Development and Characterization of 14 Microsatellite Markers for Indigofera pseudotinctoria (Fabaceae)

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Primer Note

Development and characterization of 14 microsatellite markers for *Indigofera pseudotinctoria* (Fabaceae)[1]

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- *Premise of the study:* Microsatellite markers can be used to evaluate population structure and genetic diversity in native populations of *Indigofera pseudotinctoria* (Fabaceae) and assess genetic disturbance caused by nonnative plants of the same species.
- *Methods and Results:* We developed 14 markers for *I. pseudotinctoria* using next-generation sequencing and applied them to test two native populations, totaling 77 individuals, and a transplanted population, imported from a foreign country, of 17 individuals. The mean number of alleles was 3.310, observed heterozygosity was 0.242, and expected heterozygosity was 0.346. The fixation index in the transplanted population was 0.469, which was higher than in the native populations (0.154 and 0.158). In addition, the transplanted population contains one allele that is not shared by the native population.
- *Conclusions:* Microsatellite markers can be useful for evaluating genetic diversity within and between populations and for studying population genetics in *I. pseudotinctoria* and related species.

Key words: 454 next-generation sequencing; Fabaceae; *Indigofera pseudotinctoria*; invasive species; microsatellites.

Genetic disturbances caused by invasive species reduce biodiversity in native populations (Byrne et al., 2011). Invasive, nonnative species are strictly regulated by species name to prevent their introduction into the native range. However, regulation is difficult when nonnative plants belong to the same species as native plants. In Japan, seeds used to vegetate roadsides after maintenance projects are usually imported from China, South Korea, and Taiwan (Uemachi et al., 2013) due to the lower cost of seed collection and transportation. In many cases, plants of the same species differ genetically between regions. Therefore, imported plants can cause genetic disturbance of native populations (McKay et al., 2005; Shimono et al., 2013), which may result in outbreeding depression between native and transplanted populations via secondary contact between these populations (Rhymer and Simberloff, 1996; Ewel et al., 1999; Allendorf et al., 2001).

Indigofera L. (Fabaceae) is a large pantropical genus containing 750 species. Among these, *I. pseudotinctoria* Matsum. is an economically important species distributed in China, Korea, and Japan (Satake et al., 1982) and used as a cover crop. The artificial migration of this species from China to Japan may affect native biodiversity. Therefore, it is important to develop a set of markers to study genetic disturbance in native populations caused by transplanted populations.

In this study, we designed microsatellite markers for *I. pseudotinctoria* and developed 14 microsatellite primers using 454 next-generation sequencing to identify appropriate transplanting zones based on genetic differentiation between nonnative and native *I. pseudotinctoria* populations.

METHODS AND RESULTS

Three populations were evaluated in this study: two native and one transplanted. The native populations were collected from Tokyo (n = 38) and Saitama Prefecture (n = 39), while the imported population was transplanted into an area in Mie Prefecture (n = 17). Individuals of the Mie population were imported from unspecified locations in China. Furthermore, we collected an additional seven species and two forms of the genus *Indigofera*: *I. bungeana* Walp., *I. tinctoria* L., *I. gerardiana* Graham ex Baker, *I. decora* Lindl., *I. decora* f. *alba* (Sarg.) Honda (for each taxon, n = 1), *I. kirilowii* Maxim. ex Palibin, *I. pseudotinctoria* f. *albiflora* Okuyama, *I. trifoliata* L., and *I. suffruticosa* Mill. (for each taxon, n = 2) (Appendix 1).

Total genomic DNA was extracted from leaf tissue using a DNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands) and the cetyltrimethylammonium bromide method (CTAB) (Porebski et al., 1997). DNA was subjected to shotgun sequencing using a Roche 454 Genome Sequencer Junior with the GS FLX Titanium Rapid Library Preparation Kit (454 Life Sciences, a Roche Company, Basel, Switzerland) according to the manufacturer's instructions. One sample of *I. pseudotinctoria* used in shotgun sequencing was obtained from the sample native to Nagano Prefecture.

