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Chemical and Biomolecular Characterization of *Artemisia umbelliformis* Lam., an Important Ingredient of the Alpine Liqueur "Genepì"

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ABSTRACT

A. umbelliformis Lam., an important Alpine plant used for preparation of flavoured beverages showed a remarkable intraspecific variability, both at genomic and gene products (secondary metabolites) level. The variability of *A. umbelliformis* Lam. currently cultivated in Piedmont (Italy, Au1) and in Switzerland (Au2) was investigated by combining the chemical analysis of essential oil and sesquiterpene lactones and the molecular characterization of the 5S-rRNA-NTS gene by PCR and PCR-RFLP. Marked differences were observed between the two plants. Au1 essential oil contained α- and β-thujone as main components while Au2 1,8-cineole, borneol and β-pinene. Au1 sesquiterpene lactone fractions contained *cis*-8-eudesmanolide derivatives and Au2 the *trans*-6-germacranolide costunolide. Specific *A. umbelliformis* Au1 and Au2 primers were designed on the sequence of the 5S-rRNA gene spacer region. Furthermore, a PCR–restriction fragment length polymorphism (PCR–RFLP) method was applied using RsaI and TaqI restriction enzymes. Chemical and biomolecular data contributed to characterize *A. umbeliformis* chemotypes.

Keywords: *Artemisia umbelliformis* Lam.; alpine liqueurs; chemotype discrimination; chemical analysis; biomolecular analysis.

INTRODUCTION

Artemisia umbelliformis Lam. is an Alpine species used to prepare "genepì", a highly praised liqueur characterized by a bitter taste and a peculiar flavour *(1)*. These properties have been traced to the volatile constituents and to the sesquiterpene lactone fraction of the plant, that are characterized by a high contents of α- and β-thujone *(2, 3)* and by the presence of the *cis*-eudesmanolide sesquiterpene lactones **1**-**3**, respectively *(4-6)*.

Thujone is a natural terpenoid also associated with common wormwood (*Artemisia absinthium* L.) and Roman wormwood (*Artemisia pontica* L.), absinthe's most widely used ingredients *(7 and reference cited there in)*. There is currently a heated debate on the toxicity of absinthe and thujones *(7 and reference cited there in)*, but the EU legislation have imposed a limit of 35 ppm to the total amount of these compounds in alcoholic beverages *(8)*. To overcome this issue, thujones-free chemotypes of *A. umbelliformis* have been selected by horticultural techniques *(9)*. Remarkably, an investigation on the sesquiterpene lactone fraction of one of these thujones-free chemotypes showed dramatic differences from the wild plant. Thus, the C-8 *cis* sesquiterpene lactones typical of *A. umbelliformis* from Western Alps (**1**-**3**) were replaced by the C-6 *trans* lactones **4**-**6a,b,** while a structurally unique sesterpene lactone (**7**) was also detected *(10)* (**Figure 1**). Chemotypes (or chemical phenotypes) are generally considered the phenotypical expression of a genotype, although different chemotypes may derive from the same genotype. This means that, according to environmental conditions, the same genotype may express different chemical patterns, or, on the other side, that different genotype may respond to the same environmental pressure with the same phenotypic expression. In this context, molecular genetic methods have recently been shown to be very effective in genotypic discrimination. Genetic methods focus on genotype rather than phenotype, and DNA based experiments are now widely used for a rapid identification (and therefore autentication) of medicinal plants. Bertea et al. *(11)* recently showed that molecular approaches are a powerful tool to distinguish the *Acorus calamus* diploid β-asarone-free cytotype from the other cytotypes containing it. The same group also used specific *Salvia divinorum* primers

designed on the sequence of the 5S-rRNA gene spacer region *(12)* to develop a Real-Time PCR-based mathematical model to quantify *S. divinorum* in commercial plant samples or hallucinogenic preparation *(13)*. Given the potential of this approach, it seemed interesting to apply a combination of biomolecular and chemical techniques to characterise the chemotypes of *A. umbelliformis* currently cultivated in Piedmont (Italy, Au1) and in Switzerland (Au2), complementing the analysis of their essential oil and sesquiterpene lactones with a molecular characterization by PCR and PCR-RFLP of the 5S-rRNA-NTS region of their genome.

