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ORIGINAL ARTICLE

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Comparative analysis of diterpene composition in the bark of the hybrid larch F_1, *Larix gmelinii* var. *japonica* × *L. kaempferi* and their parent trees

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Abstract The diterpene compositions in the bark of branches were investigated for two families of the F_1 hybrid, Kurile larch (*Larix gmelinii* var. *japonica* Pilg.) × Japanese larch [*Larix kaempferi* (Lamb.) Carr.] (hereafter F_1) and their parents clones. 13-Epimanool, larixol, larixyl acetate, 13-epitorulosyl acetate (not detected in *L. gmelinii* var. *japonica*), isopimaric acid, abietic acid, dehydroabietic acid, and neoabietic acid were detected. Larixol and abietic acid represented more than 50% of the diterpene content in *L. gmelinii* var. *japonica* and *L. kaempferi*, respectively. Larixol and abietic acid were the predominant diterpene components in the F_1, and the proportions of these diterpenes were between those of the parental species. Therefore, the diterpene compositions in the F_1 were hereditarily influenced by their parents. The ratios of labdane, pimarane, and abietane diterpenes suggested that the main diterpene biosynthesis pathway in *L. gmelinii* var. *japonica* was from copalyl diphosphate (CDP) to labdane-type diterpenes, and that in *L. kaempferi* was from CDP to abietane-type diterpenes via pimarane type. Furthermore, linear discriminant analysis suggested that the diterpene contents are effective indices for the discrimination of the hybrid seedlings.

Key words Diterpene · Hybrid larch · *Larix kaempferi* (Lamb.) Carr. · *Larix gmelinii* var. *japonica* Pilg. · Discriminant analysis

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Introduction

The species of the genus *Larix* are deciduous conifers widely distributed in subarctic zones, Europe, Siberia, northern parts of North America, high mountains at middle latitudes, and the Himalayas.[1] In Japan, Japanese larch [*Larix kaempferi* (Lamb.) Carr.] was originally distributed in the central mountainous regions of Honshu Island, central Japan. *Larix kaempferi* grows very well not only in its native area but also in eastern and northern Japan.[2] Therefore, it has been introduced into Hokkaido Island, which is the northern part of Japan, from Honshu Island and has become an important forestry species since the early part of the last century. However, the seedling and the trunk bark of *L. kaempferi* are easily damaged by the native vole (*Clethrionomys rufocanus bedfordiae* Thomas) in Hokkaido,[3] and serious damage (over 40 000 ha/year) has been recorded between 1950 and 1980.[4] On the other hand, the Kurile larch (*Larix gmelinii* var. *japonica*) was introduced to Hokkaido from its native distribution area of Sakhalin and the southern part of Kurile Island. *Larix gmelinii* var. *japonica* is superior to *L. kaempferi* in terms of vole resistance and wood quality, although its growth rate is slower than that of *L. kaempferi*. The hybrid F_1 larch [*L. gmelinii* var. *japonica* Pilg. × *L. kaempferi* (Lamb.) Carr.] exhibits the good points of Kurile larch, such as good wood quality and vole resistance,[5] and a higher growth rate of the juvenile trees than *L. kaempferi*. In addition, the selection of high CO_2-accumulating families in hybrid F_1 larch has been studied,[6,7] and the F_1 is considered one of the most important species for tree plantations in Hokkaido.

The seeds of the F_1, which were produced by open pollination in the hybridization seed orchard of *L. gmelinii* var. *japonica* and *L. kaempferi* (pollen parent), were collected from *L. gmelinii* var. *japonica* (seed parent). Because *L. kaempferi* and *L. gmelinii* var. *japonica* were able to contribute as paternal donors, the collected seeds contained those of *L. gmelinii* var. *japonica* × *L. gmelinii* var. *japonica*, as well as those of the F_1. However, it is almost impossible to discriminate the F_1 seeds from *L. gmelinii* var. *japonica* seeds. At present, after planting the seeds, F_1 seedlings are selected by their morphology (the seedling height and number of branches) and phenological characteristics (the time of winter bud formation and the leaves turning yellow). However, the frequency distributions of the F_1 characteristics are continuous,[8] and the F_1 seedlings cannot be discriminated perfectly. Therefore, improvement of the present methods or the development of more convenient discrimination methods are required.

The differences in the bark extracts between *L. gmelinii* var. *japonica*, *L. kaempferi*, and the F_1 have been discussed for resistance to vole browsing.[9,10] Sukeno and Ozawa[9] reported that diterpenes in the ether extract of the branch bark from *L. gmelinii* var. *japonica*, especially larixol and 13-epimanool, could be related to the resistance to vole attacks. Hayashi et al.[10] showed that the ether extract contents in the branch bark are quite different among the families produced by the intraspecific and interspecific crossing between these two larch species, being the greatest in the crossing combination of *L. gmelinii* var. *japonica* × *L. gmelinii* var. *japonica*, and the lowest in the combination of *L. kaempferi* × *L. kaempferi*. Moreover, the hybrids *L. gmelinii* var. *japonica* × *L. kaempferi* and *L. kaempferi* × *L. gmelinii* var. *japonica* were ranked between the former two crosses. From these reports, it was expected that the diterpene composition in the ether extracts of the larch bark could be under hereditary control according to the crossing patterns.

The discrimination of trees by diterpenes has been studied in *Cryptomeria japonica*,[11] *Thujopsis dolabrata* var. *hondae*,[12] and *Pinus pinaster*.[13] These studies suggested that diterpene compositions of these species were effective indices for the discrimination of the characteristics of the trees (geographical differentiation, diseased tree, plus trees).[11–13] In addition, it was suggested that multivariate analysis (principal component and discriminant analyses) using diterpene composition was useable for the discrimination.[11,13] However, it is not clear whether the discrimination between the F_1 hybrid and its parent species by diterpene compositions is possible. In the present study, the difference in diterpene composition in the bark of branches among *L. gmelinii* var. *japonica*, *L. kaempferi*, and F_1 mature trees was investigated and these characteristics are discussed from the viewpoint of diterpene synthesis. Moreover, the method for discrimination between F_1 and its parent species was established by linear discriminant analysis using the diterpene contents, and the seedlings from a larch hybridization seed orchard were discriminated by their diterpene contents.

Materials and methods

Plant materials

Samples for diterpene analysis were collected from mature trees at the Hokkaido Forestry Research Institute (Bibai, Hokkaido, Japan; 43°28′N, 141°88′E) in September 2005. The branches were collected from 32-year-old *Larix gmelinii* var. *japonica* clones (Kabaoka 168, two ramets; Toyooka 111, four ramets), 46-year-old *Larix kaempferi* clones (Tokachi 16, five ramets; Tokachi 35, five ramets), and two families of 11-year-old *L. gmelinii* var. *japonica* × *L. kaempferi* (Kabaoka 168 × Tokachi 16, five individuals; Toyooka 111 × Tokachi 35, six individuals).

