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ORIGINAL PAPER

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High somatic instability of a microsatellite locus in a clonal tree, *Robinia pseudoacacia*

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Abstract *Robinia pseudoacacia* L. is a clonal tree species. To investigate a mutation within eight microsatellite loci of *R. pseudoacacia*, we analyzed DNA samples obtained from different leaf samples within each ramet, leaves from ramets within the genet, and seeds. Of the eight loci, locus Rops15 (AG motif) displayed hypermutability. The mutation rates of Rops15 within each ramet, among ramets within the genet, and offspring were 6.27% (ranging from 0 to 31.1%), 6.11% (from 0 to 25.0%) and 3.78% (from 0 to 10.9%), respectively. The mutation rate increased with allele size (13–71 repeat units). The mutation patterns observed in Rops15 were distinctive in two ways. First, there was a significant bias toward additions over deletions, and both addition and deletion of single repeats were dominant at alleles with lengths less than 232 bp (63 repeats). Second, for the longest allele of 248 bp (71 repeats), the number of losses was higher than the number of gains. These observations suggest that the mutation patterns of microsatellites in *R. pseudoacacia* may follow a generalized stepwise mutation model, and that the tendency of long alleles to mutate to shorter lengths acts to prevent infinite growth. Finally, the observation of somatic hypermutability at locus Rops15 highlights the need for caution when using highly polymorphic microsatellites for population genetic structure and paternity analysis in tree species.

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Introduction

Microsatellites, comprising tandemly repeated short nucleotide sequences, are ubiquitous, abundant, and highly polymorphic in eukaryotic genomes (Tautz 1989; Leopoldino and Pena 2002). At present, microsatellites are commonly used as genetic markers for ecological and evolutionary studies because microsatellites tend to be codominant and highly polymorphic. However, we lack an understanding of the mutational mechanism that generates such a high level of variation.

Microsatellite mutation rates are estimated to occur between 10^{-2} and 10^{-6} events per locus per generation, which are very high compared with point mutation rates at coding gene loci (Li et al. 2002). The most widely accepted mechanism of microsatellite mutation is the slipped-strand mispairing model (Levinson and Gutman 1987; Tautz and Schlötterer 1994). According to this model, DNA polymerase slippage causes misaligned reassociation in replicating DNA strands, and results in the insertion or deletion of one or more repeat units. However, there is no conclusive evidence to support this model. To develop genetic models that accurately reflect microsatellite evolution, detailed information must be obtained regarding both the mechanisms that cause mutations in microsatellites and their mutation rates.

Several approaches have been used to research the origin of the variability at microsatellite loci (Wierdl et al. 1997; Anderson et al. 2000; Sturzeneker et al. 2000). The most effective approach for obtaining information on microsatellite mutation patterns is to detect allele length variations between offspring in pedigrees and parents through sexual reproductive processes. However, only limited information is available for mutations in somatic cells. Moreover, the majority of studies on patterns of microsatellite evolution have focused on a limited number of higher animals (Primmer et al. 1998; Schlötterer et al. 1998; Xu et al. 2000; Hoekert et al. 2002; Steinberg et al. 2002; Beck et al. 2003). Very little is known about somatic mutation of microsatellites in plant species in which microsatellite markers are now employed for

paternity analysis and population genetic studies. Given the increasing use of microsatellite markers in genetic analyses of long-lived plants, it is important to characterize their variability within individuals.

We have recently begun to exploit microsatellites as a tool for investigating the population genetic structure and paternity of *Robinia pseudoacacia* L., a clonal tree species. Of eight microsatellite loci used, we found a hypervariable dinucleotide repeat locus (AG motif) with an extraordinarily high mutation rate. Here, we describe the mutational characteristics of the *R. pseudoacacia* loci as determined in different leaf samples from individual ramets, ramets within individual genets, and seeds from individual mother ramets.