MATERIALS AND METHODS

Chemicals - Thujones standard mixture (mixture of α-thujone and β-thujone purity 99.9%) and all other pure reference compounds were from Sigma-Aldrich (St. Louis MO, USA). Sabinene was from Chromadex ™ (Irvine CA, USA); sabinol was kindly supplied by Robertet SA (Grasse, France); sabinyl ester homologous series were synthesized in the authors' laboratory (*2*). HPLC and analytical grade solvents were from Carlo Erba Reagenti, Rodano, Italy. The sesquiterpene lactones **1-7** were available from previous studies *(4-6, 10)*

Plant Material - Forty three samples of *Artemisia umbelliformis* Lam aerial parts from experimental cultivations run in different Alpine valleys [Val Grana (latitude 44°25'N, longitude 7°20'E), Valle Stura (44°21'N, 7°26'E), Valle Maira (44°28'N, 7°22'E), Val Chisone (44°57'N, 6°52'E)] at an height of least 1300 m a.s.l. Fresh plant material was directly indoor-dried by the farmers under controlled temperature and humidity up to a constant weight, in agreement with the WHO's guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants. Voucher specimen representative of the two chemotypes (native, defined Au1 and selected in Switzerland, defined Au2) are deposited at the Dipartimento di Scienza e Tecnologia del Farmaco (n°231 for Au1 and n°232 for Au2). For each chemotype, batches of 1 kg of aerial dried parts (see above) were supplied by the "Associazione Genepì Occitan" (Cuneo - Italy)

Essential oils and Headspace solid phase-microextraction (HS-SPME) sample preparation - Essential oils (EOs) were prepared according to the method of the European Pharmacopoeia *(14)*. Ten grams of dried aerial parts was suspended in 250 mL of water in a 500 mL flask for 1 h, and then submitted to hydrodistillation in a Clevenger micro-apparatus for 2 h *(2)*. The resulting EO was left to stabilize for 1 h, then recovered with hexane and then analyzed by GC-MS.

The SPME device and the three component CAR/PDMS/DVB fused silica fiber (2 cm long, coating volume: 1.000 μm^3) were purchased from Supelco (Bellafonte, PA, USA), *(15)*. Before use, the fiber was conditioned as recommended by the manufacturer.

Each sample (200 mg of *A. umbelliformis* dried aerial parts) hermetically sealed in a 2.0 mL vial was introduced in a thermostatic bath at 80 °C for 15 min; the SPME device was inserted in the sealed vial containing the sample, and the CAR/PDMS/DVB fiber exposed to the matrix headspace (30 min). The vial was vibrated for 10 s every 5 min with an electric engraver (Vibro-Graver V74), (Burgess Vibrocrafters Inc., Brayslake, IL). After sampling, the SPME device was immediately inserted into the GC injector and the fiber thermally desorbed. A desorption time of 5 min at 230 °C was used. Before sampling, each fiber was reconditioned for 20 min in the GC injection port at 230 °C.

Sesquiterpene lactone extraction - One gram of dried aerial parts of both chemotypes were sonicated three times with ethanol 96% (50 mL) for 10 min. The resulting total extract (150 mL) was filtered, and evaporated to dryness under vacuum; the weighed solid residue was dissolved in acetonitrile/water 20/80 at a concentration of 0.1 mg/mL and analyzed by high-performance liquid chromatography-diode array-ultraviolet detection-mass spectrometry (HPLC-DAD-UV-MS).

GC and GC-MS Analyses - GC analyses were carried out on a Shimadzu QP2010 system provided with a FID and a MS detector, and the results processed by GC Solution software and GC-MS solution software (2.51version) (Shimadzu Italia, Milano Italy). Capillary GC-FID-MS analyses were carried out on two 25 m, 0.25 mm i.d., 0.25 μm columns from MEGA (Milano – Italy), i.e. Mega5 (95% polydimethyl-siloxane, 5% phenyl) and MegaWax (polyethyleneglycol, PEG20M).