DNA library sequencing resulted in 15,243 reads of 32–662 bp. We found 572 contigs of 60–660 bp using CLC Genomics Workbench version 5.5 (QIAGEN). Microsatellite motifs were found in 533 reads using MSATCOMMANDER (Faircloth, 2008). Many microsatellite motifs had repeats that were

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TABLE 1. Characteristics of 14 microsatellite markers developed for *Indigofera pseudotinctoria*.

Locus	Primer sequences (5′–3′)	Repeat motif	Multiplex[a]	Fluorescent dye	Allele size range (bp)	T_a (°C)	*A*	DDBJ accession no.
A7G7T	F: TGCAATCTCCCGTCGCATATTC R: TGAGGAGTTGAGGTCTTGTG	$(AAT)_{12}$	1	PET	258–267	60	5	AB827329
A913S	F: CGTGTCGTGAGAGAATGAGTTTGG R: CCGTTAGGGTTTTCTGGTTGGTAG	$(AAT)_8(AAG)_2$	1	VIC	179–188	60	4	AB827330
AAJSO	F: CAAGGGCCAAGCAAGAACAAC R: GTTAGTGTCAGCCCCTCCTTCC	$(TTA)_{10}T(TTA)_3$	3	6-FAM	303–326	60	9	AB827331
ACUJQ	F: GCTGATTGTTTCCTACCTA R: AGCATCAAACTTCATCACAG	$(AAT)_{17}$	3	VIC	195–221	60	10	AB827332
AN24U	F: CATTGGAGCAGGTGTTTCGACG R: CGTCCAAAAGCTCCATTATCGTCA	$(AAC)_4…(GAA)_7$	1	VIC	246–249	60	4	AB827333
AUHSL	F: TGGGTTGCTTCATGTTGCC R: GGTTCCTCACTGCATATC	$(CCT)_8$	2	VIC	312–328	60	6	AB827334
AZQ4M	F: GGAAAATAGAGAAGGGTAGGAC R: GGTCCATCAAATCCATCTCTCTC	$(GA)_9$	2	6-FAM	261–269	60	7	AB827335
BSN57	F: AGCTCCGACACCTGTTTTGA R: GAGGAAAAAGCATTCGGGTA	$(AT)_3AATT(TA)_5(AT)_3A(ATATA)_2(ATA)_3$	3	PET	150–170	60	7	AB827337
BTPFL	F: GTCGTCGTGTCGTGAGAGAA R: ACGCCTAGGGTTTTCTGGTT	$(AAT)_8$	2	PET	188–197	60	5	AB827338
BP01Y	F: CGTTTTGTTTTCTTCTGTACTGGAC R: CACTAGTCAATCAACCGAAAAAGAG	$(TTTGCA)_6$	5	VIC	231–249	48	5	AB917736
B0L6E	F: CATTGTGGCATCGTTGATTC R: GTCATGATGATATTACTGAGGAACG	$(TTG)_9$	5	6-FAM	175–194	48	7	AB917737
AQ11D	F: ATTTTCCAGTCCACCAACTGAT R: TCTAATCCCGTGTAATGTGTGC	$(TTA)_6$	4	VIC	210–275	48	5	AB917738
A2T7M	F: AAGAGTTCACTAGCCTTCTTTGGA R: AAACTAGAACCTGGTGGTTCCTC	$(TTC)_8$	5	NED	177–199	48	6	AB917739
AHK5H	F: GATCTCCTTGGTGGTCTCTGATA R: GCGTCTTCGTGAACTTGTTACAT	$(TTC)_{10}$	4	6-FAM	198–213	48	8	AB917740

Note: *A* = expressed total number of alleles; DDBJ = DNA Data Bank of Japan; T_a = annealing temperature.
[a] Multiplex PCR primer combination.