In addition, 2-year-old seedlings were used. The seeds produced by open pollination were collected from *L. gmelinii* var. *japonica* (Nakashibetsu 660, Nakashibetsu 121, Kabaoka 484, Kabaoka 455, Rubeshibe 28) in the hybridization seed orchard, where *L. gmelinii* var. *japonica* and *L. kaempferi* were planted at random, in August 2004. The hybridization seed orchard was established at the Eastern Abashiri Forestry Center (Kunneppu, Hokkaido, Japan; 43°44′N, 143°42′E) in 1974. The tree heights of *L. gmelinii* var. *japonica* and *L. kaempferi* when the samples were collected were about 18 m and 16 m, respectively. The collected seeds were sown in a nursery garden at Hokkaido Forestry Research Institute in May 2005. The seedlings were replanted at a density of 36 trees/m^2 in May 2006. The strict discrimination of F_1 seedling is impossible by morphological traits. Therefore, *L. gmelinii* var. *japonica* and the F_1 seedlings were discriminated using DNA markers.[14] The seedlings of the F_1 and *L. gmelinii* var. *japonica* (6–25 and 24–26 individuals per seed parent, respectively) from five seed parents, a total of 209 individuals, were used. The seedling branches were collected in September 2006 and they were frozen immediately until diterpene analysis.

Diterpene analysis

Standard abietic acid and dehydroabietic acid were purchased from Wako (Osaka, Japan), while neoabietic acid and isopimaric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Helix Biotech (Richmond, Canada), respectively. These chemicals were purified by silica gel column chromatography and recrystallization appropriately. 13-Epimanool, larixol, larixyl acetate, and 13-epitorulosyl acetate were isolated in a pure form from the dichloromethane extract of *L. gmelinii* var. *japonica* bark by silica gel column chromatography, high-performance liquid chromatography (HPLC), and/or recrystallization. The structures were confirmed by gas chromatography-mass spectrometry (GC-MS; JMS-600H, JEOL, Tokyo, Japan) and nuclear magnetic resonance (NMR) spectroscopy (JNM-AL400, JEOL, Tokyo, Japan,) (Seki, unpublished).

The extract preparation from bark samples and chemical analysis were modified from the method of Hansson et al.[15] The bark samples were crushed in a homogenizer

Table 1. Characteristic mass spectrometry fragmentation of diterpenes

Diterpene	RT (min)[a]	Characteristic fragmentation (*m/z*)[b]
13-Epimanool	9.34	55 (37), 69 (44), 81 (72), 137 (100), 257 (57), 272 ([M-H_2O]$^+$, 30)
Larixol	12.33	69 (100), 71 (44), 153 (59), 195 (1), 255 (38), 270 (28), 273 (26), 288 ([M-H_2O]$^+$, 27)
Isopimaric acid methyl ester	13.12	241 (100), 257 (76), 287 (23), 301 (24), 316 (M$^+$, 65)
Dehydroabietic acid methyl ester	13.41	239 (100), 255 (7), 299 (26), 314 (M$^+$, 18)
Larixyl acetate	14.14	71 (37), 153 (100), 195 (16), 255 (65), 270 (43), 273 (9), 288 ([M-H_2O-CH_3CO]$^+$, 13)
Abietic acid methyl ester	14.31	241 (52), 256 (100), 301 (7), 316 (M$^+$, 96)
13-Epitorulosyl acetate	15.17	81 (53), 135 (100), 161 (22), 257 (50), 270 (13), 302 (12), 315 (10), 330 ([M-H_2O]$^+$, 17)
Neoabietic acid methyl ester	15.22	135 (100), 148 (26), 181 (16), 257 (12), 316 (M$^+$, 74)

RT, Retention time
[a] DB-1MS capillary column; column temperature, programmed from 170°C to 266°C at 5°C/min
[b] Relative intensity given in parentheses

(Nihonseiki, Tokyo, Japan) with liquid nitrogen and freeze-dried. Then 100 mg of bark meal was extracted with 2 ml of petroleum ether–diethyl ether (1:1, v/v) containing 400 µg heptadecanoic acid (Aldrich, Milwaukee, WI, USA) as an internal standard in an ultrasonic bath for 2 h at 10°–15°C. The extracted samples were centrifuged, and the supernatant was evaporated to dryness under a stream of nitrogen. The dried extract was methylated with diazomethane in diethyl ether prior to GC-MS analysis. The samples were analyzed by GC-MS (JMS-600H, JEOL, Tokyo, Japan) equipped with a capillary column of DB-1MS (30 m × 0.25 mm i.d., film thickness 0.25 µm; Agilent, Tokyo, Japan). The column was programmed from 170°C to 266°C at 5°C/min. Helium was used as the carrier gas. The injector temperature was maintained at 270°C, and the ionizing energy was 70 eV. Peaks were identified by the comparison of their retention times and mass spectra (Table 1) with those of standard diterpenes and quantified by calibration curves. Diterpene composition was calculated as mol% = (mole of each diterpene)/(the sum of moles of identified diterpenes) × 100. Total diterpene content was calculated as nmol/bark mg = (the sum of moles of identified diterpenes)/(bark mg). All data for diterpene analyses are presented as averages from at least three independent experiments, along with the standard deviation.

Statistical analysis

The multiple comparison with diterpene contents among *L. gmelinii* var. *japonica*, *L. kaempferi*, and the F_1 were carried out by the Tukey-Kramer HSD test ($P < 0.05$). Linear discriminant analysis was also carried out in order to determine the effect of discrimination of the F_1 by the diterpene contents. All statistical analysis was performed with the program JMP 5.0.1 J (SAS Institute, Cary, NC, USA).