GC and GC-MS conditions: injection mode: split; split ratio: 1: 20 Temperatures: injector: 230 °C, transfer line: 230 °C; ion source: 200 °C; carrier gas: He flow-rate: 1.0 mL /min in constant flow-mode. MS detector was in electron impact ionization mode (EI) at 70 eV, scan rate was 1111 amu/s and mass range of 35–350 *m/z*. Temperature program: from 50 °C (1 min) to 220 °C (5 min) at 3 °C min^{-1}.

EOs and headspace components were identified by comparison of both their linear retention indices, calculated versus a C_8-C_{25} hydrocarbon mixture, and their mass spectra with those of authentic samples or with data in the literature.

Quantitative analysis. Suitable amounts of α + β-thujone commercial standard were diluted with cyclohexane to obtain six concentration levels ranging from 0.5 ng /μL to 6 ng/μL for α-thujone and from 0.04 ng/μL to 0.5 ng/μL for β-thujone**.** Calibration curves were obtained by analyzing the resulting standard solutions three times by GC-FID using *n*-nonane as internal standard.

HPLC-DAD-UV analysis - HPLC-DAD-UV analyses were carried out on a Shimadzu 2010EV system provided with a PDA detector (Shimadzu, Dusseldorf Germany). A 150 x 4.6 mm i.d., 5 μm, Zorbax Stable Bond column (Agilent, Waldbronn Germany) was used. Analysis conditions were: mobile phase: eluent A: 20% acetonitrile/water; eluent B: 100% acetonitrile; mobile phase gradient: from 100% A to 100% B in 25 min. Injection volume: 10 μL, flow rate: 1 mL/min. UV detection wavelengths: 210nm.

Quantitative analysis – Suitable amounts of custonolide and umbellifolide were diluted with methanol to obtain concentrations ranging from 0.5, 5, 10, 25, 50 and 100 ng/μL of each marker, respectively. Umbellifolide (**3**) was adopted as standard for quantitation also for the hydroperoxytelekins **1** and **2**, because of the similarity of structures, their trace abundance and chemical instability; the results are expressed as the sum umbellifolide + hydroperoxytelekins. A calibration curve was made by analyzing the resulting standard solutions three times by HPLC-DAD-UV at 210 nm.

HPLC-MSD analysis - HPLC-MSD analyses were carried out with a single quadrupole Shimadzu 2010EV system (Shimadzu, Dusseldorf, Germany) equipped with an orthogonal atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources. The same column and mobile phase as for HPLC-DAD-UV analysis was used. Flow rate: 0.8 mL/min. MSD conditions: MS-APCI+; temperature: 400 °C; nebulizer's flow: 2.5mL/min; CDL voltage: 250 °C; Q-Array: MS-ESI+; temp:250 °C; nebulizer's flow: 1,5mL/min; CDL voltage: 250 °C.

MSD analysis conditions were optimized by direct flow injection of pure standards of costunolide (**4**) and umbellifolide (**3**) and of a fraction containing a mixture of hydroperoxytelekines

Genomic DNA extraction - Plant material employed for the chemical analyses was also used for genomic DNA extraction. . Fifty mg of dried material was frozen in liquid nitrogen and ground to a fine powder with Tissue Lyser (Qiagen, Hilden, Germany). Genomic DNA was extracted from the ground powder by using the Nucleospin Plant Kit (Macherey Nagel, Düren, Germany) following manufacturer's instruction. The quantity and quality of the DNA were assessed by spectrophotometric analysis by using the Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) from several samples of the two chemotypes.

PCR amplification, subcloning and sequencing - Approximately 20 ng of genomic DNA isolated from powdered leaf material of Au1 and Au2 were used as a template for PCR amplification with forward primer 5S-P1 (5′ - GTGCTTGGGCGAGAGTAGTA-3′) and reverse primer 5S-P2 (5′ - TTAGTGCTGGTATGATCGCA-3′) flanking the NTS of 5S-rRNA gene *(11-13, 16).* The amplification was carried out in a 50 µL reaction mixture containing 5 µL 10X PCR reaction buffer (Fermentas), 0.2 mM dNTPs, 20 pmol forward and reverse primers and 0.5 U of *Taq* DNA polymerase (Fermentas, Glen Burnie, MA, USA). The PCR reactions were carried out in a Whatman Biometra T-Gradient Thermalcycler (Whatman Biometra, Goettingen, Germany). Cycling conditions consisted of an initial 4 min at 94 °C, followed by 30 sec denaturing at 94 °C, 1 min annealing at 52 °C, and 1 min elongation at 72 °C repeated for 30 cycles and with 5 min final extension at 72 °C.

