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Identification of an EcoRI restriction site for a rapid and precise determination of β-asarone-free *Acorus calamus* cytotypes

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Abstract

Calamus (*Acorus calamus* L., Araceae) is an aromatic herb, indigenous to Central Asia and Eastern Europe. The fragrant oils obtained by alcoholic extraction of the rhizome are mainly used in the pharmaceutical and oenological industries. Nevertheless, the occurrence of β-asarone [(*Z*)-1,2,4-trimethoxy-5-prop-1-enyl-benzene] limits the possibility of its use due to the carcinogenic properties of this compound.

The aim of this work was to identify a diploid β-asarone-free *A. calamus* by using chemical and molecular approaches. For these purposes alcoholic extracts of both diploid and triploid *A. calamus* were analyzed by gas chromatography–mass spectrometry (GC–MS) and comparison of the 700 bp sequence of the non-transcribed spacer (NTS) in the 5S-rRNA gene was also performed.

Alcoholic extracts of the triploid *A. calamus* were characterized by a higher percentage of β-asarone (11%), which was the main compound, followed by higher percentages of camphene (2.27%), *E*-β-ocimene (3.28%), camphor (1.54%), calarene (1.42%), α-selinene (5.02%) and τ-cadinol (2.00%), when compared to the diploid *A. calamus*. The latter had higher percentages of *iso*-shyobunone (8.62%), β-sesquiphellandrene (3.28%), pre*iso* calamendiol (22.81%) and acorone (26.33%), and completely lacked of β-asarone.

The 5S-rRNA spacer region of both diploid and triploid *A. calamus* were amplified by PCR using a pair of primers located at the 3′ and 5′ ends of the coding sequence of 5S-rRNA gene. The resulting PCR products (about 700 bp) were gel purified, subcloned into pGEM®-T Easy vector and sequenced. By aligning the isolated nucleotide sequences of the two varieties and the sequences from different *A. calamus* chemotypes present in Genbank, sequence diversities were found in the spacer region. Furthermore, the PCR products were digested by using EcoRI. The restriction profile of the spacer domain resulted different for the two cytotypes.

Along with chemical analysis of alcoholic extracts, sequence analysis coupled to restriction mapping was demonstrated to represent a powerful tool to distinguish the *A. calamus* diploid cytotype from the others. The security and effective usage of the diploid β-asarone-free *A. calamus* was also discussed.

Keywords: *Acorus calamus* L.; Araceae; Sweetflag; Oil chemical composition; β-Asarone; Diploid; Triploid; 5S-rRNA spacer region; DNA sequence analysis; EcoRI site restriction mapping

1. Introduction

Acorus calamus L., also known as sweetflag, is a native plant of India which was introduced in Europe in the 16th century as a medicinal plant (Raina et al., 2003). The plant thus became a common littoral species of the European wetlands, which are characteristic by high trophic status (Vojtíšková et al., 2004). The rhizome contains active ingredients possessing insecticidal (Singh and Upadhyay, 1993; Schmidt and Streloke, 1994; Perrett and Whitfield, 1995; Sugimoto et al.,

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1995; Paneru et al., 1997; Rham and Schmidt, 1999; Raina et al., 2003; Lahlou, 2004), antifungal (Lee et al., 2004), antibacterial (McGraw et al., 2002), and allelopathic (Nawamaki and Kuroyanagi, 1996) properties.

