American Journal of Botany

American Journal of Botany: e490–e492. 2012.

*AJB* Primer Notes & Protocols in the Plant Sciences

# Microsatellite markers developed for *Corallodiscus lanuginosus* (Gesneriaceae) and their cross-species transferability[1]

Lian-Ming Gao[2], Zhi-Rong Zhang[3], Ping Zhou[4], Michael Möller[5,6] and De-Zhu Li[2,3,6]

[2]Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, Yunnan, People's Republic of China; [3]Plant Germplasm and Genomics Center, Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, Yunnan, People's Republic of China; [4]Xishuangbanna Tropical Botanic Garden, Chinese Academy of Sciences, Kunming 650223, Yunnan, People's Republic of China; and [5]Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR, Scotland, United Kingdom

- *Premise of the study: Corallodiscus* is widespread across China and in adjacent countries to the west and south, with *C. lanuginosus* covering the entire range of the genus. Microsatellite markers will be useful to address within-complex taxonomic and biogeographic structures of this species.
- *Methods and Results:* Fourteen markers were developed using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol. Polymorphism was assessed in 12 individuals each from two populations from West Yunnan, China. Nine primers displayed polymorphisms. The number of alleles per locus ranged from one to six (mean: 2.7). The observed and expected heterozygosities ranged from 0 to 1 and from 0 to 0.772 (average: 0.487 and 0.448). Only one of the polymorphic loci deviated from Hardy–Weinberg equilibrium. Among the four congeneric species, cross-amplification success ranged from two to eight markers.
- *Conclusions:* The markers will be useful for population genetic and evolutionary history studies for *C. lanuginosus* and the allied species *C. bhutanicus*, *C. cooperi*, and *C. kingianus*.

**Key words:** *Corallodiscus lanuginosus*; Gesneriaceae; microsatellite marker; polymorphism; population genetics.

The genus *Corallodiscus* Batalin (Gesneriaceae) was first established in 1892 (Batalin, 1892), based on a specimen of '*C. conchaefolius*' (*C. conchifolius* Batalin). Craib (1919a, b) described 16 further species, but described them as *Didissandra* C. B. Clarke. Burtt (1947) transferred all of Craib's species to *Corallodiscus*, because Craib's placement in *Didissandra* conflicted with the work of Ridley (1905), who used the name *Didissandra* for another group of plants from the Malay Peninsula. Burtt's (1947) taxonomic work resulted in 18 species in *Corallodiscus*. Of these, 16 species came from China, and *C. cooperi* (Craib) B. L. Burtt and *C. bhutanicus* (Craib) B. L. Burtt came from Bhutan. Further changes were made by Chinese taxonomists. For the *Flora of China*, Wang et al. (1990) revised *Corallodiscus* based on Craib and Burtt's taxonomy and reduced the 16 Chinese species to nine. In the later English version of the *Flora of China*, Wang et al. (1998) further reduced the species from 11 to five: *C. bhutanicus*, *C. conchifolius*, *C. cooperi*, and *C. kingianus* (Craib) B. L. Burtt were all retained, and the remaining species were included in a fifth, representing the *C. lanuginosus* complex. The authors found morphological variation in the complex in vegetative and generative characters, and suggested that "the variation in all of these characters is continuous and is not correlated with the variation in other characters. For this reason it was impossible to clearly delimit previously recognized taxa. Thus, we recognize one, highly variable species, but further studies are needed to understand the variation."

With a background of such complex morphological variation and the taxonomic uncertainty of the *C. lanuginosus* complex, population genetics approaches such as microsatellite markers seem an appropriate method to investigate the *C. lanuginosus* complex. Consequently, we have developed and characterized 14 microsatellite markers for *C. lanuginosus* (Wall. ex DC.) B. L. Burtt using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol (Zane et al., 2002).

## METHODS AND RESULTS

Total genomic DNA was isolated from silica gel–dried leaves of a single individual following the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) with minor modifications. The microsatellite loci were isolated using the FIASCO protocol. Approximately 500 ng of DNA was digested with *Mse*I (New England Biolabs, Beverly, Massachusetts, USA), and the fragments ligated to the *Mse*I AFLP adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) at 37°C for 2 h with T4 DNA ligase

[1]Manuscript received 13 April 2012; revision accepted 28 June 2012.

Laboratory work was carried out at the Germplasm Bank of Wild Species in Southwest China, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). We are grateful to Jun-Bo Yang for his technical assistance. This study was supported by a CAS Visiting Professorship for Senior International Scientists (grant no. 2011T1S20) and the Talent Project of Yunnan Province (grant no. 2008YP064). The Royal Botanic Garden Edinburgh is funded by the Rural and Environment Science and Analytical Services division in the Scottish Government.