One microliter of the amplification reaction was analyzed by capillary gel electrophoresis (CGE) using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer's instructions. The DNA 1000 LabChip Kit provides sizing and quantitation of dsDNA fragments ranging from 25 to 1000 bp. PCR products were also analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. From this gel a band of about 220 bp for Au1 and about 320 bp for Au2 was purified by using the Nucleospin Extract II Kit (Macherey Nagel) and then subcloned into pGEM-T Easy vector (Promega). The ligated products were transformed into the *Escherichia coli* Subcloning DH5α Efficiency 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 QIAprep Spin Miniprep Kit (Qiagen), following manufacturer's instructions. The plasmid DNAs were employed as a template for sequencing. Both strands of DNA were sequenced at least twice and the sequences were aligned by using ClustalX software.

PCR amplification using specific primers for Au1 and Au2 - Sequences derived from powdered leaf material of Au1 and Au2 were aligned in a unique sequence that allowed the design of two forward primers AuF 5′ -CTAGGATGGGTGACCTCCTG -3′(which is common to both chemotypes) and Au2F 5′-GCGGTGACAGAGTCGGTAAA-3′ and two reverse specific primers: Au2R1 5′ -CGTAAAATTCACCGCCTACG -3′ and Au2R2 5′-TCCTTTCTCATTGCCTATTTTTC -3′, which corresponded respectively to nucleotides 21-40, 168-187, 212-231, 253-275 of Au2 non-transcribed spacer (NTS) sequence. The internal primers were used for amplification also in combination with primer 5S-P1 and 5S-P2.

The conditions of the PCR reactions were the same as mentioned above. One microliter of the amplification products were separated by CGE with the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer's instructions.

PCR–RFLP - The purified PCR products of the 5S-rRNA gene spacer region of both Au1 and Au2 chemotypes were either digested with 10 U of RsaI (Amersham Biosciences) at 37 °C for 1h or, in a separate reaction, with 10 U of TaqI (Sigma) at 65 °C for 1 h. One microliter of both digestion reactions was fractionated by CGE using the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer's instructions.

RESULTS AND DISCUSSION

This study aims to characterize the two chemotypes of *A. umbelliformis* Lam. under investigation by combining results from chemical and genomic analyses of 43 samples from experimental cultivation (Au1, Italian native and Au2, selected in Switzerland).

Chemical analyses - Chemical analyses investigated the fractions responsible for plant odor and taste i.e. the composition of the volatile fraction including quantitation of α- and β-thujone, and that of the sesquiterpene lactone fraction (i.e. the components responsible for the liqueur bitter taste). The volatile fraction was studied by analyzing both the essential oil (EO) obtained by hydrodistillation and the headspace (HS) sampled by solid phase microextraction (HS-SPME) combined with GC and GC-MS. HS-SPME sampling was applied in view of developing a fully automatic control method, to be run in combination with GC and GC-profile multivariate analysis (Principal Component Analysis (PCA). **Table 1** reports the average percent areas normalized vs. *n*-nonane as internal standard and percent range of the characteristic components of the EOs of the samples investigated together with their Linear Retention Indices (LRI) on both GC columns. **Table 2** reports calibration curves, mean and range amounts of α- and β-thujone in the samples investigated. This Table considers only ten samples of chemotype Au2 on seventeen because the alpha and beta-thujone peak areas of the remaining seven samples were too low to be correctly used for quantitative determination. From these results it is clear that the two chemotypes are characterized by a different composition: Au1 was found to contain α- and β-thujone and an homologous series of sabinyl esters as main components, whereas in the chemotype Au2, 1,8-

cineole, borneol and β-pinene were the major compounds. Moreover, thujones were almost absent from the Au2 chemotype, their total amount accounting from 0.2 to 0.4g/100g of EO, while in the Au1 chemotype thujones ranged from 18 to about 58 g/100g of EO. The results obtained by HS-SPME-GC analysis, although not directly comparable, were in full agreement with those of the EOs, as shown by the PCA scatterplot of **Figure 2**. Each chemotype is clearly discriminated and the samples belonging to the same chemotype analysed by HS-SPME-GC and through their EO are coherently positioned in the PCA scatterplot (**Figure 2**).