The essential oil from the rhizomes is also used in production of beer and alcoholic beverages such as bitters, liqueurs, vermouths and at lower level in foods such as frozen desserts, yoghurts, cakes and confectionery (Burdock, 1970; Raina et al., 2003). Pharmacological studies on sweetflag essential oils have put into evidence, besides their beneficial effects, their toxicity too (Oprean et al., 1998). Among the essential oil constituents, the most characteristic component is β-asarone [(*Z*)-1,2,4-trimethoxy-5-prop-1-enyl-benzene] (Lander and Schreier, 1990; Oprean et al., 1998; Raina et al., 2003; Venskutonis and Dagilyte, 2003). The FDA interdicted the utilization of sweetflag owing to the potential carcinogenic effects of its essential oil, with particular reference to β-asarone (FDA, 1974). Annex II of Directive 88/388/ECC on flavourings fixed the maximum levels of β-asarone to 0.1 mg/kg in foodstuffs and beverages, with the exception of 1 mg/kg in alcoholic beverages and seasonings used in snack foods (ECC, 1968, 2002). β-Asarone has been demonstrated to be responsible for carcinogenic effects involving duodenal tumour induction (Taylor et al., 1967), unscheduled DNA synthesis in hepatocytes (Hasheminejad and Caldwell, 1994) as well as antiproliferative and immunosuppressive (Mehrotra et al., 2003), central nervous system inhibitory (Koo et al., 2003), sedative and hypothermic (Zanoli et al., 1998) effects. The determination of the β-asarone content can be achieve by means of various techniques, being the gas chromatographic–mass spectrometric (GC–MS) analysis the most reliable method (Oprean et al., 1998).

From a karyotipic point of view, sweetflag includes four cytotypes: diploid (2× = 24), triploid (2× = 36), tetraploid (4× = 48) and hexaploid (6× = 72). The essential oil composition of sweetflag rhizomes depends on ploidy. Diploid cytotypes are characterized by the absence of β-asarone, European and North American triploid cytotypes contain 3–19% β-asarone, whereas the Indian, Indonesian and Taiwan tetraploid cytotypes contain up to 96% of β-asarone (Menghini et al., 1998; Raina et al., 2003 and references cited therein). For these reasons, precise identification of *A. calamus* plants is a prerequisite for commercial applications.

The general approaches to herbal identification depend on morphological, anatomical, and chemical analyses, but these characteristics are often affected by environmental and/or developmental factors during plant growth (Cai et al., 1999).

Molecular genetic methods have several advantages over classical morphological and chemical analyses. For instance, the genetic method requires genotype instead than phenotype, therefore DNA based experiments have become widely employed techniques for a rapid identification of herbal medicine. By using PCR approaches, nanograms quantities of DNA are required to amplify and yield sufficient amounts of template DNA for molecular genetic analysis.

The first molecular approach to study sweetflag was used to support for an evolutionary isolated position of *Acorus* spp. by using sequence analysis of a portion of the chloroplast *rbcL* gene (Duvall et al., 1993). Later, the isolate position of sweetflag was confirmed by phylogenetic analysis of the cytochrome oxidase subunit II gene *cox2* (Albertazzi et al., 1998). Recently, the phylogenetic relationship of some *Acorus* species containing β-asarone has been evaluated using 700 bp sequences of a 5S-rRNA gene spacer region (Sugimoto et al., 1999).

In this work, we demonstrate that molecular approaches using sequence analysis coupled to restriction mapping represent a powerful tool to distinguish the *A. calamus* diploid β-asarone-free cytotype from the other cytotypes.

2. Results and discussion

DAPI staining of the root cell metaphase squashes revealed the presence of 2× = 24 and 3× = 36 chromosome numbers (data not shown), corresponding to the diploid and the triploid *A. calamus* cytotypes under study. The oil chemical composition of the alcoholic extracts from both sweetflag cytotypes analyzed in this work was characterized by the presence of several hydrocarbon and oxygenated monoterpenes, sesquiterpenes, triterpenes and some substituted propenylbenzenes (Table 1). The diploid cytotype was characterized by a very low percentage of monoterpene hydrocarbons and alcohols and had higher percentages of the sesquiterpenes β-funebrene, caryophyllene, α-*E*-bergamotene, prezizaene, caryophyllene, *Z*-β-farnesene, β-copaene, β-acoradiene, bicyclogermacrene, *iso*-shyobunone, β-sesquiphellandrene, *E*-nerolidol, pre*iso* calamendiol, α-cadinol, acora-3,7(11)-dien-8-one, acorone, *iso*-acorone and of 9,12-octadecadienoic acid (*Z,Z*), with respect to the triploid cytotype. On the other hand, the latter contained higher percentages of the monoterpenes camphene, *E*-β-ocimene and camphor, as well as superior percentages of the sesquiterpenes calarene, α-humulene, α-selinene, khusiol, as well as of ethyl hexadecanoate, methyl linoleate and the triterpene γ-sitosterol, when compared to the diploid cytotype. In the triploid cytotype β-asarone was the main compound and both α- and β-asarone were completely absent in the diploid cytotype (Table 1).