[6]Authors for correspondence: m.moeller@rbge.org.uk, dzl@mail.kib.ac.cn

doi:10.3732/ajb.1200178

Table 1. Primer sequences and characteristics of 14 microsatellite loci successfully amplified in *Corallodiscus lanuginosus.*

| Locus | Primer sequences (5′–3′) | Repeat motif | Size range (bp) | $T_a$ (°C) | GenBank accession no. |
|---|---|---|---|---|---|
| Cora011* | F: TATTCTATCACCCTGGTCAT | $(TC)_{25}$ | 232–250 | 50 | JQ743036 |
| | R: ATTGAAGAACACGACCCACA | | | | |
| Cora014 | F: GGTTGTGCATGTGGCTTCCT | $(TG)_9$ | 131 | 58 | JQ743037 |
| | R: CACAGACGAACTACACAAACCA | | | | |
| Cora018* | F: TGTTGGTGCTGAACTAAAGT | $(TG)_{11}$ | 145–171 | 50 | JQ743038 |
| | R: ATAGCGTGCCATTGATACAT | | | | |
| Cora029* | F: GGGAAACCAGTTGATGAGGA | $(AC)_7$ | 211–229 | 50 | JQ743039 |
| | R: GTTGTTATTTTCACTTTTGC | | | | |
| Cora032* | F: AAGATTTCCGTATTATGCAAG | $(TG)_{12}$ | 312–320 | 48 | JQ743040 |
| | R: CAAAGAAATAAATGCGTCCT | | | | |
| Cora034* | F: TGCCCAAAAAGAACAGTATG | $(AC)_{11}(ATAC)_5$ | 152–170 | 50 | JQ743041 |
| | R: TTGGAAAGGGGCAGACGCTA | | | | |
| Cora042* | F: GTCCTTGTCTTTTCTTTCCA | $(TG)_8$ | 173–177 | 48 | JQ743042 |
| | R: AGTAATGGACGCAGTAAAAA | | | | |
| Cora063* | F: ATTCTCGTTGCTTGATTGTA | $(TG)_{10}GG(TG)_5$ | 236–248 | 48 | JQ743043 |
| | R: TGACATCATTTCGCTATTGG | | | | |
| Cora065 | F: CAGAATAGCAATAAACTACG | $(AC)_7$ | 201 | 50 | JQ743044 |
| | R: CTTGTATTTTTCGCTTGTTC | | | | |
| Cora066* | F: TTGTGGCTTTGAACCTTCCT | $(AC)_7$ | 177–179 | 48 | JQ743045 |
| | R: ATTTTCAGGATGTTACAGGA | | | | |
| Cora067 | F: GTTGAAGTGCGAGCGTATTA | $(AC)_{14}$ | 178 | 48 | JQ743046 |
| | R: CAAAATGACATAAACCAGTG | | | | |
| Cora075 | F: GGTTCTGTGTAAGTGTTTTC | $(TG)_{11}$ | 143 | 52 | JQ743047 |
| | R: CTTGCGGTTTTCTCACTCAT | | | | |
| Cora091 | F: GCCCACGAAATCAAAAATAA | $(AC)_{10}$ | 254 | 50 | JQ743048 |
| | R: ATTACTTACTTCCATCTGTTAC | | | | |
| Cora093* | F: TTAGGTAGTGGAAATCTGAG | $(TG)_7$ | 176–184 | 50 | JQ743049 |
| | R: AACAACTCAATAAATAATAAGC | | | | |

*Note:* $T_a$ = PCR annealing temperature.
* Polymorphic loci.

(Fermentas, Burlington, Ontario, Canada). Five microliters of the diluted digestion-ligation mixture (1 : 10) were used for amplification reactions with the adapter-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) following the profile: 95°C for 3 min; 30 cycles of 94°C for 45 s, 52°C for 60 s, 72°C for 60 s; and a final extension step of 10 min at 72°C. The amplified fragments (200–800 bp) were enriched for microsatellite repeats by magnetic bead selection with 5′-biotinylated $(AC)_{15}$, $(AG)_{15}$, and $(AAG)_{10}$ probes. The captured fragments were reamplified with the *Mse*I-N primers. PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China), ligated into the pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA), and transformed into DH5α cells (TaKaRa Biotechnology Co., Dalian, China). Identification of recombinant clones was carried out in a blue/white selection assay. Positive clones were tested for microsatellite inserts by PCR with $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$ and T7/Sp6 primers, and analyzed on an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, California, USA).