Results

Diterpene composition of mature-tree bark

The petroleum ether–diethyl ether extract of mature-tree bark from *Larix gmelinii* var. *japonica* clones (Toyooka 111 and Kabaoka 168), *Larix kaempferi* clones (Tokachi 35 and Tokachi 16), and two families of the F_1 (Toyooka 111 × Tokachi 35 and Kabaoka 168 × Tokachi 16) were analyzed by GC-MS. The typical total-ion chromatogram of the extract of F_1 (Toyooka 111 × Tokachi 35) and the mean values of the diterpene composition and the total contents of the identified diterpenes in the bark (total diterpenes) are shown in Fig. 1 and Table 2, respectively. Common to two *L. gmelinii* var. *japonica* clones, larixol represented more than 50% of the diterpene composition, followed by abietic acid. In *L. kaempferi* clones, abietic acid represented more than 50% of the diterpene composition, followed by dehydroabietic acid. The F_1 families contained larixol or abietic acid as the predominant diterpene components, and their proportions ranked between their parents, *L. gmelinii* var. *japonica* and *L. kaempferi*. When the diterpenes were classified into labdane-type (13-epimanool, larixol, larixyl acetate, 13-epitorulosyl acetate), pimarane-type (isopimaric acid), and abietane-type (abietic acid, dehydroabietic acid, neoabietic acid) diterpenes, the labdane type represented about 70% in *L. gmelinii* var. *japonica* and the abietane type represented about 80% in *L. kaempferi*. On the other hand, in the F_1 of each family, labdane-type and abietane-type diterpenes represented 51% and 32% (Toyooka 111 family), and 40% and 59% (Kabaoka 168 family), respectively. These proportions were between the proportions of their parents.

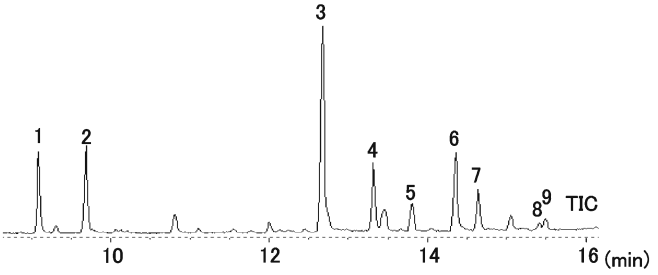

Fig. 1. Total ion chromatograms (*TIC*) of diterpenes from larch F_1 hybrid (Toyooka 111 × Tokachi 35) bark. Peaks: *1*, heptadecanoic acid methyl ester (internal standard); *2*, 13-epimanool; *3*, larixol; *4*, isopimaric acid methyl ester; *5*, dehydroabietic acid methyl ester; *6*, larixyl acetate; *7*, abietic acid methyl ester; *8*, 13-epitorulosyl acetate; *9*, neoabietic acid methyl ester

In the F_1 of two families, Toyooka 111 × Tokachi 35 and Kabaoka 168 × Tokachi 16, the total diterpene contents (184.2 and 148.5 nmol/bark mg, respectively) were not espe-

Table 2. Diterpene compositions and contents in the bark of mature-tree branches

Diterpene	*Larix gmelinii*[a,b]	F_1[c]	*Larix kaempferi*[d]	*Larix gmelinii*[a,e]	F_1[d]	*Larix kaempferi*[d]
	Toyooka 111	Toyooka 111 × Tokachi 35	Tokachi 35	Kabaoka 168	Kabaoka 168 × Tokachi 16	Tokachi 16
13-Epimanool	9.0 ± 1.9	8.5 ± 4.0	3.4 ± 1.1	7.4 ± 1.6	4.1 ± 0.7	2.7 ± 0.3
Larixol	55.3 ± 4.9	26.1 ± 14.0	3.2 ± 1.8	52.3 ± 4.0	15.7 ± 7.9	3.5 ± 0.8
Larixyl acetate	7.5 ± 0.8	6.2 ± 1.5	5.1 ± 1.3	9.3 ± 1.4	6.2 ± 2.8	8.9 ± 0.6
13-Epitorulosyl acetate	–[g]	10.4 ± 8.7	Trace[h]	–	5.5 ± 8.1	4.5 ± 0.7
Isopimaric acid	6.1 ± 0.5	9.0 ± 2.0	9.3 ± 1.3	4.7 ± 0.6	9.7 ± 1.1	1.7 ± 1.1
Abietic acid	13.9 ± 2.2	23.8 ± 8.1	52.0 ± 10.1	15.3 ± 3.0	32.6 ± 5.4	58.5 ± 4.6
Dehydroabietic acid	3.6 ± 2.4	11.9 ± 4.6	20.1 ± 7.9	4.7 ± 1.6	16.8 ± 5.8	13.6 ± 2.5
Neoabietic acid	4.7 ± 1.2	4.1 ± 1.3	7.0 ± 1.0	6.3 ± 2.2	9.4 ± 2.3	6.6 ± 0.8
Labdane type[f]	344.0 ± 50.3 A	98.2 ± 58.0 B	12.6 ± 6.4 C	333.3 ± 92.6 A	47.9 ± 18.7 B	20.7 ± 6.5 B
Pimarane type[f]	29.2 ± 4.4 A	15.3 ± 4.7 B	9.7 ± 4.4 B	22.0 ± 1.9 A	14.1 ± 2.6 B	14.8 ± 2.3 B
Abietane type[f]	108.0 ± 31.4 A	70.7 ± 29.0 A	88.0 ± 47.8 A	124.3 ± 10.2 A B	86.5 ± 20.2 B	131.3 ± 27.5 A
Total diterpenes[f]	481.2 ± 78.3 A	184.2 ± 88.3 B	110.3 ± 58.4 B	479.6 ± 104.7 A	148.5 ± 37.4 B	166.8 ± 34.4 B

Data given as mean mol% ± standard deviation
Values not sharing the same uppercase letters (A, B, C) within each family are significantly different by Tukey-Kramer HSD test ($P < 0.05$)
[a] *Larix gmelinii* var. *japonica* Pilg.
[b] $n = 4$
[c] $n = 6$
[d] $n = 5$
[e] $n = 2$
[f] Data given in units of nmol/bark mg
[g] Not detected
[h] <0.1%

cially different from those in their pollen parents, *L. kaempferi* (110.3 and 166.8 nmol/bark mg, respectively). However, they were significantly lower (Tukey-Kramer HSD test, $P < 0.05$) than those in their seed parents, *L. gmelinii* var. *japonica* (481.2 and 479.6 nmol/bark mg, respectively) (Table 2).

Discrimination of mature trees by content of each diterpene in the bark

Figure 2 shows the results of linear discriminant analysis using eight kinds of diterpene (13-epimanool, larixol, larixyl acetate, 13-epitorulosyl acetate, isopimaric acid, abietic acid, dehydroabietic acid, and neoabietic acid) contents (nmol/bark mg) in the bark from Toyooka 111, 4 ramets; Toyooka 111 × Tokachi 35, 6 individuals; Tokachi 35, 5 ramets; Kabaoka 168, 2 ramets; Kabaoka 168 × Tokachi 16, 5 individuals; and Tokachi 16, 5 ramets (total 27 individuals). The circles in Fig. 2, which indicate the 95% confidence intervals of the multivariate means, are separate from each other, indicating significant differences among the three species. The first and second axes explained 91.3% and 8.7% of the total dispersion with canonical correlations of 0.985 and 0.868, respectively. Discriminant function 1 mainly related to the contribution of neoabietic acid, 13-epimanool, and isopimaric acid, while discriminant function 2 related to isopimaric acid and abietic acid. The percentage of misjudgment by this linear discriminant analysis was 0%, suggesting that discrimination of the species is possible by diterpene contents. The percentage of misjudgment (0%) was provided by selecting three diterpenes, larixol, isopimaric acid ($P < 0.001$, Prob > F, respectively), and abietic acid ($P < 0.01$, Prob > F).