Significant differences can also be found in the composition of the non-volatile bitter fraction. **Figure 3** reports the HPLC-DAD-UV profiles of two samples belonging to Au1 and Au2 chemotypes, respectively. The bitter taste of the native Au1 chemotype is mainly due to sesquiterpene lactones of the *cis*-8-eudesmanolide type [5-deoxy-5-hydroperoxy-5-epitelekin (**1**), 5-deoxy-5-hydroperoxytelekin (**2**), umbellifolide (**3**) *(4-6)*]. On the other hand, the Au2 chemotype is characterized by high amounts of costunolide (**4**), a germacranolide typical of *A. genipi* Weber *(4)*, and by the presence of an unusual sesterpene lactone, named genepolide (**7**) *(10)*. An in depth investigation of the Au2 ethanolic extract composition after fractionation by column chromatography in combination with NMR, and analysis by HPLC-UV and HPLC-single-quadrupole-MS revealed that costunolide (**4**) was accompanied by a series of related oxygenated sesquiterpene lactones (artemorine (**5b**), santamarine (**6a**),and reynosine(**6b**)) *(4)*. On the other hand, in the Au1 chemotype the presence of both telekine hydroperoxides and umbellifolide was confirmed.

Costunolide and the sum of hydroperoxytelekins and umbellifolide were adopted as markers of the two chemotypes to evaluate quantitatively the bitter fraction of the 43 samples under investigation. These analyses showed an average amount of umbellifolide + hydroperoxytelekins expressed as umbellifolide ($y = 29.9392x - 1.6795$; R^2: 0.99999) of 0.11 g/100g of dried plant material in a range varying between 0.03 and 0.37 g/100g for the Au1 chemotype, and an average amount of costunolide ($y = 101.1709x - 31.0592$; R^2 : 0.99975) of 0.56 g/100g of dried plant material in a range

247 between 0.20 and 0.93 g/100g for the Au2 chemotype. Costunolide was also detected in very low
248 amounts in some samples of the Au1 chemotype, its percentage never exceeding 0.05 %
249 **Molecular characterization of the two A. umbelliformis chemotypes -** In higher eukaryotes, the
250 5S-rRNA gene occurs in tandemly repeated units consisting of an 120 bp coding region separated
251 by a non-transcribed spacer varies from species to species *(16)*. Thus the diversity of the spacer
252 region can be used as an identification basis *(17)*.
253 Here, two primers flanking the spacer region of 5S-rRNA, already successfully employed for
254 differing *Acorus calamus* chemotypes *(16)*, *A. calamus* cytotypes *(11)* and *Salvia divinorum* both as
255 pure plants *(12)* and in plant mixtures *(13)* were used in PCR analysis of genomic DNA isolated
256 from the two chemotypes Au1 and Au2.
257 A single fragment of approximately 220 bp was produced by Au1 (**Figure 4**, lane 1) and a single
258 fragment of about 320 bp was produced by Au2 (**Figure 4**, lane 2). Fragments derived from both
259 chemotypes were ligated into pGEM-T Easy vector and the nucleotide sequence was determined.
260 The sequenced region spans 224 bp for Au1 (NCBI GenBank Accession No. EU816950) and 327
261 bp for Au2 (NCBI GenBank Accession No. EU816951).
262 Sequence alignment of the 5S-rRNA spacer region flanked by the 3′-and 5′-ends of the coding
263 region is shown in **Figure 5**. Surprisingly, Au1 presented a difference of 103 nucleotides with
264 respect to Au2. This difference is quite consistent but not uncommon between chemotypes or
265 cytotypes, as it has been previously demonstrated with other plant species *(11, 16)*.
266 In order to characterize better the two chemotypes and to simplify the identification method,
267 nucleotide sequences of the 5S-rRNA gene spacer region were used to design four specific primers
268 (**Figure 6**).
269 PCR products derived from all possible combinations of Au1 and Au2 specific primers also used
270 with the primers designed on the coding regions of the plant 5S-rRNA gene, were analyzed. In the
271 chemotype Au1 a single fragment of 204 bp in length was amplified using the primer AuF in
272 combination with the primer 5S-P2 (**Figure 4**, lane 3). The same strategy used with the chemotype