In total, 347 clones containing inserts were sequenced, of which 229 (66%) were found to contain simple sequence repeats (SSRs); 101 of the latter with sufficient flanking regions were selected to design locus-specific primers using the program Oligo 6.0 (Offerman and Rychlik, 2003). Polymorphism of all 101 microsatellite loci was assessed in 12 individuals each from two wild *C. lanuginosus* populations (XQ20: voucher no. MMO11-1767, Lanping, Yunnan, 26°55′42″N, 99°10′02″E [E, HITBC]; and JZL: voucher no. MMO11-1772, Yunlong, Yunnan, 25°54′37″N, 99°09′06″E [E, HITBC]) collected in West Yunnan, China. The two populations were selected to span the morphological diversity found in the complex.

PCR reactions were performed in a 20-μL volume containing 30–50 ng genomic DNA, 0.6 μM of each primer, and 7.5 μL 2× *Taq* PCR MasterMix (containing 0.1 U *Taq* polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, and 3 mM $MgCl_2$ [Tiangen, Beijing, China]). The PCR cycle profile was: 95°C for 3 min, followed by 30–35 cycles at 94°C for 30 s, at a primer-specific annealing temperature (Table 1) for 45 s, and 72°C for 60 s, and a final extension step at 72°C for 7 min. The amplified fragments were separated on 6% polyacrylamide denaturing gels with a 20-bp ladder molecular size standard (Fermentas) and visualized by silver staining.

Of the 101 primer pairs tested, 14 successfully amplified (Table 1), of which nine were polymorphic (Table 2). The potentially perceived low level of successful primers stems from our very strict criteria for the success rating of the loci. We regarded PCR amplification success only for primers with PCR products visible as clear single or double bands for all samples. Primers producing multiple bands or unclear bands with shadow bands (in repeated amplifications) were treated as failure. Also discarded were primer pairs where only a few individuals failed to amplify in repeated PCRs.

Table 2. Population genetic characteristics of nine polymorphic microsatellite loci tested on two populations of *Corallodiscus lanuginosus.*[a]

| | XQ20 ($N$ = 12) | | | JZL ($N$ = 12) | | |
|---|---|---|---|---|---|---|
| Locus | $A$ | $H_o$ | $H_e$ | $A$ | $H_o$ | $H_e$ |
| Cora011 | 3 | 0.667 | 0.594 | 1 | 0.000 | 0.000 |
| Cora018 | 6 | 0.583 | 0.714 | 4 | 0.583 | 0.736* |
| Cora029 | 3 | 0.917 | 0.554 | 2 | 0.500 | 0.391 |
| Cora032 | 2 | 0.167 | 0.464 | 1 | 0.000 | 0.000 |
| Cora034 | 5 | 0.917 | 0.750 | 3 | 0.750 | 0.540 |
| Cora042 | 2 | 0.917 | 0.518 | 1 | 0.000 | 0.000 |
| Cora063 | 4 | 0.583 | 0.772 | 4 | 1.000 | 0.736 |
| Cora066 | 2 | 0.250 | 0.518 | 2 | 0.500 | 0.391 |
| Cora093 | 2 | 0.333 | 0.290 | 2 | 0.083 | 0.083 |
| Mean | 3.2 | 0.593 | 0.575 | 2.2 | 0.380 | 0.320 |

*Note:* $A$ = number of alleles revealed; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; $N$ = population sample size.
[a]Voucher/locality information for the studied populations: XQ20 = voucher no. MMO11-1767, Lanping, Yunnan, 26°55′42″N, 99°10′02″E (E, HITBC); and JZL = voucher no. MMO11-1772, Yunlong, Yunnan, 25°54′37″N, 99°09′06″E (E, HITBC).
* Significant deviation from Hardy–Weinberg equilibrium ($P$ = 0.01).

Standard genetic diversity parameters for the polymorphic loci, i.e., the number of alleles ($A$) and expected ($H_e$) and observed ($H_o$) levels of heterozygosity, were calculated, and tests for deviations from Hardy–Weinberg equilibrium and genotypic linkage disequilibrium (LD) were performed in GENEPOP version 4.0.10 (Raymond and Rousset, 1995; http://genepop.curtin.edu.au/). For the nine polymorphic primers, the number of alleles per locus ranged from one to six (mean: 2.7). $H_o$ and $H_e$ ranged from 0 to 1 and from 0 to 0.772, with an average for all samples of 0.487 and 0.448, respectively. Only one of the nine polymorphic microsatellite loci (Cora018) deviated from Hardy–Weinberg equilibrium ($P < 0.01$) (Table 2). There was no significant LD between pairs of loci at $P < 0.001$, and only for three comparisons out of the 72 pairs at $P < 0.05$.