Materials and methods

Sampling

This study was conducted in a population of *Robinia pseudoacacia* L. growing in the dry riverbed of Tamagawa River (35°41′ N, 139°24′ E), Tokyo, Japan, where *R. pseudoacacia* is distributed in several patches. Three types of samples were collected as follows: (1) to characterize mutations within the same ramet, about 80 leaves were randomly sampled from various positions in different branches of seven individual ramets with heights over 10 m selected from different patches; (2) to characterize mutations among ramets within the same genet, leaves were sampled from 458 ramets in seven patches of *R. pseudoacacia*; and (3) to characterize mutations during sexual reproduction, about 60 immature seeds were arbitrarily collected from each of nine maternal ramets. Leaf samples were desiccated using silica gel and stored at room temperature until analysis. Seed coats were peeled from immature seeds, and then the hypocotyl and radicle were excised from the embryo and stored at −30°C until use.

DNA extraction and microsatellite analysis

Crude genomic DNA was extracted from dried leaves using a modified CTAB method as reported previously (Lian et al. 2003). To obtain DNA from embryo organs, the organs were homogenized in 500 μl 2× CTAB and, after adding 3 μl of proteinase K (10 mg/ml, TaKaRa Shuzo, Tokyo, Japan), the mixture was incubated at 37°C overnight. The homogenate was then incubated at 65°C for 1 h. DNA was isolated using chloroform-isoamyl alcohol (24:1) extraction, precipitated in isopropanol, and washed with 75% ethanol. The DNA pellet was dissolved in 30 μl of sterile water.

Eight microsatellites were used to score genotypes in this study; five (Rops02, Rops05, Rops06, Rops08, Rops10) were developed by Lian and Hogetsu (2002) and three (Rops15, Rops16, Rops18) were newly prepared using a dual-suppression-PCR method (Lian and Hogetsu 2002). The characteristics of the three newly isolated microsatellites are listed in Table 1. The PCR amplification conditions and product-detection methods were described by Lian and Hogetsu (2002). PCR was performed with a thermal cycler (TP3000; TaKaRa Shuzo) in 5 μl reaction mixtures containing 0.5 μl of template DNA, 0.4 mM of each dNTP, 0.2–0.3 μM of each designed primer pair, with one primer labeled with Texas Red (Proligo Japan KK, Kyoto, Japan), 1× LA PCR buffer (Takara Shuzo), 2.5 mM Mg^{2+}, and 0.25 U of LA *Taq* DNA polymerase (Takara Shuzo). The PCR cycling conditions were as follows: 1 min at 94°C for one cycle, followed by 29 cycles of 30 s at 94°C, 30 s at the annealing temperature of the primer pair, and 30 s at 72°C, followed by 1 cycle of 30 s at 94°C, 30 s at the annealing temperature of each primer pair, and 5 min at 72°C. The reaction products were fractionated by electrophoresis on a 6% Long Ranger sequencing gel (FMC BioProducts, Rockland, Me., USA) using an SQ-5500E sequencer (Hitachi, Tokyo, Japan). Electrophoretic patterns were analyzed with FRAGLYS version 3 (Hitachi Electronics Engineering, Tokyo, Japan).

DNA samples showing a putative mutation were amplified by PCR and fractioned by electrophoresis to confirm the reproducibility of PCR amplification.

Sequencing

Four alleles confirmed as mutated in the Rops15 locus were reamplified, cloned, and sequenced to identify their mutation sites. The PCR products containing mutated alleles were purified with a SUPREC-02 filter (Takara Shuzo) and cloned using the pT7 Blue Perfectly Blunt Cloning kit (Novagen, Madison, Wis., USA). The insert in each positive clone was amplified using the M13 reverse and U19 forward primers, and its size was determined using agarose gel electrophoresis to confirm that the clone contained the full length of the PCR product. The PCR products of the target inserts were sequenced directly in both directions using a Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA) using the T7 and U19 primers labeled with Texas Red, and the products were detected with an SQ-5500E sequencer (Hitachi). To examine the other seven loci, four alleles representing the range of length variation were sequenced as described above.

Data analysis

The mutation rates within each individual ramet or among ramets within each genet were calculated as $n/2m$, where n is the number of mutations and m is the number of samples analyzed. The mutation rate in offspring from each maternal ramet was calculated as n/m, where n is the number of offspring seeds that inherited a mutated allele from its mother and m the number of seeds analyzed. To calculate allele-specific mutation rates at Rops15 for each allele in each sample group investigated, the number of mutations observed was divided by the number of samples carrying the allele with the corresponding allele-specific mutation rate.

