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Genetically-controlled leaf traits in two chemotypes of *Salix sachalinensis* Fr. Schm (Salicaceae)

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Abstract

Salix sachalinensis has two chemotypes: one biosynthesises ampelopsin as a major component of low molecular weight phenolics in their leaves (A-type), and the other biosynthesises β-D-glucopyranose-1-*trans*-*p*-coumarate (PG1) and β-D-glucopyranose-1-*trans*-cinnamate (PG2) in addition to ampelopsin (AP-type). We investigated phenotypic and genetic variations and clonal repeatabilities of the pubescence density, leaf mass per area (LMA), and concentrations of total phenolics, condensed tannin, ampelopsin, PG1 and PG2. Leaves of wild A-type trees contained significantly higher concentrations of total phenolics and ampelopsin, and lower concentration of condensed tannin than those of wild AP-type trees. In the greenhouse experiment that compared leaf traits between cloned trees obtained from wild chemotypes, there were significant between-type variations in the leaf phenolic concentrations, pubescence density, and LMA. Since chemotypes of cloned trees in the greenhouse were the same as those of wild parent trees, chemotype can be considered as a genetically controlled property. There were also significant within-chemotype variations in the pubescence density, LMA, total phenolics, ampelopsin, PG1, and PG2 concentrations, but not in concentration of condensed tannin for either chemotypes. Genetic variation of leaf traits except for LMA in AP-type was significant. PG1 and

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PG2 exhibited the highest clonal repeatabilities (0.73 and 0.78, respectively). Thus, the ability to produce and the amount of production of PG1 and PG2 are genetically controlled.

Keywords: *Salix sachalinensis*; Chemotype; Ampelopsin; β-D-Glucopyranose-1-*trans*-cinnamate; β-D-Glucopyranose-1-*trans*-*p*-coumarate; Genetic variation; Phenotypic variation; Clonal repeatability

1. Introduction

Secondary metabolites (e.g. phenolics and alkaloids) and pubescence are well-known deterrents and/or attractants of herbivores and pathogens, and can affect host use patterns (Bernays and Chapman, 1994; Schoonhoven et al., 1998). Intraspecific variation in leaf traits of wild plants is well documented, and researchers have been investigating the mechanisms that maintain such variation (Kennedy and Barbour, 1992). To elucidate the maintenance mechanism of intraspecific variation, it is necessary to investigate the relative contribution of genetic and non-genetic variation to phenotypic variation observed in nature (Simms and Rausher, 1992).

Salix sachalinensis is distributed from Japan to Russian Far East (Satake et al., 1989), and is a common willow in Hokkaido, northern Japan. It occurs along riversides and in mesic lowlands (Niiyama, 1990). In general, willow leaves contain phenolics as major secondary metabolites (Palo, 1984). *S. sachalinensis* has two distinct chemotypes (A-type and AP-type) that vary in the profile of low molecular phenolics in the leaves (Mizuno et al., 1989). The A-type produces ampelopsin that varies from 5.6 to 71.2 mg/g d.w. among A-type trees in Sapporo, Japan (Matsumoto and Tahara, 2001). The AP-type produces ampelopsin and two phenylpropanoid glycosides: β-D-glucopyranose-1-*trans*-*p*-coumarate (PG1) and β-D-glucopyranose-1-*trans*-cinnamate (PG2). In addition to these phenolic traits, the undersurface of *S. sachalinensis* leaves is pubescent (Satake et al., 1989). In spite of the existence of continuous (within-chemotype) and discontinuous (between-chemotypes) phenotypic variation, genetic control of this variation in leaf traits has never been examined.

Since willows can reproduce clonally from branch fragments (Newsholme, 1992), it is easy to make clones of parent trees by rooting replicate cuttings. Using this attribute, one can estimate genetic variation of particular traits by cultivating the clones in a uniform environment (Simms and Rausher, 1992). The objective of this study is to assess the phenotypic and genetic variations in leaf traits, both between and within chemotypes, and to investigate how much this intraspecific variation is genetically controlled. Clonal studies provide clonal repeatabilities, which is equal to upper-bound estimates of broad-sense heritabilities.

2. Materials and methods

2.1. Study site

Field sampling was carried out along the Ishikari River, which runs through central Hokkaido (43°N, 141°E). Greenhouse experiments were conducted at the Center for Ecological Research, Kyoto University, in Shiga Prefecture (35°N, 135°E).