The results of the chemical analyses are in line with previous works, confirming the complete absence of β-asarone from diploid rhizome extracts and the presence of 11.16% of this compound in the triploid cytotype

Table 1
Chemical composition of alcoholic extracts of diploid and triploid *A. calamus* L. rhizomes

Compound	KI	Diploid (%)	Triploid (%)
α-Thujene[a]	433	0.0	*tr*
α-Pinene[a]	440	*tr*	0.4
Camphene[a]	456	*tr*	2.3
Sabinene[a]	481	0.0	0.2
β-Pinene[a]	485	0.1	0.1
Myrcene[a]	499	*tr*	*tr*
γ-Terpinene[a]	531	0.0	*tr*
p-Cymene[a]	541	0.0	*tr*
Limonene[a]	547	*tr*	0.3
E-β-Ocimene[a]	559	0.0	3.3
Z-β-Ocimene[a]	572	0.0	0.3
γ-Terpinene[a]	588	0.0	*tr*
Terpinolene[a]	630	0.0	0.1
Linalool[a]	646	0.0	0.5
Camphor[a]	715	0.0	1.5
Terpinene-4-ol[a]	768	0.0	0.1
α-Terpineol[a]	789	0.0	*tr*
Bornyl acetate[a]	948	0.2	0.1
Nonanyl acetate	992	*tr*	0.0
α-Copaene	1097	*tr*	*tr*
α-Funebrene	1105	*tr*	0.1
α-Cedrene	1106	0.2	0.0
β-Elemene	1124	0.1	0.0
Dihydrocarveyl acetate[a]	1129	0.1	0.0
α-Cedrene	1138	*tr*	0.0
Z-*Iso*eugenol[a]	1149	*tr*	0.0
β-Funebrene	1160	1.6	0.9
β-Cedrene	1169	0.8	1.6
Calarene[a]	1187	0.0	1.4
α-*E*-Bergamotene[a]	1197	0.9	0.5
Prezizaene	1210	0.7	0.4
Caryophyllene[a]	1220	1.2	0.9
α-Humulene[a]	1221	0.0	0.6
Z-β-Farnesene	1231	1.7	1.2
β-Copaene	1238	0.6	0.0
β-Acoradiene[a]	1244	0.3	0.2
β-Curcumene	1252	0.1	*tr*
α-Neocallitropsene	1256	0.3	0.4
γ-Amorphene	1266	0.3	0.9
ar-Curcumene	1271	0.3	*tr*
β-Selinene	1273	0.0	0.2
allo-Aromadendr-9-ene	1277	0.2	0.4
α-Selinene	1289	1.5	5.0
Bicyclogermacrene[a]	1292	0.2	0.0
α-Muurolene	1297	0.0	0.1
α-Amophene	1298	0.1	0.0
Germacrene A[a]	1304	0.3	0.3
γ-Guaiene	1308	0.0	0.4
iso-Shyobunone	1324	8.6	6.9
β-Sesquiphellandrene	1336	3.3	2.7
E-Nerolidol	1405	1.3	0.4
4-α-Hydroxygermacra-1(10),5-diol	1415	1.6	1.8
Cedrol[a]	1431	0.5	0.5
Pre*iso*calamendiol[a]	1470	22.8	7.8
β-Asarone[a]	1488	0.0	11.2
Dehydroxy-*iso*calamendiol[a]	1497	0.3	0.0
τ-Cadinol	1514	0.3	2.0
α-Cadinol	1532	0.7	0.0
4-*epi*-Acorenone	1562	0.4	0.2
Khusiol	1573	0.1	5.9
Acorenone[a]	1585	5.3	9.3
Torilenol	1607	0.1	1.8
Acora-3,7(11)-dien-8-one	1630	0.5	0.0
Squamulosone	1686	0.1	0.0
Acorone[a]	1750	26.3	8.4
iso-Acorone	1779	1.3	0.1
n-Hexadecanoic acid	1954	0.2	0.0
Ethyl hexadecanoate	1989	0.0	2.4
9,12-Octadecadienoic acid (*Z Z*)-	2164	0.4	0.0
Methyl linoleate[a]	2191	0.5	4.6
9.12.15-Octadecatrienoic acid, ethyl ester. (*Z.Z.Z*)-	2197	0.1	0.8
γ-Sitosterol[a]	3269	0.1	2.6