Table 1 Characteristics of three microsatellites isolated from *Robinia pseudoacacia*. *Ta* Annealing temperature of the primer pair, *HE* expected heterozygosity, *HO* observed heterozygosity

Locus	Repeat	Primer sequence(5′–3′)	*Ta.* (°C)	Size range (bp)	No. of alleles	*HO*	*HE*	GenBank accession no.
Rops15	(CT)20	GCCCATTTTCAAGAATCCATATATTGG TCATCCTTGTTTTGGACAATC	54	112–254	43	0.950	0.910	AB120731
Rops16	(CT)13	AACCCTAAAAGCCTCGTTATC TGGCATTTTTTGGAAGACACC	56	195–223	15	0.889	0.910	AB120732
Rops18	(AC)8	AGATAAGATCAAGTGCAAGAGTGTAAG TAATCCTCGAGGGAACAATAC	54	135–219	13	0.856	0.845	AB120733

The chi-squared test was used to analyze the differences between the numbers of repeat units gained or lost at a mutated Rops15 locus. To test differences between mutation rates within the individual ramet, ramets within the genet, and seeds collected from the same genet, the paired Student's *t*-test was employed.

Results

Mutations within individual ramets

Different leaves sampled from each of seven individual ramets were genotyped using eight microsatellite loci. Leaves obtained from different ramets had different genotypes, whereas leaves sampled from the same ramet differed only at the Rops15 locus. Of seven individual ramets investigated, Rops15 mutations were scored in six ramets (Table 2). Repeated PCR amplifications showed complete reproducibility of the electrophoretic bands for Rops15, confirming the presence of mutations at the locus. In addition, sequence analysis of the mutant alleles showed no variations in the flanking regions of the microsatellites. This indicated that the mutations occurred in the region of dinucleotide repeats. Variation in the number of dinucleotide repeats ranged from 13 to 75. All leaf samples except those from ramet 7 showed one or two electrophoretic bands at Rops15, corresponding to paired alleles. However, 30 of 74 leaves taken from ramet 7 showed a faint band in addition to two stronger bands. The faint bands were considered to result from the presence of a small number of cells carrying a different allele from the dominant paired alleles in the leaf tissues. Therefore, such faint bands were ignored in the following analysis.

Assuming that the allele with the highest frequency within each ramet was the progenitor allele, the progenitor with high frequency (>91.7%) was found in all ramets except for ramet 7, in which the longer alleles included seven variants (Table 2). Of these seven alleles found in ramet 7, three alleles of 244 bp, 248 bp, and 254 bp occupied similar relatively high frequencies of 37.8%, 21.6%, and 27.0%, respectively. Based on the general assumption that mutated alleles are produced as the sum of differences between mutated alleles and the progenitor is least (Primmer et al. 1998), we concluded that the progenitor allele of this ramet was the 248 bp allele, the middle allele of the three. The mutation rates within individual ramets varied from 0 to 31.1%. The overall mutation rate of the examined samples was 6.27% (Table 2).

Mutation rates among ramets within individual genets

We used the eight markers to genotype all leaves from 458 individual trees in the study site. Based on genotype identity at seven loci other than Rops15, the ramets were grouped into 19 genotypes, or genets. Mutant alleles at the Rops15 locus were found in ten genets (Table 3). Only the longer allele of the allele pair showed mutations in most genets, while the shorter allele did not (Table 3). Fifty-six mutated alleles were recorded in the leaf samples. Assuming that the allele with the highest frequency among Rops15 alleles in each genet was the progenitor allele, mutation rates among ramets within the same genet were calculated to range from 0 to 25.0%. The overall mutation rate of the examined samples was 6.11%.

Mutation rates among seeds from individual ramets

In 508 seeds collected from nine maternal trees, 19 seeds did not share the maternal alleles at Rops15. Among these, four seeds had only the alleles of the maternal ramet at seven loci except Rops15, strongly suggesting that these seeds were produced by self-fertilization (Table 4). The mutation rate among seeds from the same maternal ramet ranged from 0 to 10.9%. The mutation rate in the total collection of seeds investigated was 3.78%.