2.2. Discrimination of wild chemotypes

In mid-April 2001, we determined the chemotype of 231 willow trees (~2 m in height) in the field using the criteria of Mizuno et al. (1989). Since the two chemotypes cannot be distinguished morphologically, we assayed each plant for the two phenylpropanoid glycosides, PG1 and PG2, using thin-layer chromatography (TLC). Presence of PG1 and PG2 indicated that the plant was AP-type. Samples were run on a reverse-phase silica gel RP-18F_{254S} plate (Merck Co, Art.15389, layer thickness 0.25 mm) in H_2O–MeOH (3:2). The methods of extraction and development of the leaf constituents are described below. If PG1 and/or PG2 were present at Rf 0.54 and/or Rf 0.28, respectively, both quenched under a UV lamp (254 nm) and gave pinkish red spots when sprayed with thymol reagent and heated (Krebs et al., 1969). Authentic PG1 and PG2 were prepared according to Mizuno et al. (1989), and identified by spectroscopic methods (MS, UV, $[\alpha]_D$, ^{1}H NMR and ^{13}C NMR).

2.3. Leaf trait analyses

We measured the following leaf traits: pubescence density, LMA (leaf mass per area) as an indicator for leaf thickness, and the concentrations of total phenolic, condensed tannin, ampelopsin, PG1, and PG2. It is well known that these leaf traits affect host plant selection and performance of herbivores (Bernays and Chapman, 1994; Schoonhoven et al., 1998).

2.3.1. Chemical traits

We collected leaves from five AP-type and 15 A-type trees growing in close proximity (Fig. 1). Five current shoots were randomly collected from each tree on 30 July 2001, immediately transported to the laboratory, placed in paper bags (10 × 15 cm), within plastic zip bags (25 × 30 cm) containing silica gel, and allowed to desiccate at room temperature for five days. Silica gel was changed daily. After desiccation, three mature leaves (completely expanded leaves along the middle part of a shoot) from each shoot were manually crumbled together. The resulting mixture of three leaves was regarded as a sample of a current shoot, and each of the five shoots was sampled as described above. Five samples per individual tree were used for measuring total phenolics, condensed tannin, ampelopsin, PG1, and PG2 concentrations.

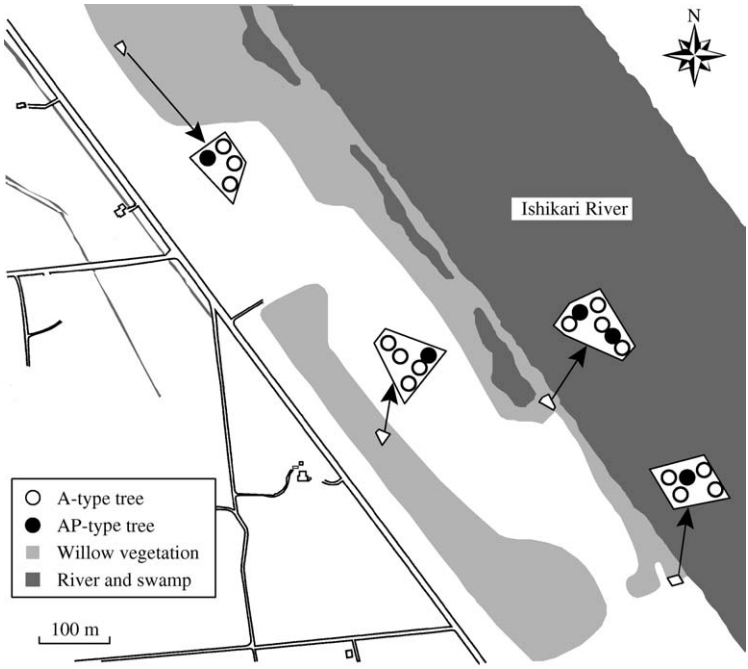

Fig. 1. Map of the study site with the position of studied trees. Open and filled circles show A-type and AP-type trees, respectively. Fifteen A-type trees and five AP-type trees were used for analyses (see text).