either too long or too short or comprised single base pair repeats. Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012) was used to design primers for 63 loci, of which 25 were polymorphic. Eleven loci were excluded because of weak PCR amplification or difficulty in identifying the peaks. Therefore, a total of 14 microsatellite loci were selected, and the fragments were amplified by touchdown PCR using a QIAGEN Multiplex PCR Kit with fluorescent primer pairs for each microsatellite (Table 1). The 14 loci include AAJSO, ACUJQ, AN24U, AUHSL, AZQ4M, A7G7T, A913S, BSN57, BTPFL, BP01Y, B0L6E, AQ11D, A2T7M, and AHK5H. The number of microsatellite motifs was detected by resequencing the *I. pseudotinctoria* sample from Nagano Prefecture. Fluorescent primer pairs were labeled using Dye Set G5 (Applied Biosystems by Thermo Fisher Scientific, Waltham, Massachusetts, USA). Primer combinations used in a multiplex PCR are given in Table 1. PCR was conducted in 10-μL reactions containing 20 ng/μL genomic DNA, 5 μM each primer, and 5 μL QIAGEN Multiplex PCR Master Mix. The following touchdown PCR profile was used for all multiplex PCR reactions: initial denaturation for 15 min at 94°C; followed by three cycles of 3 s at 94°C, 90 s at 65°C (54°C), and 1 min at 72°C; three cycles of 3 s at 94°C, 90 s at 62°C (51°C), and 1 min at 72°C; 30 cycles of 3 s at 94°C, 90 s at 59°C (48°C), and 1 min at 72°C; and final elongation for 30 min at 60°C. PCR products were analyzed using an ABI Prism 3130 sequencer and visualized with GeneMapper (Applied Biosystems, Grand Island, New York, USA). The size standard was GeneScan 600 LIZ Size Standard (Applied Biosystems). The significance of linkage disequilibrium (LD) was calculated with CERVUS 3.03 (Kalinowski et al., 2007); the number of alleles (A), allelic richness (A_R), and fixation index (F_{IS}) were determined with FSTAT 2.9.3 (Goudet, 2001); and observed heterozygosity (H_o), expected heterozygosity (H_e), and Hardy–Weinberg equilibrium (HWE) were analyzed with GenAlEx 6.5 (Peakall and Smouse, 2012). The allele-sharing distance (ASD) matrix was calculated using Excel Microsatellite Toolkit 3.3.1 (Park, 2001).

No LD was observed in any of the studied populations. The mean number of alleles across populations was 3.310, H_o was 0.000–0.718 (mean 0.242), and H_e was 0.000–0.820 (mean 0.346). The Mie population showed higher H_e (e.g., 0.78 for the B0L6E locus) than H_o (e.g., 0.06 for the B0L6E locus) (Table 2).

The Mie population also deviated from HWE because the seeds from this population did not result from natural breeding but originated from different locations in China and were translocated to the Mie Prefecture. The mean number of alleles per population was 2.142 for the Tokyo population, 3.071 for the Saitama population, and 4.714 for the Mie population. The mean F_{IS} was 0.154 for the Tokyo population, 0.158 for the Saitama population, and 0.469 for the Mie population, as analyzed by FSTAT (Goudet, 2001).

A and F_{IS} were low in the Tokyo and Saitama populations (Table 2). Higher levels of polymorphism were found in the Mie population (2–7, mean 4.714) than in the Saitama (1–3, mean 2.07) or Tokyo populations (1–5.80, mean 2.74) (Table 2). The ASD matrix was calculated from the number of shared alleles among individual pairwise allele-sharing distances; the resulting values were 0.429–0.893, 0.071–0.464, and 0.071–0.429 for the Tokyo–Saitama, Mie–Saitama, and Mie–Tokyo populations, respectively. *Indigofera pseudotinctoria* and *I. pseudotinctoria* f. *albiflora* were amplified using 14 markers, whereas *I. trifoliata*, *I. decora* f. *alba*, and *I. kirilowii* were amplified using 10 markers and *I. tinctoria*, *I. gerardiana*, and *I. decora* were amplified using nine markers (Table 3).

CONCLUSIONS

Population assessment with 14 microsatellite markers revealed that the nonnative population was highly polymorphic, included alleles different from those in native populations, and the difference was not due to neutral variation. Our results indicate that the transplanted and native populations did not share the few same alleles and that the Mie population had few unique alleles. To compare the origin of Chinese and Japanese populations, natural populations of China should be analyzed. Overall, microsatellite markers developed in this study could be used to discriminate native from nonnative *I. pseudotinctoria* individuals in Japan and determine genetic disturbance in native populations caused by imported plants.