Characteristics of mutations

Rops15 mutation rates within individual genets were not significantly different from those within individual ramets (t=1.89<$t_{0.05}$, df=4) and seeds (t=1.23<$t_{0.05}$, df=4) (Table 5). The difference in mutation rates between the latter two was also not significant (t=2.72<$t_{0.05}$, df=3).

Two trends were observed in the mutations within individual ramets. When the allele lengths were less than 232 bp, there was a markedly significant bias toward gain versus loss of repeat units (19 versus 3; χ^2=11.8, df=1, P<0.001). Twenty of 22 mutations involved the gain or loss of a single repeat unit from the progenitor allele,

Table 2 Rops15 mutations observed in different leaves within a *R. pseudoacacia* ramet. The number in parenthesis is the number of leaves showing each genotype

Ramet	Leaf no. sampled	Genotypes						
1	80	128/172 (79)	128/174 (1)	–	–	–	–	–
2	69	132/160 (69)	–	–	–	–	–	–
3	80	172/226 (78)	172/228 (2)	–	–	–	–	–
4	86	176/224 (1)	176/232 (79)	176/234 (5)	178/232 (1)	–	–	–
5	81	128/170 (79)	128/172 (2)	–	–	–	–	–
6	72	176/210 (1)	178/208 (1)	178/210 (62)	178/212 (3)	178/214 (2)	180/210 (3)	–
7	74	132/242 (3)	132/244 (28)	132/246 (2)	132/248 (16)	132/252 (4)	132/254 (20)	132/256 (1)

Table 3 Rops15 mutations observed among ramets within a *R. pseudoacacia* genet. Each genet was identified based on allele patterns from seven loci (Rops02, Rops05, Rops06, Rops08, Rops10, Rops16, Rops18). The number in parenthesis is the number of ramets showing each genotype

Genet	Ramet no.	Genotypes						
1	35	112/172 (34)	112/174 (1)	–	–	–	–	–
2	3	120/126 (3)	–	–	–	–	–	–
3	18	120/132 (18)	–	–	–	–	–	–
4	29	126/128 (29)	–	–	–	–	–	–
5	42	128/128 (42)	–	–	–	–	–	–
6	19	128/164 (19)	–	–	–	–	–	–
7	19	128/170 (2)	128/172 (14)	128/174 (2)	128/176 (1)	–	–	–
8	70	128/170 (60)	128/172 (10)	–	–	–	–	–
9	13	128/208 (13)	–	–	–	–	–	–
10	8	128/132 (8)	–	–	–	–	–	–
11	46	132/152 (1)	132/158 (1)	132/160 (44)	–	–	–	–
12	9	132/226 (6)	132/228 (2)	132/230 (1)	–	–	–	–
13	15	132/244 (1)	132/252 (2)	132/254 (11)	132/256 (1)	–	–	–
14	3	134/142 (3)	–	–	–	–	–	–
15	2	134/210 (2)	–	–	–	–	–	–
16	30	154/166 (1)	154/174 (26)	154/176 (3)	–	–	–	–
17	12	172/224 (5)	172/226 (6)	174/226 (1)	–	–	–	–
18	56	174/226 (1)	176/228 (2)	176/230 (38)	176/232 (11)	176/236 (1)	178/230 (2)	178/234 (1)
19	29	178/210 (26)	178/212 (2)	180/210 (1)	–	–	–	–

Table 4 Rops15 mutations observed in seeds from a *R. pseudoacacia* ramet. The number in parenthesis is the number of mutation events

Maternal genotype	Seed no. sampled	Alleles not matching with mothers	
178/210	55	180 (4)	208 (2)
176/228	70	230 (7)[a]	
172/226	58	170 (1)	228 (2)
128/170[b]	259	172 (3)	
128/132	60	0	

[a] Mutation events contained 4 seeds produced by self-fertilization
[b] Data from five maternal ramets within the same genet were summed up

Table 5 Comparison of Rops15 mutation rates between the ramet, ramets within the genet, and seeds. Three kinds of samples compared were collected from the same genet. – indicates no analysis