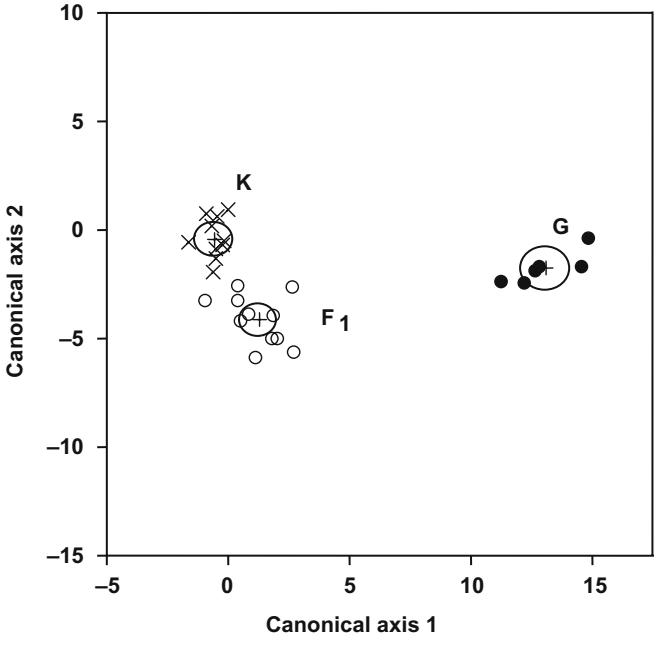

Fig. 2. Linear discriminant analysis using diterpene compositions in the branch bark of mature *Larix gmelinii* var. *japonica* (*G*), *Larix kaempferi* (*K*), and the F_1 (*L. gmelinii* var. *japonica*, 6 ramets; F_1, 11 individuals; *L. kaempferi*, 10 ramets). *Filled circles*, *L. gmelinii* var. *japonica*; *crosses*, *L. kaempferi*; *open circles*, F_1. *Large circles* show the 95% confidence intervals of the multivariate means

Diterpene composition of seedling bark

The diterpene compositions in the bark of the seedling branches were analyzed in five families (Nakashibetsu 660, Nakashibetsu 121, Kabaoka 484, Kabaoka 455, and Rubeshibe 28). Seven kinds of diterpene, 13-epimanool,

larixol, 13-epitorulosyl acetate, isopimaric acid, abietic acid, dehydroabietic acid, and neoabietic acid were detected but larixyl acetate was not detected (Table 3). Larixol was the predominant diterpene component in *L. gmelinii* var. *japonica* seedlings (42%–48%). In the F_1, abietic acid (36%–53%) was the predominant diterpene component followed by larixol (16%–33%). *Larix gmelinii* var. *japonica* seedlings contained labdane-type diterpenes at levels of 51%–59%, which was lower than in mature trees (ca. 70%). The F_1 contained abietane-type diterpenes at levels of 50%–65%.

The total diterpene content in the F_1 was 110–215 nmol/bark mg, which was lower than that in *L. gmelinii* var. *japonica* (141–351 nmol/bark mg). In *L. gmelinii* var. *japonica*, the total diterpene content in the seedling was considerably lower than that in mature trees (480–481 nmol/bark mg) (Table 3).

Discrimination of seedlings by content of each diterpene in the bark

Figure 3 shows the results of linear discriminant analysis using seven kinds of diterpene (13-epimanool, larixol, 13-epitorulosyl acetate, isopimaric acid, abietic acid, dehydroabietic acid, and neoabietic acid) contents (nmol/bark mg) in the seedlings of *L. gmelinii* var. *japonica* and the F_1 of each family. The percentage of misjudgment in the analyses of Nakashibetsu 660, Nakashibetsu 121, Kabaoka 484, Kabaoka 455, and Rubeshibe 28 were 0%, 2.0%, 2.5%, 8.1%, and 15.6%, respectively. The reason why Rubeshibe 28 showed a high percentage of misjudgment was the small number of F_1 individuals and considerable variation in diterpene content. Although the percentages of misjudgment were different in each family, these results showed that discrimination between *L. gmelinii* var. *japonica* and the F_1 was possible for the seedlings from intrafamilies.

Linear discriminant analysis of all individuals in the five families (*L. gmelinii* var. *japonica*, 127; F_1, 82) showed a misjudgment rate of 7.7% (Fig. 4). The result suggested that discrimination between *L. gmelinii* var. *japonica* and the F_1 was possible for the seedlings from interfamilies with a very high probability.

Ratio of diterpene structural types

The labdane/(pimarane + abietane) and abietane/pimarane ratios were calculated to compare the labdane, pimarane, and abietane-type diterpene biosynthetic abilities in the