A 50 mg sample was put into a 8 ml screw-capped tube with 0.5 ml of 70% aqueous acetone, and allowed to stand for 1 h in a 40 °C ultrasonic bath. Total phenolic concentration (mg/g d.w.) in 2 μl of the extract was measured using the Folin–Ciocalteu method as described in Julkunen-Tiitto (1985), and condensed tannin concentration (mg/g d.w.) in 10 μl of the extract was measured using the butanol–HCl method as described in Waterman and Mole (1994). Absorbance and UV spectra were measured using a UV–Vis (HITACHI U 3210 for wild tree samples and SHIMADZU UV-2500PC for greenhouse samples).

Ampelopsin concentration (mg/g d.w.) was measured by TLC. Ampelopsin whose structure had been identified by mass, UV, and nuclear magnetic resonance (NMR) spectroscopes (Matsumoto and Tahara, 2001) was used as the standard. The extract (20 μl) was applied to TLC plates (Merck, Art.5715, silica gel $60F_{254}$, layer thickness 0.25 mm) as a 10 mm band in parallel with known amount of ampelopsin dissolved in methanol. Plates were developed in a mixture of toluene–ethyl acetate–isopropanol–formic acid (40:30:20:1). Both spots of ampelopsin standard and the extracts were visualized under UV (254 nm), and the silica gel layer of each relevant spot was scrapped off the plate. The silica gel was placed in a screw-capped tube along with 10 ml of MeOH. Samples were placed in a refrigerator for 12 h, and then the absorbance of the methanolic supernatant was measured at λ_{max} 292 nm of

ampelopsin. The blank for this quantification was prepared by using 10 ml of MeOH and approximately similar amounts of silica gel to that of ampelopsin spot scrapped off from the thin-layer plate developed in solvent alone. Calibration curve was made using standard ampelopsin.

The amounts of PG1 and PG2 were also determined as described for ampelopsin quantification. As with ampelopsin analysis, 20 μl of the leaf extracts and standard of PG1 and PG2 were separated on reverse-phase silica gel RP-18F_{254S} plates (Merck, Art.15389, layer thickness 0.25 mm) developed in a solvent (H_2O–MeOH 3:2) and examined at 254 nm. Spots corresponding to PG1 and PG2 were scraped off. Again the silica gel and 10 ml of MeOH were added to screw-capped tubes and placed in a refrigerator for 12 h. Absorbance of the supernatant was measured at λ_{max} 316 nm (PG1) and 281 nm (PG2). Calibration curves were made using the standard of PG1 and PG2.

2.3.2. Physical traits

We used the same five AP-type and 15 A-type trees used in chemical trait analyses for physical trait analyses. We collected one mature leaf from 10 current shoots from each tree on 30 July 2001. The leaves were brought back to the laboratory, their shapes were determined using a photocopying machine, and the leaf areas were measured using the NIH image program (http://rsb.info.nih.gov/nih-image/). Pubescence density was estimated by counting the number of pubescence within two squares (3.8×2.8 mm^2) located between the margin and midvein of the middle part of the undersurface of a leaf. The average density in the two squares was used as the value of pubescence density (No./mm^2) of the leaf. After these procedures, leaves were placed in a paper bag (10×15 cm) within a plastic zip bag (25×30 cm) containing silica gel, and kept at room temperature for five days. Silica gel was changed daily. After desiccation, we measured leaf dry mass and calculated the LMA by dividing leaf dry mass by leaf area.

2.4. Greenhouse experiment

To investigate the genetic variation and clonal repeatability of leaf traits between and within chemotypes, we cultivated clones propagated from the same five AP-type and 15 A-type trees from which leaves had been sampled. Clones originating from the same parent were treated as a genotype. On 5 April 2002, 20 1-year shoots (30 cm in length) were taken from each parent tree and wrapped with wet paper towel to avoid desiccation and transported to Center for Ecological Research, Kyoto University. Shoots were rooted in water containing 10% MENEDAEL Fe(II) aq. fertilizer (Menedael Chemical Laboratory, Co) for a week. On 15 April, 10 shoots with roots from each genotype were transplanted individually into 2.5 L pots containing compost with 4.5 g MAGAMP plant fertilizer (N:P:K:M = 6:40:6:15) (HYPONeX JAPAN, Co.). Potted clones were grown in a greenhouse ($4.5 \times 10.0 \times 2.6$ m) made from plastic sheeting and mesh under natural light and temperature conditions. The greenhouse was ventilated by replacing the lower 1/3 of the plastic sheeting with fine mesh. Each genotype was grouped in two rows, and

A-type and AP-type genotype groups were randomly assigned on three tables. Potted clones were watered twice a day for 15 min by an auto-watering system. All potted plants were checked every other day, and herbivores found on the plants were removed to avoid any effects they might cause.