TABLE 2. Polymorphism analysis of 14 microsatellite markers in three populations of *Indigofera pseudotinctoria*.

	Tokyo (N = 38)					Saitama (N = 39)					Mie (N = 17)				
Locus	A	A_R	H_o	H_e	F_{IS}[a]	A	A_R	H_o	H_e	F_{IS}[a]	A	A_R	H_o	H_e	F_{IS}[a]
AAJSO	1.000	1.000	0.000	0.000	NA	6.000	1.000	0.385	0.526	0.280***	7.000	7.000	0.471	0.820	0.451**
ACUJQ	3.000	2.942	0.211	0.278	0.255***	6.000	5.807	0.564	0.776	0.285***	7.000	7.000	0.588	0.787	0.281***
AN24U	1.000	1.000	0.000	0.000	NA	1.000	1.000	0.000	0.000	NA	4.000	4.000	0.412	0.469	0.152**
AUHSL	1.000	1.000	0.00	0.000	NA	4.000	3.120	0.308	0.3106	0.008	5.000	5.000	0.353	0.666	0.493
AZQ4M	2.000	1.913	0.105	0.100	−0.042	6.000	5.364	0.718	0.646	−0.098	2.000	2.000	0.059	0.161	0.652**
A7G7T	2.000	1.913	0.105	0.100	−0.042	1.000	1.000	0.000	0.000	NA	5.000	5.000	0.588	0.701	0.190
A913S	3.000	2.993	0.395	0.405	0.040*	2.000	1.949	0.077	0.120	0.370*	3.000	3.000	0.294	0.503	0.441
BSN57	3.000	2.953	0.316	0.351	0.115	2.000	1.826	0.077	0.074	−0.027	4.000	4.000	0.235	0.559	0.599**
BTPFL	3.000	2.992	0.395	0.389	0.001	2.000	1.949	0.077	0.120	0.370*	4.000	4.000	0.176	0.618	0.729***
BP01Y	3.000	2.992	0.474	0.658	0.293	4.000	3.436	0.538	0.641	0.172	2.000	2.000	0.059	0.057	0.000
B0L6E	3.000	2.401	0.158	0.147	−0.060	2.000	1.436	0.026	0.025	0.000	6.000	6.000	0.059	0.777	0.929**
AQ11D	1.000	1.000	0.00	0.000	NA	2.000	1.436	0.026	0.025	0.000	5.000	5.000	0.118	0.396	0.718***
A2T7M	1.000	1.000	0.026	0.000	NA	2.000	1.905	0.051	0.197	0.483**	5.00	5.000	0.588	0.709	0.200
AHK5H	3.000	2.913	0.342	0.481	0.30*	3.000	2.973	0.487	0.541	0.113**	7.00	7.00	0.294	0.521	0.459

Note: A = number of alleles; A_R = allelic richness; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; NA = not analyzed.
[a] Significant deviation from Hardy–Weinberg equilibrium in each population is indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Table 3. Cross-amplification and polymorphism analysis of 14 microsatellite markers developed for *Indigofera pseudotinctoria* in nine species of *Indigofera*.[a]

Locus	*I. bungeana* (*N* = 1)	*I. decora* (*N* = 1)	*I. decora* f. *alba* (*N* = 1)	*I. gerardiana* (*N* = 1)	*I. kirilowii* (*N* = 1)	*I. pseudotinctoria* f. *albiflora* (*N* = 2)	*I. suffruticosa* (*N* = 2)	*I. tinctoria* (*N* = 1)	*I. trifoliata* (*N* = 2)
AAJSO	—	—	++	+	++	+	—	—	—
ACUJQ	—	++	—	++	—	+	—	ba	—
AN24U	—	++	+	—	+	+	ba	ba	—
AUHSL	—	—	++	—	++	+	—	—	—
AZQ4M	ba	—	—	—	—	+	ba	ba	+
A7G7T	—	—	+	+	+	+	+	+	+
A913S	++	+	+	+	+	+	+	+	+
BSN57	+	+	++	—	+	+	+	+	+
BTPFL	+	—	—	—	—	+	+	++	+
BP01Y	+	+	—	+	+	++	—	+	+
B0L6E	+	+	+	+	+	+	+	+	+
A2T7M	+	+	+	+	+	+	+	+	+
AHK5H	+	+	+	+	+	+	—	++	+
AQ11D	+	+	+	+	+	+	+	+	+

Note: — = no amplification product; + = single band; ++ = two bands; ba = stuttered amplification; *N* = number of individuals sampled.
[a]The expected single band in three populations of nine *Indigofera* spp.