Values are the mean of at least three injections. KI = Kovats Index.
[a] These compounds were identified by direct comparison with pure standards; *tr* = traces.

(which lies in the range as expected) (Singh and Upadhyay, 1993; Menghini et al., 1998; Ozcan et al., 2002; Raina et al., 2003 and references cited therein; Venskutonis and Dagilyte, 2003). The quality of the diploid essential oil was also evaluated by a panel of experts who defined it of high quality and suitable for applications in the food and beverage industry.

Having assessed the complete lack of β-asarone in the diploid cytotype, molecular biological approaches were then used in order to simplify the correct identification of β-asarone-free *A. calamus* cytotypes. This procedure could result particularly suitable when low quantities of β-asarone-containing sweetflag rhizomes are mixed with other medicinal plants or when adulteration of the diploid cytotype occurs. Moreover, identification of herbs by using genetic sequences has been shown to be faster and reliable, by meeting the needs of the present market (Ma et al., 2001).

In higher eukaryotes, the 5S-rRNA gene occurs in tandemly repeated units consisting of an ≈120 bp coding region separated by a non-transcribed spacer of various sizes. Although the coding region is highly conservative, the non-transcribed spacer varies from species to species in both sequence and length since it is apparently not under the same rigorous selection pressure as in the coding region (Sugimoto et al., 1999). Thus, the diversity of the spacer region can be used as an identification basis. Here, two primers flanking the spacer region of 5S-rRNA, already employed for differing *A. calamus* chemotypes (Sugimoto et al., 1999) were used in PCR analysis of genomic DNA isolated from diploid and triploid cytotypes. A single fragment of ≈700 bp was produced from each *A. calamus* cytotype (Fig. 1). Fragments were ligated into pGEM®-T Easy vector and the nucleotide sequence was determined as shown in Fig. 2. Over 3 individual clones of the same

PCR product were sequenced to avoid any mutation introduced by *Taq* polymerase.

The sequenced region spans 713 bp for the triploid (NCBI Genbank Accession No. AY812747) and 695 bp for the diploid (NCBI Genbank Accession No. AY214463) cytotype. Sequence alignment of the 5S-rRNA spacer region flanked by the 3′- and 5′-ends of the coding region is shown in Fig. 2. The 5S-RNA spacer region of the two varieties was clearly different in both length and sequence. Surprisingly, the diploid cytotype presented a shorter nucleotide sequence (695 bp) with respect to the triploid cytotype and other *A. calamus* chemotypes (713 bp) (Sugimoto et al., 1999).

The sequences were further analyzed by the neighbour joining (NJ) method to infer phylogenetic relationship among diploid, triploid, and the *A. calamus* chemotypes deposited in the NCBI Genbank (Sugimoto et al., 1999). Fig. 3 shows the phylogenetic tree where the diploid and triploid cytotypes analyzed in this study form independent clusters, which robustness is supported by high bootstrap scores. By considering that the chemotype A of Fig. 3 possess a high percentage, chemotype B has low percentages and chemotype M has intermediate percentages of β-asarone (Sugimoto et al., 1999), the positions of the triploid (low level β-asarone) and of the diploid (no β-asarone) cytotypes correlate well with the phylogenetic relationship predicted by the spacer region data (Fig. 3).