On 13 August, four mature leaves were collected from each clone and dried using the same method described above. Four dried leaves were manually crumbled together. A mixture of four leaves was treated as a sample from one clone and used for chemical trait analyses. Another four mature leaves were collected from five clones from each genotype, and pubescence density and LMA were measured using the same methods described above. An average of four leaves was treated as measure of each clone.

2.5. Statistical and genetic analyses

Mann–Whitney *U* test was performed to test for between-chemotype differences in leaf traits of wild trees and of greenhouse clones. Differences in leaf traits within each chemotype were tested on the field trees and the greenhouse clones separately using one-way analyses of variance. We performed a contingency table analysis to test the relationship between field chemotype and chemotypes of clones in the greenhouse. The chemotypes of clones were distinguished by the presence or absence of PG1 and PG2. Clonal repeatabilities, which are equal to upper-bound estimates of broad-sense heritabilities, of leaf traits were estimated by using among- and within-genotype mean squares (MS_{among} and MS_{within}) obtained from one-way ANOVAs on greenhouse clones. The formulae used for clonal repeatability are (Lynch and Walsh, 1998):

$$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{ES}^2} \tag{1}$$

$$MS_{within} = \sigma_{ES}^2 \tag{2}$$

$$MS_{among} = \sigma_{ES}^2 + n\sigma_G^2 \tag{3}$$

where σ_{ES}^2 and σ_G^2 are environmental variance and total genetic variance, respectively, and n is the number of replications within a genotype. The limitation of this clonal analysis is that maternal and possible residual environmental effects are not factored out.

3. Results

3.1. Chemotype ratio in the field

Five AP-type trees (two male and three female) and 226 A-type trees (65 male, 141 female, and 25 unknown) were identified by screening 231 wild *S. sachalinensis* trees (Fig. 1). The proportion of A-type trees to all screened wild trees was 97.8%.

3.2. Leaf trait variations between chemotypes

Field A-type plants exhibited significantly higher ampelopsin and total phenolic concentrations and lower condensed tannin concentration than those of AP-type, whereas pubescence density and LMA did not differ significantly between chemotypes (Table 1). All leaf traits differed significantly between cloned chemotypes that were determined by the presence or absence of PG1 and PG2 in wild parent trees (Table 2). Any cloned trees obtained from A-type parent trees did not contain PG1 and PG2, while all AP-type clones contained PG1 and PG2. Proportion of chemotypes of potted clones was significantly different between A- and AP-type parent trees ($\chi^2 = 200.00$, $p < 0.0001$). AP-type clones also had significantly lower ampelopsin and total phenolic concentrations and pubescence densities, and higher condensed tannin concentration and LMA than those of A-type clones (Table 2).

3.3. Leaf trait variations within A-type trees

Wild A-types had significant among-tree differences in pubescence density, LMA, and total phenolic and ampelopsin concentrations (Table 3). However, condensed tannin concentration did not differ significantly. On the other hand, all leaf traits exhibited significant differences among A-type genotypes in the greenhouse. The clonal repeatabilities of leaf traits among A-type genotypes ranged from 0.18 to 0.76.

Table 1
Leaf traits (mean ± SD) of two chemotypes growing in the field

Leaf traits	Chemotype		*U*	*p*
	A ($N = 15$)	AP ($N = 5$)		
Pubescence density (no./mm^2)	4.5 ± 3.74	2.0 ± 2.01	17	0.0736
LMA (mg/mm^2)	7.1 ± 0.85	7.3 ± 0.54	34	0.7600
Total phenolics (mg/g d.w.)	151.1 ± 22.97	86.9 ± 25.27	0	0.0011
Condensed tannin (mg/g d.w.)	7.0 ± 1.70	18.0 ± 3.08	0	0.0011
Ampelopsin (mg/g d.w.)	49.7 ± 14.14	17.7 ± 13.44	4	0.0035
PG1 (mg/g d.w.)	0.0 ± 0.00	5.0 ± 2.77	0	<0.0001
PG2 (mg/g d.w.)	0.0 ± 0.00	16.3 ± 9.32	0	<0.0001

