# SYSTEMATICS OF *JUNIPERUS CHINENSIS* AND *J. TSUKUSIENSIS* FROM JAPAN AND TAIWAN: DNA SEQUENCING AND TERPENOIDS.

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## ABSTRACT

Analyses of nrDNA, petN-psbM, trnD-trnT and trnS-trnG revealed that *Juniperus chinensis* var. *tsukusiensis* and *J. c.* var. *taiwanensis* are not conspecific with *J. chinensis*. In addition, analyses of the leaf oils (terpenoids) also revealed numerous differences. Based on these new data, *J. c.* var. *tsukusiensis* is recognized as *J. tsukusiensis* Masam. and *J. c.* var. *taiwanensis* as ***J. tsukusiensis* var. *taiwanensis* (R. P. Adams and C-F. Hsieh) R. P. Adams**, **comb. nov.** *Phytologia 93(1): 118-131 (April 1, 2011).*

**KEY WORDS**: *Juniperus chinensis, J. tsukusiensis, J. tsukusiensis* var. *taiwanensis, J. jarkendensis,* DNA, terpenoids, systematics.

Adams et al. (2002) examined the RAPDs from putative *J. chinensis* from Japan and Taiwan and found (Fig. 1) that *J. chinensis* L. (Japan) was quite distinct from *J. c.* var. *sargentii* Henry (both high and low bornyl acetate types), and *J. c.* var. *tsukusiensis* Masam. (Yakushima, Japan) and *J. c*. var. *taiwanensis* R. P. Adams and C-F. Hsieh (Taiwan). Notice that *J. c.* var. *taiwanensis* was well resolved from *J. chinensis* and *J. c.* var. *tsukusiensis* (Fig. 1).

In his newest monograph of *Juniperus* (Adams, 2011) recognized *J. chinensis* with three varieties: var. *sargentii*, Japan, var. *taiwanensis*, endemic to Mt. Chingshui, Taiwan, and var. *tsukusiensis*., endemic to the off shore island of Yaku Shima, Japan.

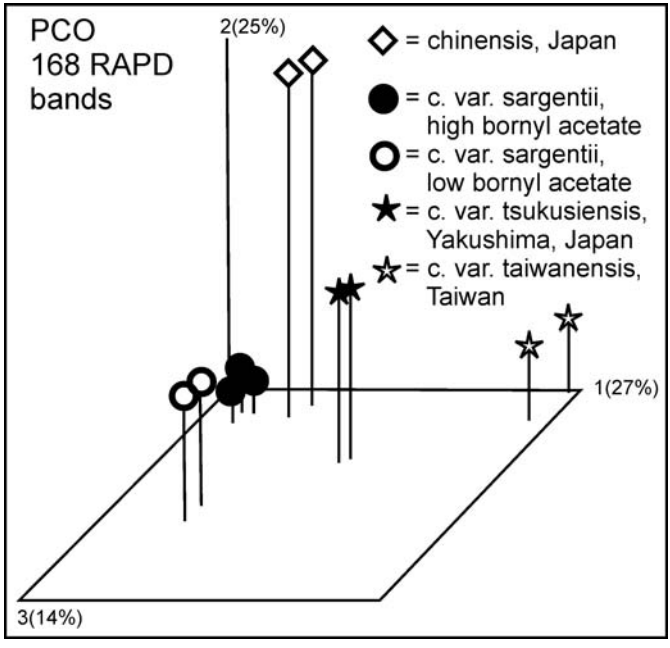


Figure 1. PCO based on 168 RAPD bands for *J. chinensis* taxa from Japan and Taiwan (adapted from Adams et al., 2002).

Recent DNA sequencing in our labs indicate that *J. c.* var. *taiwanensis* and var. *tsukusiensis* are more closely related to *J.*

*jarkendensis* than to *J. chinensis*. In order to understand the relations, we have sequenced additional regions and also analyzed the leaf terpenoids. The purpose of this paper is to present the sequencing and leaf oil analyses to resolve the relationships of *J. c.* var. *taiwanensis* and var. *tsukusiensis* to other *J. chinensis* taxa.