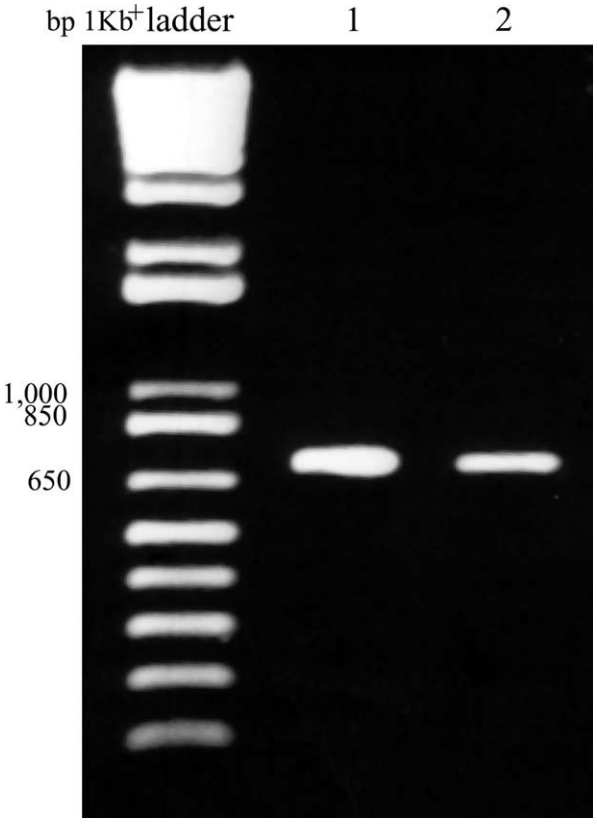

Fig. 1. Agarose gel electrophoresis of PCR products: lane 1, triploid *A. calamus*; lane 2, diploid *A. calamus*.

In order to characterize better the diploid from the triploid cytotype and to simplify the identification method, purified PCR products of the 5S-rRNA gene spacer region were digested with EcoRI, using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP). From the identified sequences, an EcoRI site, absent into the diploid cytotype, could be found in the triploid cytotype at 586 bp position (Fig. 2). As expected, PCR products from the triploid cytotype could be digested by EcoRI, while the diploid cytotype was insensitive to the digestion (Fig. 4). Two fragments of 586 and 127 bp, represented by a very faint band on the gel (Fig. 4) were created from digested triploid *A. calamus* DNA, while no digestion fragments were revealed from the diploid *A. calamus* DNA. This EcoRI site could then be used for a rapid and precise identification of *A. calamus* cytotypes.

In conclusion, biomolecular analysis supported by GC–MS chemical characterization allowed differentiating cytotypes belonging to the species *A. calamus*. To our knowledge this work represents the first report about a rapid and precise method for the identification of a commercial cytotype of β-asarone-free *A. calamus* based on 5S-rRNA nucleotide sequence analysis following restriction enzyme profiling. Owing to the importance of sweetflag utilization as a medicinal plant as well as in the food and beverage industry, and considering the health hazard depending on the presence in triploid, tetraploid and hexaploid cytotypes of the carcinogenic β-asarone, the identification of an EcoRI site to be used for a rapid and precise identification of β-asarone-free diploid *A. calamus* cytotypes can be considered a valuable tool for the effective and safe usage of these plants.

3. Experimental

3.1. Plant material

Plants of clonal populations of *A. calamus* (2× = 24 and 3× = 36), determined by chromosome counting using DAPI (4′-6-diamidino-2-phenylindole), were grown for several years in the Botanical Gardens of the University of Turin. Diploid and triploid plants were used for essential oil analysis and DNA extraction.

