Isolation and characterization of chloroplast microsatellite markers in the invasive tree species *Robinia pseudoacacia* L.

By M. K. KIMURA^{1,*}, C. LIAN¹ and T. HOGETSU²

(Received 20th December 2012)

Abstract

Robinia pseudoacacia is one of the most problematic invasive alien species in Japan. Information on genetic diversity and population structure is urgently required in order to generate effective management strategies. To assist such efforts we have identified five chloroplast microsatellite (cpSSR) markers for *R. pseudoacacia* by amplifying noncoding regions of chloroplast DNA (cpDNA) using universal chloroplast primers. Among 857 individuals these cpSSR markers showed substantial polymorphism, with three to eight alleles per locus and gene diversity ranging from 0.387 to 0.713. These cpSSR makers will be useful for analyzing maternal lineages and population genetic structure of *R. pseudoacacia*.

Key words: cpSSR; Maternal lineage; Black locust.

Introduction

Black locust (*Robinia pseudoacacia* L.), native in south-eastern North America, is one of important tree for forestry in many countries as a multi-purpose tree (e.g. LIESEBACH and SCHNECK, 2012). On the other hand, this species is a globally invasive tree species that has been expanding rapidly in temperate habitats with moist climates and loose silty or sandy soils in Europe and various other parts of the world (KERESZTESI, 1988; SABO, 2000). The species was introduced into Japan about 140 years ago as a valuable tree for planting in disturbed areas. However, it was listed as an “Uncategorized Alien Species” by the Ministry of the Environment of Japan in 2006 and among “the top 100 invasive alien species in Japan” by the Ecological Society of Japan (2002). The information of invasive process is important to management of alien species. Chloroplast DNA markers, the so-called cpDNA haplotype, are potentially a very good marker type to describe source populations and number of maternal lineage of invasive populations. In the present study, we developed chloroplast microsatellite (cpSSR) markers for *R. pseudoacacia* to assist efforts to elucidate its invasion processes and formulate strategies for effectively managing the species in Japan.

Materials and Methods

We amplified the sequences of several non-coding regions in chloroplast DNA (cpDNA) in *R. pseudoacacia* and identified the SSR regions as previously reported by LIAN et al. (2003), ISLAM et al. (2008) and GENG et al. (2009). The cpSSR regions were isolated as follows. Genomic DNA was extracted from silica gel-dried leaves using a modified cetyltrimethyl ammonium bromide (CTAB) method (LIAN et al., 2003). cpDNA fragments were then amplified using eight universal chloroplast primer pairs developed by TABRLET et al. (1991), DEMESURE et al. (1995) and DUMOLINLAPEGUE et al. (1997) (Table 1), in 25 µL reaction mixtures containing 1 µL of DNA solution, 0.4 mM of each dNTP, 1× LA PCR buffer (Takara Shuzo, buffer composition unknown), 2.5 mM Mg²⁺, 1.25 U LA Taq DNA polymerase (Takara Shuzo, city, Japan) and 0.2 µM of each universal chloroplast primer pair. The PCR program consisted of 1 min at 94°C, then 29 cycles of 30 s at 94°C, 1 min at the optimal annealing temperature for the primer pair and 3 min at 72°C, followed by 30 s at 94°C, 1 min at the optimal annealing temperature, and 10 min at 72°C. Information on the universal chloroplast primers and amplified fragments is presented in Table 1. The single band PCR products amplified were digested separately with four blunt-end restriction enzymes (*AluI*, *EcoRV*, *HaeIII*, and *SspI*). The digested fragments were electrophoretically separated in a 1.5% agarose gel, and then the fragments that were digested into lengths shorter than 1,500 bp by each of these enzymes were subcloned and sequenced using the protocol described by LIAN et al. (2003). We screened the microsatellite repeats in all the sequences, and designed a primer pair from the sequences flanking each of the cpSSR loci (Table 2).

To assess the polymorphism of the isolated cpSSR loci, leaf samples were collected from 614 individuals in 29 populations from Hokkaido (43°17'24.8"N, 141°51'14.1"E) to Hiroshima (34°16'29.0"N, 132°15'56.7"E) in Japan and one population in China (2–61 individuals per population). These populations were invasive forests, not planting forest. Genomic DNA was extracted from the samples using the modified CTAB method (LIAN et al., 2003), and the loci were then amplified in 5 µL PCR reaction mixtures containing about 5 ng of template DNA, 0.4 mM of each dNTP, 1× LA PCR buffer (Takara Shuzo), 2.5 mM Mg²⁺, 0.25 U LA Taq DNA polymerase (Takara Shuzo), and 0.2 µM of each cpSSR primer (forward primer Texas Red-labeled and reverse primer non-tailed). The thermal program consisted of 1 min at 94°C, then 30 cycles of 30 s at

¹) Asian Natural Environmental Science Center, The University of Tokyo, Midori-cho 1-1-8, Nishitokyo, Tokyo 188-0002, Japan.

²) Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan.

^{*}) Corresponding author: MEGUMI K. KIMURA. Asian Natural Environmental Science Center, The University of Tokyo, Midori-cho 1-1-8, Nishitokyo, Tokyo 188-0002, Japan. Tel.: +81-29-829-8261, Fax: +81-29-874-3720. E-Mail: kimegu@affrc.go.jp

Table 1. – Universal chloroplast primers and characteristics of fragments amplified in *R. pseudoacacia*.