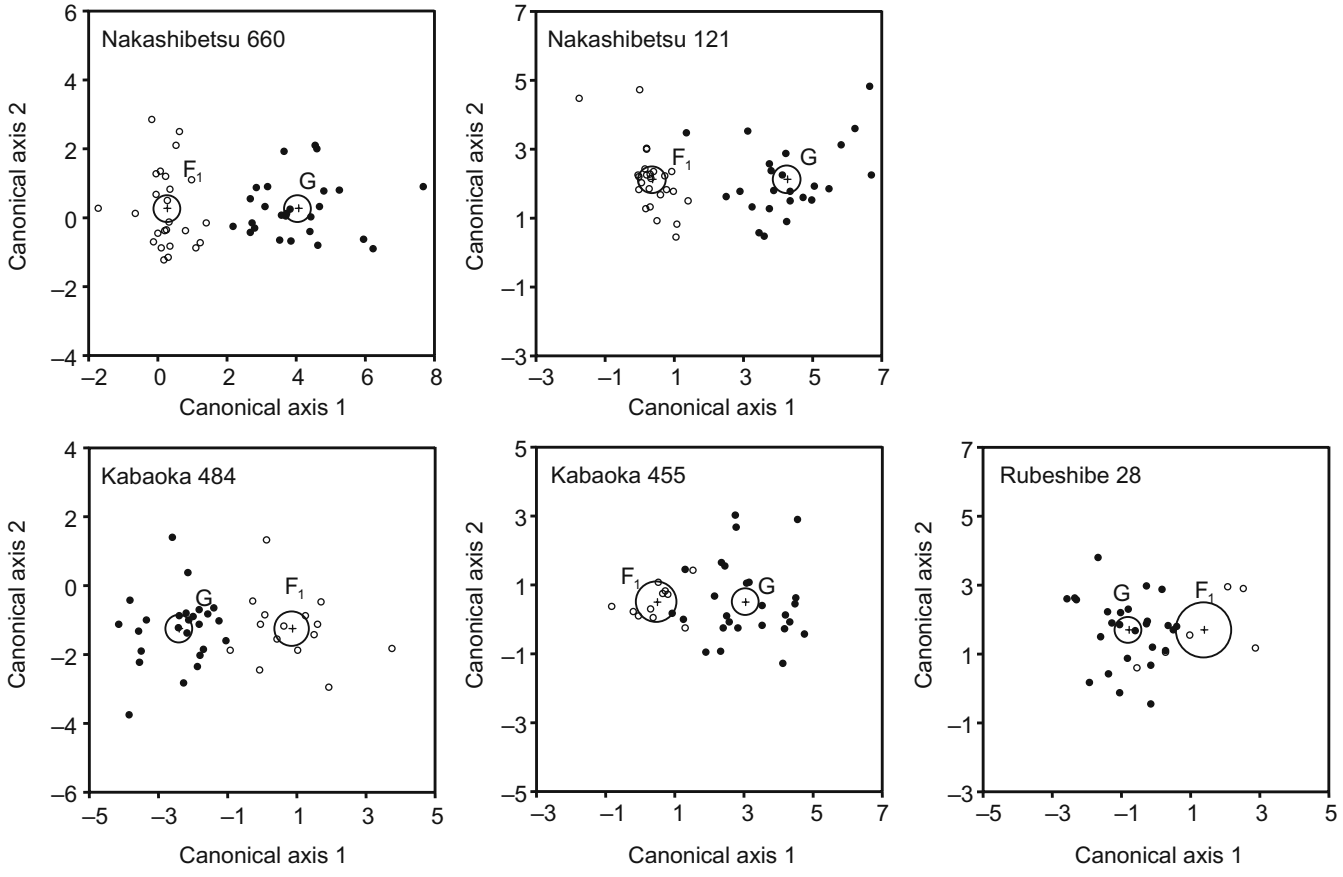

Fig. 3. Linear discriminant analysis using diterpene compositions in the seedlings of *L. gmelinii* var. *japonica* and the F_1 in each family (Nakashibetsu 660: *L. gmelinii* var. *japonica*, 26 individuals; F_1, 25 individuals; Nakashibetsu 121: *L. gmelinii* var. *japonica*, 24 individuals; F_1, 25 individuals; Kabaoka 484: *L. gmelinii* var. *japonica*, 25 individuals; F_1, 15 individuals; Kabaoka 455: *L. gmelinii* var. *japonica*, 26 individuals; F_1, 11 individuals; Rubeshibe 28: *L. gmelinii* var. *japonica*, 26 individuals; F_1, 6 individuals). *Filled circles*, *L. gmelinii* var. *japonica* (*G*); *open circles*, F_1; *large circles*, 95% confidence intervals of the multivariate means

Table 3. Diterpene compositions and contents in the bark of seedling branches

Diterpene	Mother tree									
	Nakashibetsu 660		Nakashibetsu 121		Kabaoka 484		Kabaoka 455		Rubeshibe 28	
	L. gmelinii[a,b]	F_1[c]	*L. gmelinii*[a,d]	F_1[c]	*L. gmelinii*[a,c]	F_1[e]	*L. gmelinii*[a,b]	F_1[f]	*L. gmelinii*[a,b]	F_1[g]
13-Epimanool	13.4 ± 4.3	3.7 ± 2.2	9.7 ± 3.3	2.8 ± 1.4	10.7 ± 3.0	6.5 ± 4.1	8.9 ± 2.5	8.1 ± 4.1	7.5 ± 2.2	5.6 ± 2.9
Larixol	41.6 ± 8.2	16.0 ± 8.2	41.6 ± 8.8	18.0 ± 7.0	47.9 ± 7.3	29.6 ± 10.0	42.2 ± 8.3	25.3 ± 13.7	47.9 ± 7.5	33.0 ± 11.6
13-Epitorulosyl acetate	0.1 ± 0.2	0.5 ± 0.7	0.1 ± 0.3	0.2 ± 0.4	0.1 ± 0.2	0.5 ± 0.8	0.0 ± 0.1	0.2 ± 0.4	0.2 ± 0.8	0.8 ± 2.0
Isopimaric acid	11.7 ± 2.0	15.2 ± 3.8	14.3 ± 3.3	14.5 ± 3.9	12.4 ± 1.8	12.2 ± 2.6	14.0 ± 3.5	12.0 ± 4.6	10.1 ± 2.9	10.9 ± 1.9
Abietic acid	16.4 ± 8.2	52.8 ± 11.6	11.2 ± 8.8	53.3 ± 10.8	12.9 ± 4.4	40.5 ± 10.8	17.7 ± 10.1	40.5 ± 13.2	24.2 ± 7.3	36.2 ± 17.5
Dehydroabietic acid	14.2 ± 7.4	8.9 ± 6.9	19.6 ± 10.7	6.4 ± 5.7	13.1 ± 5.3	7.3 ± 4.7	13.8 ± 6.6	11.9 ± 8.9	7.5 ± 3.5	10.6 ± 9.4
Neoabietic acid	2.5 ± 1.7	2.8 ± 1.4	3.5 ± 2.0	4.8 ± 2.9	2.9 ± 1.4	3.4 ± 2.5	3.3 ± 2.2	2.2 ± 1.2	2.6 ± 1.7	2.9 ± 2.7
Labdane type[h]	90.2 ± 28.5 A	23.8 ± 11.7 B	73.1 ± 27.6 A	22.1 ± 8.0 B	115.0 ± 60.2 A	60.0 ± 34.6 B	86.8 ± 36.1 A	43.8 ± 28.8 B	202.1 ± 106.1 A	82.1 ± 32.7 B
Pimarane type[h]	18.7 ± 6.0 A	18.9 ± 11.9 A	20.4 ± 7. 8 A	15.4 ± 5.6 B	23.1 ± 9.2 A	20.5 ± 12.4 A	48.1 ± 43.1 A	14.3 ± 5.5 B	32.5 ± 10.4 A	22.6 ± 8.5 A
Abietane type[h]	52.8 ± 20.3 B	86.7 ± 60.7 A	47.1 ± 14.4 B	72.5 ± 32.3 A	53.7 ± 23.1 B	85.5 ± 56.4 A	46.2 ± 25.0 A	68.9 ± 29.9 A	116.1 ± 44.9 A	110.2 ± 68.5 A
Total diterpenes[h]	159.4 ± 44.0 A	129.4 ± 75.3 A	140.6 ± 38.3 A	110.1 ± 37.8 B	191.8 ± 87.2 A	166.0 ± 95.4 A	182.0 ± 70.1 A	127.0 ± 44.7 B	350.7 ± 162.0 A	214.9 ± 95.7 A