3.2. Chromosome counts

Root tips of both *A. calamus* cytotypes were fixed with the Carnoy reagent (EtOH:$CHCl_3$:acetic acid, 6:3:1) and then stored at 4 °C for 48 h. Root tips were then rinsed several times and then cell walls were digested with an enzymatic mixture (2–6% cellulase + 1–2% pectolyase) at 37 °C for 70 min. Root tips were then rinsed with

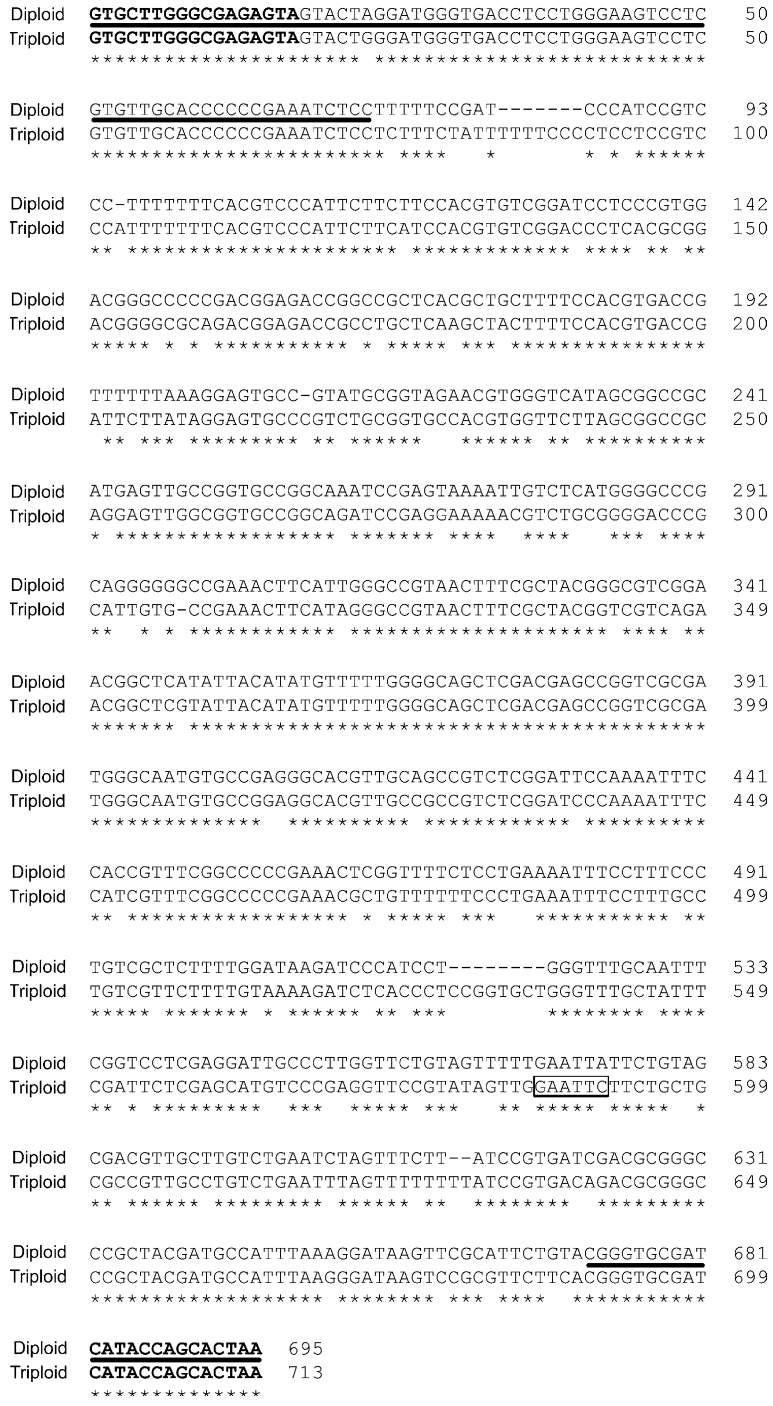

Fig. 2. Aligments of the nucleotide sequences of 5S-rRNA gene spacer region of diploid and triploid *A. calamus* cytotypes. The coding regions are underlined. Primer sequences are indicated in bold. Identical sequences are indicated by (*). Gaps (-) are introduced for the best alignment. EcoRI site is evidenced in the squared box.

water, transferred to a microscopic slide, stained with 10 μM DAPI in the dark for 30 min, squeezed and observed under a Nikon E400 epifluorescence microscope.