Genotype in genet	Mutation rate (%)		
	Within ramet	Ramets within genet	Seeds
128/132	–	0	0
132/160	0	2.18	–
128/170	1.24	7.15	1.16
172/226	1.25	25.0	5.17
176/228	4.07	16.1	10.0
178/210	6.95	5.15	10.9
Average	2.70	9.25	5.45

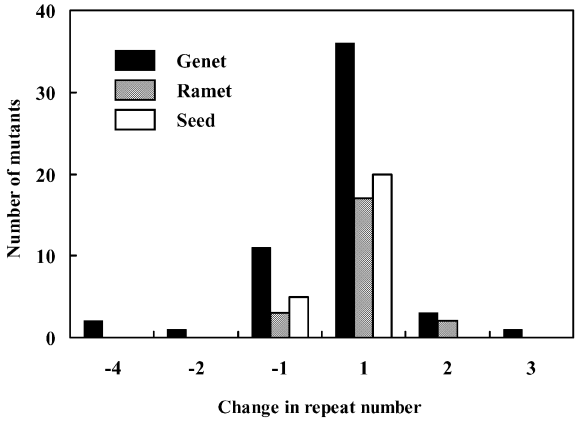

Fig. 1 Frequency distribution of magnitude and direction of change in repeat number at locus Rops15

although two mutations showed variation by two repeat units (Fig. 1). The longer alleles found in ramet 7 did not show the same pattern. Repeat unit variation by mutation ranged from 1 to 4, and the number of deletions (33) was higher than the number of additions (25).

Among ramets, there was also a significant bias toward gain versus loss of repeat units (41 versus 17; χ^2=43.0, *df*=4, *P*<0.001), and 50 of 58 mutant alleles differed from the progenitor allele by an increase or decrease of one repeat unit (Fig. 1).

Within the seeds, there was a marked bias toward gains against losses (16 vs. 3, respectively) (Fig. 1). All mutated alleles differed from the maternal alleles by addition or deletion of one repeat unit (Fig. 1).

The mutation rate at Rops15 was markedly influenced by allele length. Considering the data on mutation within individual ramets, among ramets and in seeds, mutations did not occur at alleles smaller than 160 bp; these alleles

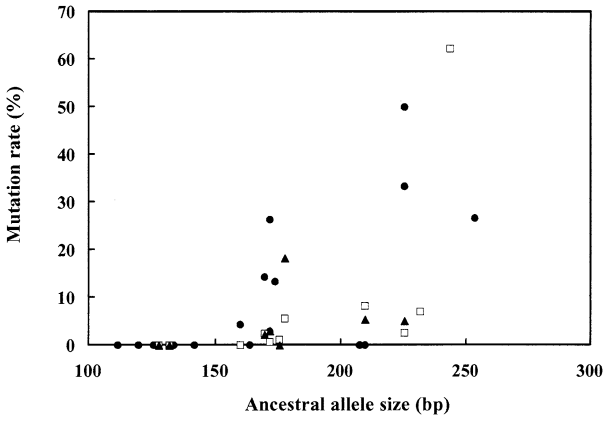

Fig. 2 Rops15 mutation rates of ancestral alleles detected within individual ramets (❑), individual genets (●), and seeds (▲). The mutation rate was calculated by dividing the number of mutations in each allele size by the total number of samples carrying that allele

had 3–24 repeats (Tables 2, 3, 4). The mutations occurred at alleles equal to or greater than 160 bp (27 repeats), and a significant positive relationship was found between the mutation rate and allele size (linear regression, y=0.2399x –33.88; r^2=0.3762; P<0.05) (Fig. 2).

Discussion

This is the first report of somatic mutation processes at microsatellites in different leaves within the same plant ramet. The average somatic mutation rate at Rops15 in individual *R. pseudoacacia* ramets was estimated to be 6.27×10^{-2}, which is significantly greater than any trans-generation mutation rate reported to date for plant microsatellites. For example, maize dinucleotide microsatellites mutate at a rate of 7.7×10^{-4} (Vigouroux et al. 2002) and the chickpea (TAA)*n* microsatellite locus mutates at a rate from 1.0×10^{-2} to 3.9×10^{-3} (Udupa and Baum 2001). Furthermore, no somatic variants were detected within Eastern white pine (Cloutier et al. 2003). The mutation rate at Rops15 is also higher than rates reported for microsatellite somatic mutation within an individual animal, which vary from 10^{-2} to 6.3×10^{-6} (Primmer et al. 1998; Schlötterer et al. 1998; Xu et al. 2000; Hoekert et al. 2002; Beck et al. 2003).