## MATERIALS AND METHODS

Specimens collected: *J. chinensis*, *Adams8535-8537,* Shizuoka Prefecture, Osezaki Point, 3m, Japan, 16 June 1998, *J. c.* var. *sargentii*, *Adams 8688*, collected by Naotoshi Yoshida at the Medicinal Bot. Gard., Hokkaido Univ., Japan, *J. c*. var. *taiwanensis*, *Adams 9061-9063*, Mt. Chingshui, *ex situ* Taiwan Forestry Institute, 24 June 2000, *J. c.* var. *tsukusiensis*, *Adams 8805-8808*, collected by K. Miyazaki via Jin Murata, Mt Kuromidake, 1500m, Yaku Shima, Japan, 4 Aug. 1999; *J. jarkendensis*, *Adams 7820-7825*, Kunlun Mtns., 2600 m, Oeetak, above Akto forestry station, Xinjiang, China, 28 July 1996; *J.* occidentalis, *Adams 8592-8594*, 0.2 km nw of Sisters, OR, USA, 17 Oct. 1998. Voucher specimens are deposited at BAYLU.

*Isolation of Oils* - Fresh leaves (200 g) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

Chemical Analyses - Oils from 10-15 trees of each of the taxa were analyzed and average values reported. The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1 sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see 5 for operating details). Identifications were made by library searches of our volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

Data Analysis - Terpenoids (as per cent total oil) were coded and compared among the species by the Gower (1971) metric. Principal coordinate analysis was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967).

DNA Analysis - One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20$^{o}$ C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). PCR amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM, trnDT, trnSG) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams et al. (2011) for the ITS, petN-psbM, trn D-trnT and trnS-trnG primers utilized. The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.). In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit. The gel purified DNA band with the appropriate primer was sent to McLab Inc. (South San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.). Alignments and NJ trees were made using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/). Minimum spanning networks were constructed from SNPs data using PCODNA software (Adams et al., 2009). Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the maximum observed value for that compound over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

## RESULTS AND DISCUSSION

The leaf oils exhibited considerable differences among the taxa (Table 1). *Juniperus chinensis* (Japan) was dominated by sabinene (27.5%), and bornyl acetate (19.7%) with moderate amounts of myrcene (5.5%), limonene (6.1%), β-phellandrene (4.1%) and elemol (6.1%).

This oil differs from the others by having pregeijerene B, (E)-caryophyllene, cis-cadina-1,4-diene, epi-zonarene, 10-epi-cubebol and 8-α-acetoxyelemol (Table 1). The amount of bornyl acetate is polymorphic with a range of 2.5 to 30.2%.

The oils of *J. c.* var. *tsukusiensis* and var. *taiwanensis* are very similar (Table 1). Both have large amounts of α-pinene (33.2, 13.4%), sabinene (11.5, 1.4%), myrcene (5.6, 11.6%), bornyl acetate (8.4, 22.5%), δ-cadinene (5.2, 4.0%) and α-cadinol (4.7, 7.4%). These two taxa share ten compounds not found in the other taxa: α-copaene, β-cubebene, trans-muurola-3,5-diene, trans-muurola-4(14),5-diene, trans-cadina-1,4-diene, α-cadinene, β-oplopenone, 1-epi-cubenol, α-muurolol and α-cadinol. The oil of *J. c.* var. *taiwanensis* had no unique components (greater than a trace) and var. *tsukusiensis* had one component (naphthalene). The amount of bornyl acetate was nearly constant in var. *taiwanensis* ranging from 6.2 to 9.6 %, but wide ranging in var. *tsukusiensis* from 11.7 to 32.3%.

The oil of *J. jarkendensis* is very different from the other oils (Table 1) and is dominated by sabinene (57.7%) and cedrol (9.1%). The presence of cedrol (a major component of *Juniperus* wood oils, Adams, 1991, 2009; Adams and Lu, 2008) is found in the leaf oils of only a few species in the world (Adams, 2011). Several other typical wood oil components were present: α-cedrene, β-cedrene, cis-thujopsene, allo-cedrol and cedryl acetate. It seems likely that the pathway to these compounds is activated in the leaves of *J. jarkendensis*, along with the typical leaf oil components. This makes the oil appear very different from the other taxa (Table 1). Aside from the 'wood oil' components, the leaf oil is still quite different in having cis- and trans-thujone, methyl citronellate, trans-sabinyl acetate, methyl geranate, as well as lacking in sesquiterpenes.

Overall, the oils of *J. c.* var. *taiwanensis* and var. *tsukusiensis* are very similar but differ from *J. chinensis* and *J. jarkendensis* oils.