3.3. Essential oil extraction and GC–MS analysis

Extracts were prepared by chopping dried rhizomes in 70% ethanol (1:4 drug dry weight: solvent ratio). The material was incubated for 1 week at room temperature in the dark and then filtered by passage through 8 layers of cheesecloth. Fifty millilitres of the solution were extracted by using the same amount of hexane and the hexane phase was then concentrated to 5 ml by N_2 flux. The extracts were then dried by passing over a column packed with anhydrous $MgSO_4$.

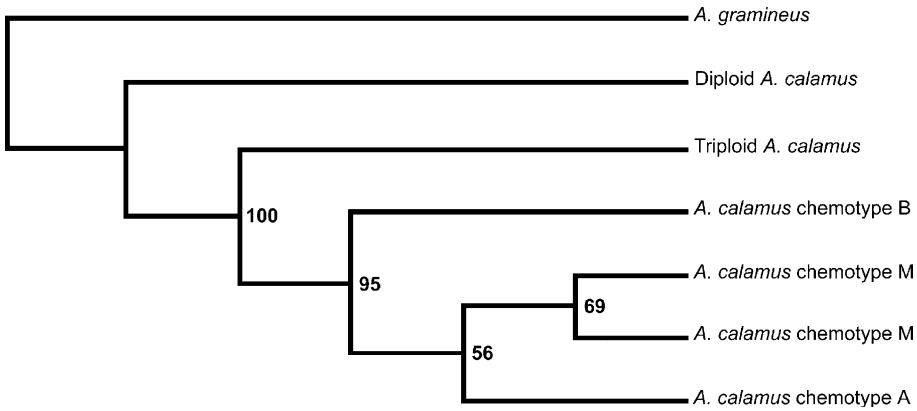

Fig. 3. Cladogram of Clustal X alignment of diploid, triploid and different chemotypes of *A. calamus* plants based on the nucleotide sequences of 5S-rRNA gene spacer region using the NJ method. Bootstrap values higher than 85 are likely to be significant. NCBI accession numbers of the sequences of *A. calamus* chemotypes: A017421 (chemotype A, rich in β-asarone); A017422 (chemotype B, rich in sesquiterpenoids); A017423 (chemotype M, intermediate); A017424 (chemotype M, intermediate) and *Acorus gramineus*: A017425 (Sugimoto et al., 1999).

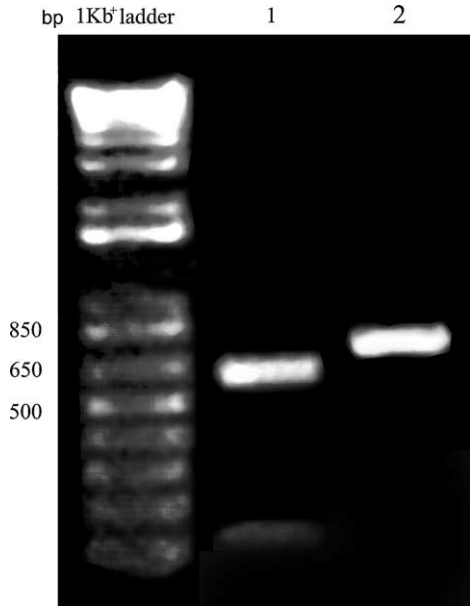

Fig. 4. PCR-RFLP analysis using EcoRI: lane 1, digested products from triploid *A. calamus* 5S-rRNA gene spacer region; lane 2, digested products from diploid *A. calamus* 5S-rRNA gene spacer region. These restriction digestions were reproduced from three different samples.