Primer pairs	Reference	650	T_a (°C)	Size (bp)	No. of cpSSR regions
<i>trnT/trnF</i>	TABRIET et al. (1991)		52	-	
<i>trnD/trnT</i>	DEMESURE et al. (1995)		52	1500	1
<i>trnH/trnK</i>	DEMESURE et al. (1995)		58	1700	2
<i>trnK1/trnK2</i>	DEMESURE et al. (1995)		58	3000	0
<i>psbC/trnS</i>	DEMESURE et al. (1995)		56	1600	0
<i>trnK2/trnQr</i>	DUMOLIN-LAPEGUE et al. (1997)		58	-	
<i>trnT/psbC</i>	DUMOLIN-LAPEGUE et al. (1997)		54	3700	2
<i>trnV2/rbcL</i>	DUMOLIN-LAPEGUE et al. (1997)		54	3800	3

Table 2. – Characteristics of each of the five amplified microsatellite loci in *R. pseudoacacia*: region, forward (F) and reverse (R) primer sequences, repeat motif, optimal annealing temperature (T_a), size range of fragment, no. of alleles, gene diversity and accession number.

Locus	Region	Primers(5'-3')	Repcat motif	T_a (°C)	Size Range (bp)	No. of alleles	Gene diversity	GeneBank accession No.
Ropscp03	<i>trnD/trnT</i>	F: GGATCTTTCTGAATTCCTAG R: CAGCAAATCAATCATTTCCTG	A ₁₀	54	155-164	6	0.660	AB854291
Ropscp04	<i>trnT/psbC</i>	F: TACGATCTTGTAATTTCC R: ACTACTCTCCTTTCATCAAAG	T ₅ AT ₁₀	54	99-113	3	0.477	AB854292
Ropscp06	<i>trnV2/rbcL</i>	F: CCATGGTATTTGATTTACCAA R: TCAAGGTCGAGAGTGAATTTC	T ₁₀ AT ₅	54	137-140	4	0.387	AB854293
Ropscp07	<i>trnV2/rbcL</i>	F: AGTACCGGACCAATTATTTGG R: TTAACCGATCAACTTGCTTTG	T ₁₀	54	171-176	6	0.698	AB854294
Ropscp08	<i>trnV2/rbcL</i>	F: GAACACGAGTCGAGGCTATC R: GATACCATGGATTCTGGTATC	A ₁₁	54	186-201	8	0.713	AB854295

94°C, 30 sec at the optimal annealing temperature (Table 2) for each designed primer and 30 sec at 72°C (5 min at 72°C in the final cycle). The reaction products were electrophoretically separated in 6% Long Ranger sequencing gels (FMC Bioproducts Co., ME, USA) using an SQ-5500E sequencer (Hitachi, Tokyo), the resulting patterns were analyzed using FRAGLYS version 3

(Hitachi Co.), and gene diversities were calculated using GenAlEx 6.41 (PEAKALL and SMOUSE, 2006). Haplotypes were defined using all cpSSR primers. Associations between haplotypes were analyzed by principal coordinate (PCO) analysis based on estimates of pairwise genetic similarities, calculated using the haploid distance in GenAlEx 6.41 (PEAKALL and SMOUSE, 2006).

Figure 1. – Genetic similarity among 21 haplotypes, as revealed by principal coordinate (PCO) analysis using 5 cpSSR.

Results and Discussion

Of the eight universal chloroplast primers used, five pairs (*trnD/trnT*, *trnH/trnK*, *psbC/trnS*, *trnT/psbC* and *trnV2/rbcL*) clearly amplified a single band from cpDNA of *R. pseudoacacia*. The other three primer pairs (*trnT/trnF*, *trnK1/trnK2* and *trnK2/trnQr*) did not amplify any detectable fragments. Each of the amplified fragments without *psbC/trnS* pair contained one or more microsatellite repeats (SSR) with more than eight A/T base pairs. These sequences were selected for primer design. Finally, five microsatellite primer pairs were selected that successfully amplified DNA sequences in *R. pseudoacacia* which are polymorphic (Table 2). Numbers of alleles of these five cpSSR loci ranged from three to eight and gene diversity values ranged from 0.387 to 0.713 (average, 0.587) among 614 *R. pseudoacacia* individuals sampled from 29 populations in Japan and one population in China. Totally 21 haplotypes were found in 614 samples using 5 cpSSR markers. Top two haplotypes account for 55.3% of total frequency (32.1% and 24.2%, respectively). The frequencies of other haplotypes were less than 10%. Total haplotype diversity was 0.816 in 614 samples. The first two axes of the PCO analysis explained 98.5% of the total variation (Fig. 1). Twenty-one haplotypes were separated three groups by the PCO analysis (Fig. 1). LIESEBACH and SCHNECK (2012) found 11 haplotypes belonging to 2 clearly separated groups from European and North American populations using PCR-RFLP method. cpSSR makers found many more haplotypes and genetic groups, but our results weren't inconsistent with previous reports.

These cpSSR makers will be useful for analyzing distributions of maternal lineages and population genetic structure of *R. pseudoacacia*, thus assisting efforts to breed and to elucidate the invasion processes and formulate strategies for effectively managing this species.

Acknowledgments

This work was supported by Grants-in-Aid from the Japan Society for the Promotion of Science (JSPS).

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