.00684.x