GC–MS analyses were made using an Agilent Technologies 6890 series II gas chromatograph equipped with a capillary HP-5MS (30 m × 0.25 mm, film thickness of 0.25 μm) coupled to an Agilent Technologies 5973N MSD mass spectrometer. One microlitre of hexane extract was injected in split mode (1:50). The GC/MSD was operated under the following conditions: injector temperature 250 °C; transfer line 280 °C; oven temperature programmed with a initial temperature of 60 °C and final of 290 °C with an increment of 3 °C/min, final time was 20 min; carrier gas was He under a constant flow of 1 ml/min during the entire analysis. The MSD was operated with the following parameters: MS Source 280 °C, MS Quad 150 °C; auto-tune set was as follows: emission 34.6, El Energy 69.9, EMVolts 1247, AmuGain 2422, AmuOffs 131, MassGain 236, MassOffs −9, Repeller 31.46, IonFcus 90.2, EntLens 9.5, EntOffs 18.32, DC Pol NEG. The acquisition was set in scan mode and the scan range was from 30 to 800 amu; the solvent delay was 5 min.

Peak identification was performed with the Nist mass spectral search program v2.0 using the libraries NIST 98 and Adams (2001), as well as with direct comparison with some pure standards as indicated in Table 1.

3.4. Genomic DNA extraction

One gram of fresh leaves was frozen in liquid nitrogen and ground to a fine powder in a chilled mortar.

Genomic DNA was extracted from the ground powder by using Qiagen DNA Maxi Kit following manufacturer's instruction. The quality of the DNA was assessed by spectrophotometric and gel electrophoresis analyses.

3.5. PCR amplification, subcloning and sequencing

Approximately 20 ng of genomic DNA were used as a template for PCR amplification with forward primer P1 (5′-GTGCTTGGGCGAGAGTAGTA-3′) and reverse primer P2 (5′-TTAGTGCTGGTATGATCGCA-3′) flanking the spacer region of 5S-rRNA (Sugimoto et al., 1999). The amplification was carried out in a 50 μl reaction mixture containing 5 μl 10× PCR buffer (Amersham Biosciences), 0.2 mM dNTPs, 20 pmol forward and reverse primers and 0.5 units of *Taq* DNA polymerase (Amersham Biosciences). The PCR reactions were carried out in a Gene Cycler (Biorad).

Cycling conditions consisted of an initial 5 min at 94 °C followed by 1 min denaturing at 94 °C, 2 min annealing at 57 °C and final 2 min elongation at 72 °C

repeated for 35 cycles and with 10 min extension at 72 °C.

PCR products were analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. A band of about 700 bp was purified by using agarose Gel DNA extraction Kit (Amersham Biosciences) and then subcloned into pGEM®-T Easy vector (Promega). The ligated products were transformed into *E. coli* DH5α F^- competent cells (Invitrogen). Colonies containing DNA inserts of the correct size were picked and grown overnight in 3 ml of Luria–Bertani (LB) liquid medium. The mini-preparation of plasmid DNAs were performed using Qiagen Plasmid Mini Kit, following manufacturer's instructions. The plasmid DNAs were employed as a template for sequencing. ABI Prism, BigDye Terminator and Cycle Sequencing Ready Reaction Kit were used for sequence reaction with T7 and SP6 primers (Applied Biosystems). Sequences were detected by an ABI 377 automated sequencer according to the manufacturer's protocol (Applied Biosystems). Both strands of DNA were sequenced at least twice and the sequences were aligned by using ClustalX software.

3.6. PCR-RFLP

The purified PCR products of the 5S-rRNA gene spacer region were digested with 10 units of EcoRI at 37 °C for 1 h. Digestion products were fractionated by 2% gel electrophoresis and visualized by ethidium bromide staining under UV.

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