Our data revealed several features of the mutational process at Rops15 in *R. pseudoacacia*. First, mutation frequency was dependent on the number of dinucleotide repeats; mutation occurred in larger alleles at higher frequencies. The longest allele mutated at a rate over 62%. This is consistent with the hypothesis that microsatellite alleles with a larger number of repeats tend to have higher mutation rates (Wierdl et al. 1997; Primmer et al. 1998; Brohede et al. 2002; Beck et al. 2003).

Second, our study of a large set of alleles at Rops15 revealed interesting results about the directionality and magnitude of mutations. There was a significant bias toward additions over deletions at alleles with lengths less than 232 bp (63 repeats). This bias, however, was not observed at the longest allele, 248 bp (71 repeats); in this latter allele, the number of deletions was higher than that of additions. There still remains considerable controversy about the directionality and magnitude of microsatellite mutations. Certain studies have indicated that there is a significant bias toward additions over deletions (Amos et al. 1996; Primmer et al. 1998; Ellegren 2000). In contrast, no clear differences in the number of gains vs. losses were observed in several recent studies (Xu et al. 2000; Brohede et al. 2002; Leopoldino and Pena 2002; Beck et al. 2003). Although they presented no conclusive evidence, Primmer et al. (1998) hypothesized that a mechanism counteracting gradual microsatellite expansion involves large deletions of 50 to 100 repeat units, which sets a ceiling on microsatellite length. In the present study, repeat loss at the longest allele was indeed higher than gain (33 versus 25, respectively). Although the deletion was not as large as suggested by Primmer et al. (1998), our results seem to substantiate their hypothesis.

Third, the majority of mutations observed in this study involved length changes of only one or a few repeat units. This result favors a revised stepwise mutation model (Di Rienzo et al. 1994) where single step changes dominate. The somatic mutation at Rops15 in *R. pseudoacacia* was not consistent with the type of mutation found in human minisatellites, which appear to occur by separate pathways involving replication slippage and, more likely, intra-allelic unequal crossing-over (Jeffreys and Neumann 1997).

The mechanism for the high mutation rate at Rops15 is unclear. Various factors have been found to influence the rate of microsatellite mutations, including GC content in flanking DNA, repeated motif, allele size, types of cell division (mitotic vs meiotic), sex, genotype, and chromosome position (Li et al. 2002; Beck et al. 2003). Several researchers noted that GC content in the flanking DNA influences the mutation rate of microsatellites (Glenn et al. 1996; Vorechovsky et al. 2001). In the present study, seven of eight microsatellite loci showed no mutations among leaves in the same ramet. As we found almost no differences in GC content between flanking sequences of Rops15 and other loci, the GC content in the flanking DNA does not appear to influence the mutation rate for this locus. Moreover, the repeat motif, allele size, sex, and genotype were equally identical for Rops15 and the other seven loci in the present study. Therefore, the high mutation rate at Rops15 could not be explained by these factors.

Another factor that influences mutation rate is the position of the locus in the chromosome. Li et al. (2003) examined microsatellite diversity in wild emmer wheat, and they found a strong effect of the interaction between mean repeat length and SSR locus distance from the centromere on the number of alleles, and variance in repeat size at SSR loci. Future studies should be performed to determine the location of Rops15 in the *R. pseudoacacia* genome.

Finally, the somatic hypermutability of a microsatellite locus in the clonal tree *R. pseudoacacia* cautions against readily applying highly polymorphic microsatellites to genetic analyses. Microsatellites have rapidly become the preferred markers for individual assignment, pedigree or parentage analysis, and gene-mapping studies, as well as for studies of population genetics. Checking for hypermutability of microsatellite markers would increase confidence in the above types of analyses.

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