Table 2
Leaf traits (mean ± SD) of two chemotypes growing in the greenhouse

Leaf traits	Chemotype		*U*	*p*
	A ($N = 15$)	AP ($N = 5$)		
Pubescence density (no./mm^2)	2.4 ± 2.21	0.5 ± 0.41	6	0.0060
LMA (mg/mm^2)	6.1 ± 0.46	6.8 ± 0.37	15	0.0495
Total phenolics (mg/g d.w.)	130.6 ± 16.94	78.4 ± 16.13	0	0.0011
Condensed tannin (mg/g d.w.)	8.2 ± 0.79	12.0 ± 1.96	2	0.0019
Ampelopsin (mg/g d.w.)	89.7 ± 21.94	14.8 ± 4.42	0	0.0011
PG1 (mg/g d.w.)	0.0 ± 0.00	13.9 ± 5.15	0	<0.0001
PG2 (mg/g d.w.)	0.0 ± 0.00	43.2 ± 33.71	0	<0.0001

3.4. Leaf trait variations within AP-type trees

Wild AP-types exhibited significant among-tree differences in pubescence density, LMA, total phenolics, ampelopsin, PG1, and PG2, but not in condensed tannin concentration (Table 3). On the other hand, among-genotype differences in leaf traits (with the exception of LMA) were significant in the greenhouse. Our estimates of clonal repeatabilities for the AP-type plants ranged from 0.22 to 0.78.

4. Discussion

Mizuno et al. (1989) distinguished the chemotypes of *S. sachalinensis* by the presence or absence of PG1 and PG2. This study indicated that the difference between two chemotypes was characterized not only by the presence or absence of PG1 and PG2, but also by the concentrations of total phenolics, condensed tannins, and ampelopsin.

The variation among cloned trees and between chemotypes was observed in the controlled conditions of the greenhouse. This indicates that significant genetic variation exists. If cloned trees have the same chemotypes as field parent trees, it can be concluded that the chemotypes of *S. sachalinensis* are genetically controlled. But if the chemotypes of cloned trees are different from those of field parent trees, one can assume that non-genetic factors determine the chemotypes of *S. sachalinensis*. In this study, chemotypes of cloned trees were in accord with those of wild parent trees. In other words, any cloned trees that originated from AP-type wild trees contained PG1 and PG2, and vice versa. Phenolic compounds other than PG1 and PG2 also showed a consistent relationship between wild parent and cloned trees. Phenolic compound that exhibited higher concentrations in a wild parent chemotype also exhibited higher concentrations in the cloned chemotypes (Tables 1 and 2). These results suggest that the difference between the two chemotypes in *S. sachalinensis* is genetically determined. Furthermore, since both chemotypes were represented by both male and female plants, the chemotypes are not a sex-based property. Berenbaum and Zangerl (1992) argued that qualitative traits (e.g. presence or

Table 3
ANOVAs of leaf traits among A-type trees and among AP-type trees growing in the field and greenhouse

Leaf traits	Field				Greenhouse					
	A-type ($N = 15$)		AP-type ($N = 5$)		A-type ($N = 15$)			AP-type ($N = 5$)		
	$MS_{(tree)}$	$MS_{(error)}$	$MS_{(error)}$	$MS_{(tree)}$	$MS_{(within)}$	$MS_{(among)}$	h^2	$MS_{(within)}$	$MS_{(among)}$	h^2
Pubescence density	8.81	87.90***	7.27	24.78*	1.31	21.93***	0.76	0.10	0.85**	0.59
LMA	0.65	3.29***	0.66	2.39*	0.20	1.95***	0.64	0.28	$0.68^{n.s.}$	0.22
Total phenolics	491.04	2637.81***	237.33	3192.77***	202.99	1575.06***	0.40	104.38	1185.60***	0.51
Condensed tannin	11.18	$14.49^{n.s.}$	118.41	$47.41^{n.s.}$	0.98	3.20**	0.18	2.13	23.85***	0.51
Ampelopsin	110.63	999.43***	105.86	902.88**	247.89	2425.08***	0.47	21.02	113.93*	0.31
PG1			3.21	38.34***				4.26	121.26***	0.73
PG2			21.47	434.67***				138.11	5090.19***	0.78