NJ analyses, based on combined nrDNA, petN-psbM, trnD-trnT and trnS-trnG sequences, is shown in Figure 2. There is support for the separate clades of *(J. jarkendensis*, *J. c.* var. *taiwanensis*, *J. c.* var. *tsukusiensis*) and (*J. chinensis*, *J. c.* var. *sargentii*). There is also support

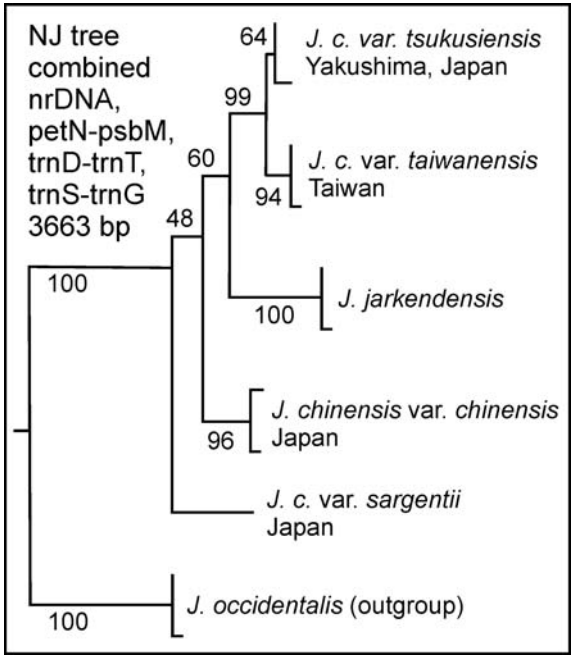


Figure 2. NJ tree based on combined sequence data. The numbers are bootstrap percentages (1000 reps).

for *J. c*. var. *taiwanensis* and *J. c*. var. *tsukusiensis* belonging to separate clades. Of course, merely being in separate clades does not indicate if these taxa are distinct species or varieties. One should note that parsimony analysis gave conflicting trees which appears to be due to the inconsistent evolution among the data sets (see SNPs analyses below).

A different method to view the sequence data is by utilizing SNPs (including indel information). Figure 3 shows minimum spanning networks based on nrDNA and petN-psbM. The SNPs from nrDNA show *J. chinensis* var. *chinensis*, *J. c.* var. *taiwanensis* and *J. c.* var. *tsukusiensis* differ by only one SNP (Fig. 3, left). Interestingly, *J. c.* var. *sargentii* differs from *J. c.* var. *chinensis* by 7 SNPs which is greater than the 5 SNPs that separate *J. jarkendensis* from *J. c.* var. *taiwanensis* and var. *tsukusiensis* (Fig. 3, left).

The pattern for petN-psbM SNPs (including indel data) (Fig. 3, right) is quite different as both *J. c.* var. *taiwanensis* and var. *tsukusiensis* are shown more related to *J. jarkendensis* than to each other or to *J. chinensis*. *Juniperus chinensis* var. *sargentii* differs by 10 SNPs from *J. jarkendensis* but by only 1 SNP from *J. c.* var. *chinensis* (Fig. 3, right).

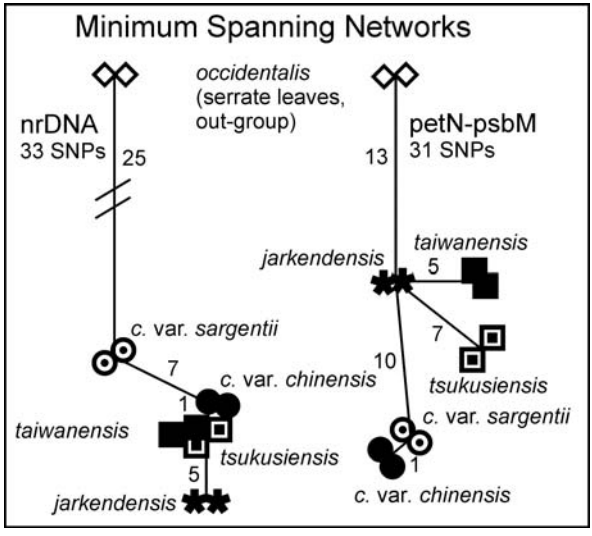


Figure 3. Minimum spanning networks based on nrDNA and on petN-psbM. The numbers next to lines are the number of SNPs.