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APPENDIX 1. Voucher information for 10 taxa in the genus *Indigofera* used in this study.

Species	Locality	Geographic coordinates/Location	Origin of sample	Institute[a]	Herbarium	Herbarium voucher no.
I. bungeana Walp.	Tokyo	1833-81 Todori, Hachioji City, Tokyo	Herbarium	Tama Forest Science Garden	Tama Forest Science Garden	TFA51609
I. decora Lindl.	Unknown	648 Matsudo, Matsudo City, Chiba Prefecture	Purchased	Graduate School of Horticulture, Chiba Univ.	Graduate School of Horticulture, Chiba Univ.	MTDO4130
I. decora Lindl. f. *alba* (Sarg.) Honda	Tochigi	1842 Hanaishi, Nikko City, Tochigi Prefecture	Cultivated	Koishikawa Botanical Garden	Graduate School of Horticulture, Chiba Univ.	MTDO4124
I. gerardiana Graham ex Baker	Tokyo	1833-81 Todori, Hachioji City, Tokyo	Herbarium	Tama Forest Science Garden	Tama Forest Science Garden	TFA014490
I. kirilowii Maxim. ex Palib.	Ibaragi	4-1-1 Amakubo, Tukuba City, Ibaragi Prefecture	Cultivated	Tsukuba Botanical Garden	Graduate School of Horticulture, Chiba Univ.	MTDO4128
I. pseudotinctoria Matsum.	Nagano	648 Matsudo, Matsudo City, Chiba Prefecture	Purchased	Graduate School of Horticulture, Chiba Univ.	Graduate School of Horticulture, Chiba Univ.	MTDO4134
I. pseudotinctoria Matsum.	Tokyo	35°35′29.0″N, 139°25′45.1″E	Local	—	Graduate School of Horticulture, Chiba Univ.	MTDO4137
I. pseudotinctoria Matsum.	Saitama	36°08′17.5″N, 139°19′55.9″E	Local	—	Graduate School of Horticulture, Chiba Univ.	MTDO4140
I. pseudotinctoria Matsum.	Mie	34°20′02.8″N, 136°48′31.1″E	Local	—	Graduate School of Horticulture, Chiba Univ.	MTDO4143
I. pseudotinctoria Matsum. f. *albiflora* Okuyama	Kanagawa	35°19′22.60″N, 139°34′16.15″E	Cultivated	Zyomiyouzi Temple in Kamakura City	Graduate School of Horticulture, Chiba Univ.	MTDO4120
I. suffruticosa Mill.	Toyama	Kamikutuwada 42, Toyama City, Toyama Prefecture	Cultivated	Botanic Gardens of Toyama[b]	Graduate School of Horticulture, Chiba Univ.	MTDO4151
I. tinctoria L.	Toyama	Kamikutuwada 42, Toyama City, Toyama Prefecture	Cultivated	Botanic Gardens of Toyama[b]	Graduate School of Horticulture, Chiba Univ.	MTDO4146
I. trifoliata L.	Okinawa	35°35′29.0″N, 139°25′45.1″E	Local	—	Graduate School of Horticulture, Chiba Univ.	MTDO4129

[a] Institute whose personnel collected the samples. Koishikawa Botanical Garden = Koishikawa Botanical Garden, University of Tokyo; Tama Forest Science Garden = Tama Forest Science Garden, Forestry and Forest Products Research Institute; Tsukuba Botanical Garden = Tsukuba Botanical Garden, National Science Museum.

[b] Accession numbers of the Botanic Gardens of Toyama: 48223 (*I. suffruticosa*) and BGT45639 (*I. tinctoria*).