Data given as mean mol% ± standard deviation
Values not sharing the same uppercase letters (A, B) within each family are significantly different by Tukey-Kramer HSD test ($P < 0.05$)
[a] *Larix gmelinii* var. *japonica* Pilg.
[b] $n = 26$
[c] $n = 25$
[d] $n = 24$
[e] $n = 15$
[f] $n = 11$
[g] $n = 6$
[h] Data given in units of nmol/bark mg

Table 4. Ratios of the diterpene type of bark of mature-tree branches based on results shown in Table 2

Ratio of diterpene type	*L. gmelinii*[a]	F_1	*L. kaempferi*	*L. gmelinii*[a]	F_1	*L. kaempferi*
	Toyooka 111	Toyooka 111 × Tokachi 35	Tokachi 35	Kabaoka 168	Kabaoka 168 × Tokachi 16	Tokachi 16
Labdane/(abietane + pimarane)	2.5 A	1.1 B	0.1 C	2.3 A	0.5 B	0.1 C
Abietane/pimarane	3.7 B	4.6 B	9.1 A	5.6 B	6.1 B	8.9 A

Values not sharing the same uppercase letters (A, B, C) within each family are significantly different by Tukey-Kramer HSD test ($P < 0.05$)
[a] *Larix gmelinii* var. *japonica* Pilg.

Table 5. Ratios of the diterpene type of bark of seedling branches based on results shown in Table 3

Ratio of diterpene type	Mother tree									
	Nakashibetsu 660		Nakashibetsu 121		Kabaoka 484		Kabaoka 455		Rubeshibe 28	
	L. gmelinii[a]	F_1	*L. gmelinii*[a]	F_1	*L. gmelinii*[a]	F_1	*L. gmelinii*[a]	F_1	*L. gmelinii*[a]	F_1
Labdane/(pimarane + abietane)	1.3 A	0.2 B	1.1 A	0.3 B	1.5 A	0.6 B	0.9 A	0.5 B	1.4 A	0.6 B
Abietane/pimarane	2.8 B	4.6 A	2.3 B	4.7 A	2.3 B	4.2 A	1.0 B	4.8 A	3.6 B	4.9 A

Values not sharing the same uppercase letters (A, B) within each family are significantly different by Tukey-Kramer HSD test ($P < 0.05$)
[a] *Larix gmelinii* var. *japonica* Pilg.

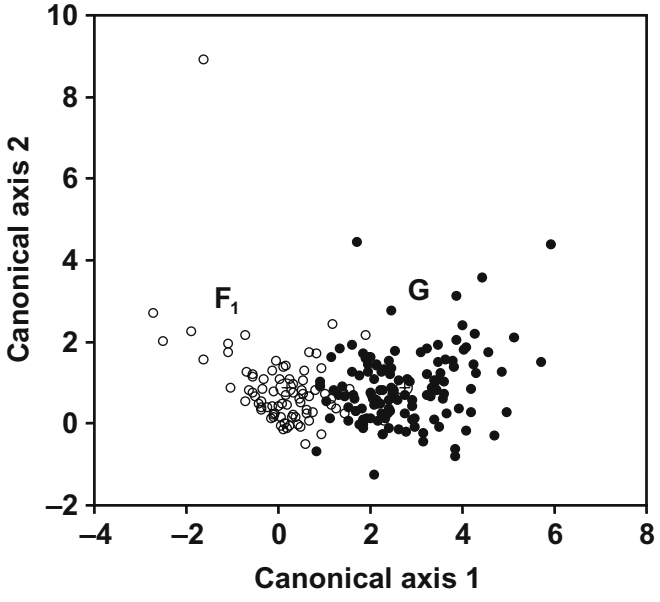

Fig. 4. Linear discriminant analysis using diterpene compositions in the seedlings of *L. gmelinii* var. *japonica* and the F_1 in five families (*L. gmelinii* var. *japonica*, 127 individuals; F_1, 82 individuals). *Filled circles*, *L. gmelinii* var. *japonica* (*G*); *open circles*, F_1; *large circles*, 95% confidence intervals of the multivariate means

species examined (Table 4). The labdane/(pimarane + abietane) ratios of mature trees were 2.3–2.5 in *L. gmelinii* var. *japonica* and 0.1 in *L. kaempferi*, which indicated significant differences between these species (Tukey-Kramer HSD test, $P < 0.05$). On the other hand, the abietane/pimarane ratios in *L. kaempferi* were 8.9–9.1 and those in *L. gmelinii* var. *japonica* were 3.7–5.6, showing that those in *L. kaempferi* were significantly higher (Tukey-Kramer HSD test, $P < 0.05$). In addition, the labdane/(pimarane + abietane) ratio and the abietane/pimarane ratio of the F_1 were the middle range of their parents in common with the two families.

In the seedlings, the labdane/(pimarane + abietane) ratio of *L. gmelinii* var. *japonica* and the F_1 were 0.9–1.5 and 0.2–0.6, respectively, and the abietane/pimarane ratio of *L. gmelinii* var. *japonica* and the F_1 were 1.0–3.6 and 4.2–4.9, respectively, showing a similar trend to the mature trees (Table 5).

Discussion

The presence of larixol and larixyl acetate was reported in *Larix decidua*[16] and Dahurian larch,[17] the typical variety of *Larix gmelinii*.[1] In the current study, these diterpenes were detected in the bark of Kurile larch, *L. gmelinii* var. *japonica* (Table 2). In the oleoresin from *Larix kaempferi*, Bol'shakova et al.[18] reported the presence of larixol and larixyl acetate, while Mills[16] did not detect these diterpenes. In the present study, the results that either larixol or larixyl acetate existed in the bark of all individuals of *L. kaempferi* (Table 2, the data for each individual are not shown) agreed with the report of Bol'shakova et al.[18] Mills[16] described that epitorulosol and epitorulosyl acetate existed in the species in which larixol and larixyl acetate were absent (*L. gmelinii* var. *japonica* etc.). In the present study, *L. gmelinii* var. *japonica* contained larixol but did not contain epitorulosyl acetate as reported by Mills.[16] However, in *L. kaempferi* (Tokachi 16), four out of five ramets contained larixol (or larixyl acetate) with epitorulosyl acetate, suggesting that the contents of larixol, larixyl acetate, and epitorulosyl acetate in *L. kaempferi* might be different between the strains or individuals.