The SNPs from trnD-trnT (Fig. 4, left) show a similar pattern as seen for petN-psbM (Fig. 3, left) in that *J. c.* var. *chinensis* and var. *sargentii* differ by only one SNP. However, *J. c*. var. *taiwanensis* and var. *tsukusiensis* are nearly identical (1 SNP, Fig. 4, left) and only 2 SNPs removed from *J. jarkendensis*.

The evolution within trnS-trnG (Fig. 4, right) is similar to the pattern of trnD-trnT in that *J. c.* var. *chinensis* and var. *sargentii* have no differences and *J. c*. var. *taiwanensis* and var. *tsukusiensis* are nearly identical (1 SNP, Fig. 4, right). *Juniperus jarkendensis* is much more distinct (5 SNPs, Fig. 4) than seen in analysis of trnD-trnT (Fig. 4, left).

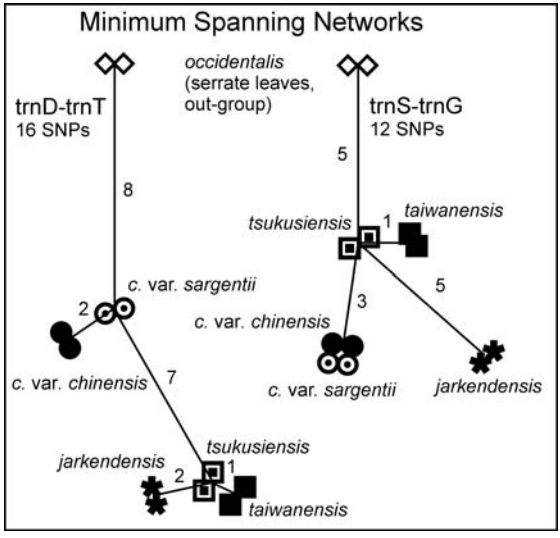


Figure 4. Minimum spanning networks based on trnD-trnT and trnS-trnG.

The overall minimum spanning network based on 92 SNPs shows considerable differentiation between *J. c.* var. *chinensis* and var. *sargentii* (10 SNPs, Fig. 5) and *J. c.* var. *taiwanensis* and var. *tsukusiensis* (10 SNPs, Fig. 5). The c*hinensis-sargentii* group is separated by 24 SNPs from the *taiwanensis-tsukusiensis* group (Fig. 5). The *taiwanensis-tsukusiensis* group is a little closer to *J. jarkendensis* (19 SNPs, Fig. 5) than the c*hinensis-sargentii* group (24 SNPs, Fig. 5).

The finding by DNA sequencing that the Yaku Shima and Taiwan junipers are not as closely related to *J. chinensis* (Japan) as to *J. jarkendensis* (w. China) was unexpected. The leaf oils are more like *J. chinensis* than *J. jarkendensis* (Table 1). Adams (2011) noted that *J. c.* var. *taiwanensis* and var. *tsukusiensis* differ from *J. chinensis* in being procumbent shrubs, with scale leaves that are very short and wide (appearing as a sting of beads), and with glands that are raised (vs. sunken in *J. chinensis*).

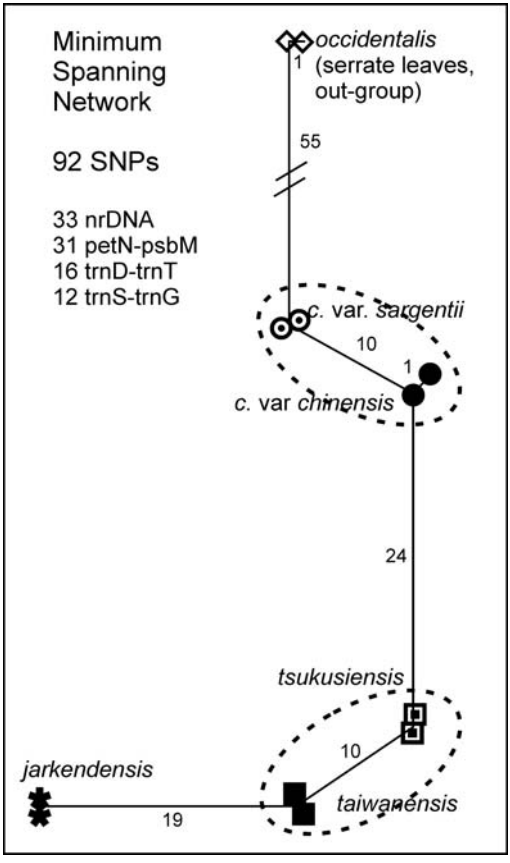


Figure 5. Minimum spanning network based on combined data from four sequences. Numbers on lines are the number of SNPs (including indels).