Mean squares and clonal repeatabilities (h^2) are presented. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

absence of secondary compounds) are usually oligogenic and controlled by dominant alleles. For example, the cucumber plant *Curcumas sativus* (Cucurbitaceae) varies qualitatively in the intraspecific presence or absence of cucurbitacin production. Cucurbitacin production is controlled by a two-allele, single-locus gene, where it is dominant (Andeweg and de Bruyn, 1959). In the ponderosa pine, *Pinus ponderosa* (Pinaceae), five chemotypes vary in the relative amounts of three monoterpene (α-pinene, β-pinene, Δ-3-carene), and single gene allelic variation appears to control them (Latta et al., 2003). Future studies should examine the genetic pattern of inheritance for the two chemotypes of *S. sachalinensis*.

Intraspecific continuous variations in phenolic compounds have been reported in some boreal deciduous plants, including willows (Julkunen-Tiitto and Meier, 1992; Price et al., 1989; Veteli et al., 2002), aspen (Lindroth and Hwang, 1996), and birch (Laitinen et al., 2000). For example, concentrations of two phenolic glycosides, salicortin and 2′-cinnamoyl salicortin, in the leaves of the willow, *Salix sericea*, differ significantly among clones (Nichols-Orians et al., 1993). Some of these variations are affected by environmental factors, such as temperature, CO_2 concentration, and water or nutrient availability (Price et al., 1989; Veteli et al., 2002). For instance, nutrient fertilization decreased salicortin concentration in *S. sericea* (Orians et al., 2003). The clonal repeatabilities differed among leaf traits, showing highest values in PG1 and PG2. Thus, the amount of production of the two phenolics may be less affected by environmental factors, and under a stronger genetical control. On the other hand, condensed tannin showed low repeatability, suggesting a greater effect of environmental factors. Berenbaum and Zangerl (1992) pointed out in a review of the literature that heritabilities vary widely among secondary metabolites. Orians et al. (1996) showed a difference in narrow-sense heritabilities between salicortin and 2′-cinnamoyl salicortin of *S. sericea*. This variation might be caused by differences in bioactivity and the period of time under which this leaf trait has been subjected to natural selection.

Secondary metabolites of plants are well known as attractants or deterrents for herbivores and as antifungal agents. Feeding by the leaf beetle *Plagiodera versicolora* (Chrysomelidae), one of the major herbivorous insects on *S. sachalinensis*, is stimulated by PG1 but not by PG2 (Matsumoto and Tahara, unpublished data). Ampelopsin is a major antifungal constituent not due to high specific activity, but due to its high concentration in *S. sachalinensis* leaves (Matsumoto and Tahara, 2001). Ampelopsin also acts as a feeding stimulant and arrestant for adult *P. versicolora* (Matsumoto and Tahara, unpublished data). In general, tannins act as digestion inhibitors and toxins for herbivores (Harborne, 1993). Ayres et al. (1997) performed bioassays of the effects of condensed tannins in 20 species of woody plants (including six salicaceous species) on six folivorous insect species, and showed that these tannins decreased the insect growth rate. Moreover, Zvereva et al. (1998) showed that the leaf pubescence of *Salix borealis* reduced the amount of leaf consumption by the willow leaf beetle *Melasoma lapponica*. Since two chemotypes differ also in leaf traits other than PG1 and PG2 that affect the preference and performance of herbivores, an integrated consideration of leaf trait effects is necessary to understand the differences of herbivore activity between chemotypes. In

this context, the community structure of herbivorous insects on potted clones differed significantly between two chemotypes (Hayashi and Ohgushi, unpublished data), which may have resulted from species-specific responses of herbivorous insects to the leaf phenolics in different chemotypes.

The proportion of A-types was 97.8% at the Ishikari study site in Hokkaido. In contrast, no A-type *S. sachalinensis* trees were found in Sendai, northeast Japan (Mizuno et al., 1989). Because of the genetic control of chemotypes, this geographic variation in chemotype frequencies is not caused by phenotypic plasticity, but by differences in genotype frequencies. Detection of biotic and/or abiotic factors that are correlated with chemotype frequencies and the effects of these factors on the fitness of chemotypes would be valuable to understand the maintenance mechanism of these two chemotypes in *S. sachalinensis*.

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