It was reported that the monoterpene composition was constitutively steady in most strains of *Chamaecyparis obtusa*, but considerable proportional variation in the

amount of sabinene was observed between clones.[19] In *L. gmelinii* var. *japonica* clones, larixol composition showed considerable variation (Table 2, Toyooka 111, 48.0%–58.9%; Kabaoka 168, 49.5%–55.1%, the data for each individual are not shown).

Mills[16] reported that *Larix eurolepi*s Henry, the hybrid F_1 larch of *L. decidua* (containing both larixol and larixyl acetate but neither epitorulosol nor epitorulosyl acetate) and *L. kaempferi* (containing both epitorulosol and epitorulosyl acetate but neither larixol nor larixyl acetate) showed a resin composition essentially intermediate between those of its parents, containing epitorulosol, larixol, and larixyl acetate together. The present study showed that the larixol and abietic acid compositions in the F_1 are affected by the seed parent, *L. gmelinii* var. *japonica*, and their pollen parent, *L. kaempferi*, respectively (Table 2), suggesting that diterpene compositions are hereditarily affected by the parents, as reported by Mills.[16] Gallis and Panetsos[20] demonstrated that no qualitative difference, but rather quantitative differences were found in monoterpenes and sesquiterpenes between the hybrid pine F_1 (*Pinus brutia* × *Pinus halepensis*) and the level of oleoresin in the parents, and most of the terpene composition in the F_1 was intermediate between their parental species. The present results suggest that the diterpene compositions showed intermediate characteristics between the parents as with the phenological characteristics (except the seedling height) and vole resistance.[5,10]

Hayashi et al.[10] showed that the correlation coefficient between the survival rate of trees against vole attack and the ether-extract content in the branch bark was highly significant in larch crossing families from *L. gmelinii* var. *japonica* and *L. kaempferi*, indicating that the ether-extract content may be used as a possible index for resistance to vole browsing. Sukeno and Ozawa[9] reported that larixol and 13-epimanool in the ether extract of the branch bark of *L. gmelinii* var. *japonica* could be associated with resistance to vole browsing. The present study also showed that the abundance of labdane-type diterpene in these families is in the order *L. gmelinii* var. *japonica* > F_1 > *L. kaempferi* (Table 2), which suggests a relationship with vole resistance. In the present study, the larixol composition in the F_1 showed considerable variation even at an intrafamily level (Toyooka 111 × Tokachi 35, 11.9%–46.5%; Kabaoka 168 × Tokachi 16, 4.7%–23.8%, the data for each individual are not shown); therefore, it may be possible to select more resistant individuals to vole browsing according to the labdane-type diterpene content.

The contents of labdane-type, pimarane-type, and abietane-type diterpenes were different among *L. gmelinii* var. *japonica*, *L. kaempferi*, and the F_1 (Table 2). Therefore, these characteristics are discussed from the viewpoint of the biogenesis pathway of diterpene structural types (Fig. 5).[21] The ratio of labdane/(pimarane + abietane) in *L. gmelinii* var. *japonica* was higher than that in *L. kaempferi* (Table 4), suggesting that the fluxes of labdane-type diterpene synthesis from copalyl diphosphate in the former were higher than those in the latter. On the other hand, the ratio of abietane/pimarane in *L. kaempferi* was higher than that

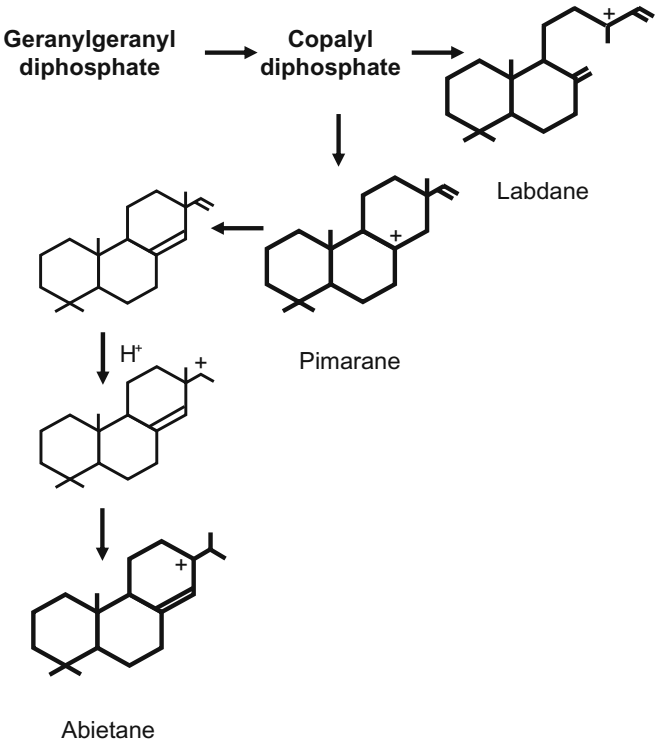

Fig. 5. Biogenesis of diterpene structural types

in *L. gmelinii* var. *japonica*, suggesting that the fluxes of abietane-type diterpene synthesis via pimarane in the former were higher than those in the latter. In addition, as for the F_1 both the labdane/(pimarane + abietane) and abietane/pimarane ratios were intermediate with respect to its parents, suggesting that the fluxes of diterpenes were under hereditary control.

In seedlings, the labdane/(pimarane + abietane) ratios in *L. gmelinii* var. *japonica* were higher than those in the F_1 and abietane/pimarane ratios in the F_1 were higher than those in *L. gmelinii* var. *japonica*, showing a similar trend to mature trees (Table 5). In *L. gmelinii* var. *japonica*, mature trees contained much higher levels of labdane-type diterpene than seedlings, suggesting that the biosynthesis of labdane-type diterpene increases with maturity.

It was demonstrated that discrimination of plus trees was possible using neutral diterpenes and the resin acid composition in oleoresin of *Pinus pinaster*.[13] With the mature-tree branches of two families, Kabaoka 168 × Tokachi 16 and Toyooka 111 × Tokachi 35, the current study used linear discriminant analysis among the F_1 and parent species using eight kinds of diterpene component in the bark, and the percentage of misjudgment was 0% (Fig. 2). In addition, discrimination between *L. gmelinii* var. *japonica* and F_1 seedlings in each family was possible as well as in mature trees, suggesting that diterpenes were effective components for the discrimination of the hybrid at the early stage of growth (Fig. 3). In order to obtain a percentage of misjudgment lower than 10%, 13-epimanool, larixol, abietic acid, and isopimaric acid were used in the discrimination (except Rubushibe 28). In particular, the *P* values of 13-epimanool and larixol were low ($P < 0.05$, Prob > *F*).