Considering all the data available at present, its seems prudent to follow Masamune's original species concept [Bot. Mag. Tokyo 44: 50 (1930)] and recognize *J. c*. var. *tsukusiensis* (Masam.) Masam. as a distinct species: ***J. tsukusiensis***, Type: Japan, Yaku Shima, G. Masamune s. n. (syntype IT), known only from steep rocks on Yaku Shima. In addition, the relationship between var. *tsukusiensis* and var. *taiwanensis* seems, at present, appropriately characterized as being conspecific at the

variety level. This warrants the recognition and moving of *J. c.* var. *taiwanensis* to a variety of *J. tsukusiensis* as:

***Juniperus tsukusiensis*** Masam. var. ***taiwanensis*** (R. P. Adams and C-F. Hsieh) R. P. Adams, **comb. nov.**

**Basionym**: *Juniperus chinensis* L. var. *taiwanensis* R. P. Adams and C-F. Hsieh (Taiwan). Biochem. Syst. Ecol. 30: 235 (2002), Taiwan juniper, Type: Taiwan, Mt. Chingshui, 200 m, Sheng-you Lu 14498 (HOLOTYPE: TAIF).
Distribution: Known only from the type locality, about 100 m below the summit of Mt. Chingshui, Taiwan. The currently recognized distribution of *J. tsukusiensis* is shown in Figure 6.

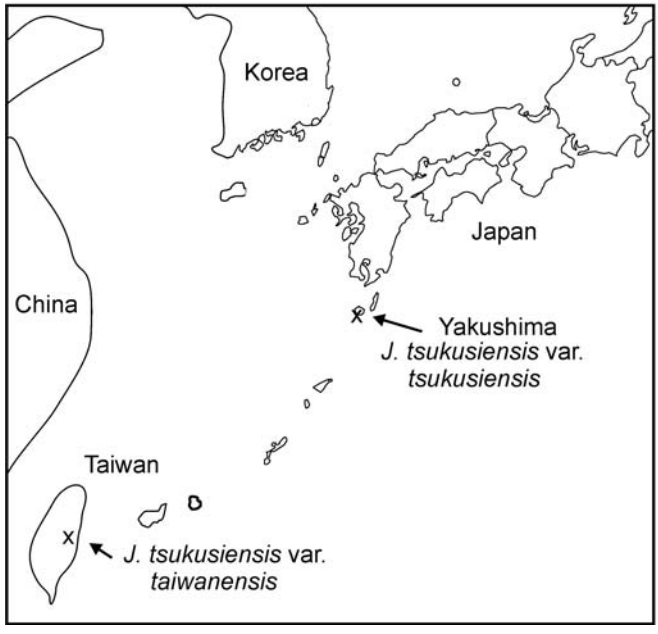


Figure 6. Distribution of *J. tsukusiensis* var. *tsukusiensis* (endemic to Yakushima) and *J. t.* var. *taiwanensis* (endemic to Taiwan).

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Table 1. Comparison of leaf essential oils of *J. chinensis* (Chin), *J. c. var. taiwanensis* (Taiw), *J. c.* var. *tsukusiensis* (Tsuk) *and J. jarkendensis* (Jark). Compounds in bold appear to separate the taxa. t = trace, < 0.1%, RI = retention index on DB-5.