Cross-amplification was tested using the PCR method described above using the nine polymorphic primer pairs on individual samples of the four other species in *Corallodiscus*, *C. kingianus* (voucher no.: MMO 09-1585, Batang, Sichuan, 30°12′29″N, 99°17′10″E; E, HITBC), *C. cooperi* (voucher no.: *F. Ludlow, G. Sherriff & J. H. Hicks 16939*, Dhur valley, Bhutan, 27°38′00″N, 90°40′00″E; E), *C. bhutanicus* (voucher no.: *A. J. C. Griersen & D. G. Long s.n.*, Thimphu, Bhutan, 27°40′00″N, 89°54′00″E; E), and *C. conchifolius* (voucher no.: ZP2010-006, Wenchuan, Sichuan, 31°31′09″N, 103°14′54″E; E, HITBC). In *C. kingianus*, eight primers amplified a PCR product, six in *C. cooperi*, four in *C. bhutanicus*, and only two in *C. conchifolius* (Table 3). Thus, some primers developed here for *C. lanuginosus* will also be useful for population genetic studies of its allied species.

## CONCLUSIONS

Of the 14 microsatellite markers developed for *C. lanuginosus* in this study, nine showed polymorphisms, indicating that these markers will be suitable for investigations of the genetic structure at the population level of this widespread and taxonomically difficult complex. Such studies will allow insight into its evolutionary history to understand the origin of the morphologically complicated pattern and perhaps suggest the presence of cryptic taxonomic units. These SSR markers developed for *C. lanuginosus* have a high potential for analyzing allied species in the genus, such as *C. kingianus* in China, because of their high cross-amplification rate.

TABLE 3. Result of cross-amplification tests for nine polymorphic microsatellite loci designed for *Corallodiscus lanuginosus*.

| Locus | *C. kingianus* | *C. cooperi* | *C. bhutanicus* | *C. conchifolius* |
|---|---|---|---|---|
| Cora011 | + | — | — | — |
| Cora018 | + | + | + | — |
| Cora029 | + | + | + | + |
| Cora032 | + | — | — | — |
| Cora034 | + | + | + | + |
| Cora042 | + | + | — | — |
| Cora063 | + | + | + | — |
| Cora066 | — | — | — | — |
| Cora093 | + | + | — | — |

*Note*: + = successful amplification; — = no amplification.

## LITERATURE CITED


BATALIN, A. 1892. [Gesneriaceae]. *In* Notae de plantis Asiaticis. *Trudy Imperatorskago S.-Petersburgsk Botaniceskago Sada* 12(6): 176–177.

BURTT, B. L. 1947. *Didissandra* and *Corallodiscus. Gardeners Chronicle, ser. 3* 122: 204, 212.

CRAIB, W. G. 1919a. *Didissandra* and allied genera in China and N. India. *Notes from the Royal Botanic Garden Edinburgh* 11: 255–268.

CRAIB, W. G. 1919b. Gesneracearum novitates. *Notes from the Royal Botanic Garden Edinburgh* 11: 233–254.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of leaf tissue. *Phytochemical Bulletin* 19: 11–15.

OFFERMAN, J., AND W. RYCHLIK. 2003. Oligo primer analysis software. *In* S. Krawetz and D. Womble [eds.], Introduction to bioinformatics: A theoretical and practical approach, 345–356. Humana Press, Totowa, New Jersey, USA.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249.

RIDLEY, H. N. 1905. The Gesneriaceae of the Malay Peninsula. *Journal of the Straits Branch of the Royal Asiatic Society* 44: 1–92 [incorrectly indicated as vol. 43].

WANG, W. T., K. Y. PAN, AND Z. Y. LI. 1990. Gesneriaceae. *In* W. T. Wang [ed.], Flora Reipublicae Popularis Sinicae, vol. 69, 125–581. Science Press, Beijing, China.

WANG, W. T., K. Y. PAN, Z. Y. LI, A. L. WEITZMAN, AND L. E. SKOG. 1998. Gesneriaceae. *In* Z. Y. Wu and P. H. Raven [eds.], Flora of China, vol. 18, 244–401. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

ZANE, L., L. BARGELLONI, AND T. PATARNELLO. 2002. Strategies for microsatellite isolation: A review. *Molecular Ecology* 11: 1–16.