Generally, in hybridization seed orchards, several types of parental clones are planted, and the seeds are obtained by open pollination to generate genetically diverse seeds. In the present study, the F_1 seedlings from five families were discriminated by their diterpene contents and the percentage of misjudgment was 7.7%, suggesting that the discrimination was not complete but acceptable for this method at a practical level (Fig. 4). The percentage of misjudgment (7.7%) was obtained from 13-epimanool, abietic acid, and isopimaric acid ($P < 0.001$, Prob > F). From these results, it is considered that 13-epimanool, larixol, abietic acid, and isopimaric acid are effective indices for the discrimination of the hybrid seedlings.

The current study also analyzed the diterpene compositions in leaves (results not shown). The diterpene compositions of seedlings showed similar trends to those of bark. However, the percentage of misjudgment by the diterpene contents of seedling leaves was over 14% in each family, which was higher than the percentage of those from bark. These results suggested that the bark is more appropriate as a sample for the discrimination using diterpenes than leaves.

The diterpene contents are effective indices for the discrimination of the hybrid seedlings and useable for the discrimination of the hybrids from plural families. However, further studies will be required to determine the influence on the diterpene content and composition of environmental factors such as the growth area and season.

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References

1. Farjon A (1990) Pinaceae: drawings and descriptions of the genera Abies, Cedrus, Pseudolarix, Keteleeria, Nothotsuga, Tsuga, Cathaya, Pseudotsuga, Larix and Picea. Koeltz, Königstein, pp 193–219
2. Hoshi H (2004) Forest tree genetic resources conservation stands of Japanese larch (*Larix kaempferi* (Lamb.) Carr.). For Tree Gen Res Inf Special Issue No. 1
3. Ueda M, Higuchi S, Igarashi B, Maeda M, Kuwahata T, Ota K, Abe H, Fujimaki Y, Fujikura J, Takayasu T (1966) Historical review of studies on the Bedford's red-backed vole, *Clethrionomys rufocanus bedfordiae* (Thomas) (in Japanese with English summary). Bull Gov Forest Exp Stat (Tokyo) 191:1–100
4. Kaneko Y, Nakata K, Saitoh T, Stenseth NC, Bjørnstad ON (1998) The biology of the vole *Clethrionomys rufocanus*: a review. Res Popul Ecol 40:21–37
5. Takahashi N, Nishiguchi C (1966) Studies on the resistance of forest trees to the red-backed vole, *Clethrionomys rufocanus bedfordiae* (Thomas). (2) Relative feeding preference of the vole for seedling of larch F_1 hybrids under laboratory conditions (in Japanese with English summary). Bull Tokyo Univ Forest 62: 173–188
6. Kita K, Fujimoto T, Uchiyama K, Kuromaru M (2005) The estimation of 31-year-old hybrid larch for selection of high CO_2 accumulating families (in Japanese). Abst 116th Annu Mtg Jpn For Soc 116:103
7. Kita K, Kuromaru M, Uchiyama K (2006) The effect of family selection in hybrid larch on carbon accumulation (in Japanese). Abst 117th Annu Mtg Jpn For Soc 117:104
8. Oshima T, Hatakeyama S, Kubota Y (1974) Characteristics on each generation of hybrid larch (I) – *Larix gmelinii* var. *japonica* × *L. kaempferi* hybrid larch F_1 and their double cross species (in Japanese). Trans 85th Mtg Jpn For Soc 169–171
9. Sukeno S, Ozawa S (1997) Identification of compounds found in the larch associated with vole resistance, and its application for early test (II) – identification of compounds associated with vole resistance (in Japanese). Abst 108th Annu Mtg Jpn For Soc 198
10. Hayashi E, Iizuka K, Sukeno S, Kohno K (1998) Relationship between resistance to vole browsing and content of ether extract in the bark of larch species and hybrids. J For Res 3:119–122
11. Yasue M, Ogiyama K, Suto S, Tsukahara H, Miyahara F, Ohba K (1987) Geographical differentiation of natural cryptomeria stands analyzed by diterpene hydrocarbon constituents of individual trees. J Jpn For Soc 69:152–156
12. Hanari N, Yamamoto H, Kuroda K (2003) Distinction of resin compounds between the healthy bark and the resinous stem canker of *Thujopsis dolabrata* var. *hondae*. J Wood Sci 49:548–552
13. Arrabal C, Cortijo M, Fernandez de Simon B, Garcia-Vallejo MC, Cadahia E (2002) *Pinus pinaster* oleoresin in plus trees. Holzforschung 56:261–266
14. Moriguchi Y, Kita K, Uchiyama K, Kuromaru M, Tsumura Y (2008) Enhanced hybridization rates in a *Larix gmelinii* var. japonica × *L. kaempferi* inter-specific seed orchard with a single maternal clone revealed by cytoplasmic DNA markers. Tree Genet Genom 4:637–645
15. Hansson L, Gref R, Lundren L, Theander O (1986) Susceptibility to vole attacks due to bark phenols and terpenes in *Pinus contorta* provenances introduced into Sweden. J Chem Ecol 12:1569–1578
16. Mills JS (1973) Diterpenes of *Larix* oleoresins. Phytochemistry 12:2407–2412
17. Chernenko GF, Ivanova EE, Demenkova LI, Shmidt EN (1991) Extractive substances in the bark of *Larix dahurica*. Khim Prir Soedin 4:580–582
18. Bol'shakova VI, Khan VA, Dubovenko ZhV, Shmidt EN, Pentegova VA (1985) Terpenoids of the oleoresin of *Larix leptolepis*. Khim Prir Soedin 6:839
19. Katoh S, Noda A, Furuno T (2006) Tree-to-tree and clone-to-clone variations of monoterpenes emitted from needles of hinoki (*Chamaecyparis obtusa*). J Wood Sci 52:84–89
20. Gallis AT, Panetsos P (1997) Use of cortical terpenes to discriminate *Pinus brutia* (Ten.), *Pinus halepensis* (Mill.) and their hybrids. Silvae Genet 46:2–3
21. Croteau R, Johnson MA (1985) Biosynthesis of terpenoid wood extractives. In: Higuchi T (ed) Biosynthesis and biodegradation of wood components. Academic, Orlando, pp 379–439