| RI | Component | Chin | Taiw | Tsuk | Jark |
|---|---|---|---|---|---|
| 921 | tricyclene | 0.9 | 0.4 | 0.8 | t |
| 924 | α-thujene | 0.9 | 0.4 | t | 1.3 |
| **932** | **α-pinene** | **1.8** | **33.2** | **13.4** | **2.5** |
| 946 | camphene | 0.9 | 0.7 | 0.8 | 0.1 |
| **969** | **sabinene** | **27.5** | **11.5** | **1.4** | **57.7** |
| **974** | **β-pinene** | **0.3** | **4.0** | **1.8** | **t** |
| **988** | **myrcene** | **5.5** | **5.6** | **11.6** | **3.1** |
| 1001 | δ-2-carene | 0.1 | 0.8 | 0.2 | - |
| 1002 | α-phellandrene | - | - | t | 0.1 |
| 1008 | δ-3-carene | - | t | - | t |
| 1014 | α-terpinene | 0.7 | 0.4 | 0.1 | 1.2 |
| 1020 | p-cymene | 0.1 | t | t | 0.6 |
| 1024 | limonene | 6.1 | 2.6 | 3.0 | 1.7 |
| 1025 | β-phellandrene | 4.1 | 2.6 | 3.0 | 0.4 |
| 1044 | (E)-β-ocimene | 0.4 | - | t | 0.2 |
| 1054 | γ-terpinene | 1.0 | 0.6 | 0.2 | 2.0 |
| 1065 | cis-sabinene hydrate | 0.6 | 0.2 | 0.1 | 1.0 |
| 1086 | terpinolene | 1.0 | 0.6 | 0.5 | 0.9 |
| **1096** | **trans-sabinene hydrate** | **0.2** | **0.1** | **-** | **0.6** |
| **1097** | **linalool** | **1.6** | **0.2** | **-** | **1.1** |
| 1100 | n-nonanal | - | t | t | - |
| **1101** | **cis-thujone** | **-** | **-** | **-** | **0.2** |
| 1102 | isopentyl-isovalerate | - | - | t | - |
| **1112** | **trans-thujone** | **-** | **-** | **-** | **1.6** |
| 1112 | 3-methyl-3-buten-methyl- | | | | |
| | butanoate | - | - | t | - |
| 1118 | cis-p-menth-2-en-1-ol | 0.1 | 0.1 | t | 0.3 |
| 1134 | iso-3-thujanol | - | - | - | 0.1 |
| 1136 | trans-p-menth-2-en-1-ol | t | t | t | 0.3 |
| **1141** | **camphor** | **0.2** | **0.2** | **0.9** | **-** |
| 1145 | camphene hydrate | 0.1 | t | t | - |
| 1148 | citronellal | - | - | - | 0.1 |
| 1154 | sabina ketone | - | - | - | 0.1 |
| 1155 | isoborneol | - | - | t | - |
| 1165 | borneol | 0.2 | 0.1 | 1.1 | - |
| 1174 | terpinen-4-ol | 0.2 | 1.0 | 0.7 | 4.9 |

| RI | Component | Chin | Taiw | Tsuk | Jark |
|---|---|---|---|---|---|
| **1178** | **naphthalene** | **-** | **-** | **0.5** | **-** |
| 1186 | α-terpineol | 0.1 | 0.1 | 0.5 | 0.2 |
| 1195 | cis-piperitol | - | t | - | 0.1 |
| 1207 | trans-piperitol | - | t | - | 0.1 |
| 1218 | endo-fenchyl acetate | - | t | - | - |
| 1219 | coahuilensol | - | - | - | 0.1 |
| **1223** | **citronellol** | **-** | **0.1** | **t** | **0.8** |
| 1235 | neral | - | - | - | t |
| 1249 | piperitone | - | t | t | - |
| 1253 | trans-sabinene hydrate | | | | |
| | acetate | - | - | - | 0.1 |
| **1257** | **methyl citronellate** | **-** | **-** | **-** | **1.2** |
| 1260 | 3-methyl-3-butenol, | - | | | |
| | hexanoate | t | - | t | - |
| **1274** | **pregeijerene B** | **1.5** | **-** | **-** | **-** |
| **1287** | **bornyl acetate** | **19.7** | **8.4** | **22.5** | **0.1** |
| **1289** | **trans-sabinyl acetate** | **-** | **-** | **-** | **2.7** |
| **1322** | **methyl geranate** | **-** | **-** | **-** | **0.8** |
| 1345 | α-cubebene | - | t | t | - |
| **1374** | **α-copaene** | **-** | **0.1** | **0.1** | **-** |
| 1380 | daucene | - | t | - | - |
| **1387** | **β-cubebene** | **-** | **0.1** | **0.1** | **-** |
| **1410** | **α-cedrene** | **-** | **-** | **-** | **0.5** |
| **1417** | **(E)-caryophyllene** | **0.1** | **-** | **-** | **-** |
| **1419** | **β-cedrene** | **-** | **-** | **-** | **0.2** |
| 1429 | cis-thujopsene | - | - | - | 0.1 |
| 1448 | cis-muurola-3,5-diene | 0.6 | 0.1 | 0.1 | - |
| **1451** | **trans-muurola-3,5-diene** | **-** | **0.1** | **0.1** | **-** |
| 1452 | α-humulene | 0.2 | t | - | - |
| 1461 | cis-cadina-1(6),4-diene | - | - | - | - |
| 1465 | cis-muurola-4(14),5-diene | 1.3 | 0.3 | 0.4 | - |
| 1475 | trans-cadina-1(6),4-diene | - | - | 0.2 | - |
| 1478 | γ-muurolene | - | - | 0.4 | - |
| 1480 | germacrene D | 0.3 | 0.2 | 0.1 | - |
| **1493** | **trans-muurola-4(14),5-** | | | | |
| | **diene** | **-** | **0.1** | **0.3** | **-** |
| 1493 | epi-cubebol | 0.1 | 0.4 | 0.5 | - |
| 1495 | epi-cubebene | - | - | - | - |
| **1495** | **cis-cadina-1,4-diene** | **0.1** | **-** | **-** | **-** |
| **1500** | **epi-zonarene** | **0.1** | **-** | **-** | **-** |

| RI | Component | Chin | Taiw | Tsuk | Jark |
|---|---|---|---|---|---|
| **1501** | **α-muurolene** | **0.1** | **1.4** | **1.2** | **-** |
| **1513** | **γ-cadinene** | **0.2** | **1.2** | **1.4** | **-** |
| **1513** | **cubebol** | **0.1** | **0.5** | **0.7** | **-** |
| 1522 | δ-cadinene | 1.1 | 5.2 | 4.0 | 0.1 |
| **1533** | **10-epi-cubebol** | **1.7** | **-** | **-** | **-** |
| **1533** | **trans-cadina-1,4-diene** | **-** | **0.2** | **0.1** | **-** |
| **1537** | **α-cadinene** | **-** | **0.4** | **0.4** | **-** |
| **1548** | **elemol** | **6.1** | **t** | **-** | **0.2** |
| 1550 | cis-muurola-5-en-4-β-ol | - | - | t | - |
| 1559 | cis-muurola-5-en-4-α-ol | 0.5 | t | t | - |
| 1559 | germacrene B | - | - | - | t |
| 1574 | germacrene-D-4-ol | 0.8 | 3.7 | 6.8 | 0.1 |
| **1589** | **allo-cedrol** | **-** | **-** | **-** | **0.4** |
| **1600** | **cedrol** | **-** | **-** | **-** | **9.1** |
| **1607** | **β-oplopenone** | **-** | **0.5** | **0.9** | **-** |
| 1618 | 1,10-di-epi-cubebol | 1.7 | 0.1 | 0.1 | - |
| **1627** | **1-epi-cubenol** | **-** | **0.2** | **0.2** | **-** |
| 1630 | γ-eudesmol | 0.4 | - | - | - |
| 1638 | epi-α-cadinol | 0.2 | 1.8 | 1.1 | t |
| 1638 | epi-α-muurolol | 0.3 | 1.7 | 4.2 | t |
| **1644** | **α-muurolol** | **-** | **0.7** | **1.0** | **-** |
| 1649 | β -eudesmol | 0.6 | - | - | t |
| 1652 | α-eudesmol | 0.7 | - | - | - |
| **1652** | **α -cadinol** | **1.5** | **4.7** | **7.4** | **0.1** |
| 1670 | bulnesol | 0.5 | - | - | - |
| 1688 | shyobunol | - | - | t | - |
| **1767** | **cedryl acetate** | **-** | **-** | **-** | **0.1** |
| **1792** | **8-α-acetoxyelemol** | **0.8** | **-** | **-** | **-** |
| 1958 | iso-pimara-8(14),15-diene | 0.2 | t | - | - |
| 1988 | manoyl oxide | 0.2 | 0.3 | - | t |
| 2055 | abietatriene | 0.3 | 0.1 | 0.1 | t |
| 2087 | abietadiene | t | t | - | t |
| **2282** | **sempervirol** | **0.8** | **0.2** | **2.1** | **-** |
| 2298 | 4-epi-abietal | 0.3 | 0.3 | 0.3 | 0.1 |
| **2314** | **trans-totarol** | **0.4** | **0.1** | **0.9** | **-** |
| **2331** | **trans-ferruginol** | **0.1** | **t** | **0.2** | **-** |