Au2 produced a single fragment of 307 bp in length (**Figure 4**, lane 4). The three additional specific primers designed for Au2 gave a combination of single fragments as follows: 231 bp with 5S-P1 and Au2R1 (**Figure 4**, lane 5), 275 bp with 5S-P1 and Au2R2 (**Figure 4**, lane 6), 64 bp with Au2F1 and Au2R1 (**Figure 4**, lane 7), 108 bp with Au2F1 and Au2R1 (**Figure 4**, lane 8), 213 bp with AuF and Au2R1 (**Figure 4**, lane 9) and 255 bp with AuF and Au2R2 (**Figure 4**, lane 10). All amplifications occurred only in Au2 when the Au2 specific primers were used and no PCR products were detected when Au1 DNA was employed as a template.

In addition, a PCR–RFLP method was applied. From the identified sequences, two RsaI sites could be found in both chemotype 5S-rRNA spacer regions at 18 bp and 141 bp position in Au1 and at 18 and 301 bp in Au2 (**Figure 5**). Purified PCR products obtained by using 5S-P1 and 5S-P2 primers were digested with Rsa I. As expected, PCR products from the chemotype Au1 could be digested by RsaI giving two major fragments of 123 bp and 82 bp respectively, and a minor fragment of 19 bp not visible in the gel because out of the resolution capacity of the instrument (**Figure 4**, lane 11). When purified PCR products from the chemotype Au2 were digested using RsaI, a completely different RFLP profile was observed. RsaI cleaved Au2 5S-rRNA spacer region giving a major fragment of 283 bp and two minor fragments of 25 bp (barely visible in the gel) and 19 bp (not visible in the gel) (**Figure 4**, lane 12). A TaqI site was also identified in the sequence of the chemotype Au2 (see **Figure 5**). As expected, PCR products from the chemotype Au1 could not be digested by Taq I (**Figure 4**, lane 13), whereas purified PCR products from the chemotype Au2.

Thus, our biomolecular characterization provides a useful tool for the unequivocal characterization of the two chemotypes. If some intermediate chemotypes due to meiotic rearrangements were already present in the population, they would be detected by using universal primers (different fragment size or different nucleotide composition), but this was not the case, since all samples gave the same results. Besides, this marker has been successfully used for *Acorus calamus* chemotype determination *(16)* and it represents a powerful tool to deduce genetic relationship of medicinal plants, especially at the intra-specific level.

In conclusion, these results clearly support the view that *A. umbelliformis*, a valuable plant for Alpine agriculture, shows a remarkable intraspecific variabilty, both at the genomic and gene products (secondary metabolites) levels. This multidisciplinary study, by showing remarkable chemical variation in the terpenoid profile, and consistent genomic difference in the 5S-rRNA spacer regions, has identified two chemotypes of *A. umbelliformis*. A multidisciplinary approach based on the combination of metabolome- and genome-derived product analysis enabled the unequivocal chemical and biomolecular fingerprinting of these two chemotypes. Combined "omics" approaches are becoming a useful tool not only for basic science but also for industrial plant characterization. Owing to the commercial relevance of *A. umbelliformis* and to the regulatory issues related to the presence of thujones, the identification of a RsaI and Taq sites can be used for a rapid and precise chemotype identification of the plant chemotypes, complementing the chemical analysis of the essential oil and the sesquiterpene lactones.

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369 Bertea and M.E. Maffei are indebted with Centre of Excellence CEBIOVEM of the University of
370 Turin.

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FIGURES CAPTIONS

Figure 1 - Structures of the sesquiterpene lactones identified in both chemotypes of *A.umbelliformis* Lam. **1**: 5-desoxy-5hydroperoxy-5-epitelekin; **2**: 5-desoxy-5-hydroperoxytelekin; **3**: umbellifolide; **4**: costunolide; **5a**: verlotorin; **5b**: artemorine; **6a**: santamarine; **6b**: reynosine; **7**: genepolide

Figure 2 - PCA scatterplot of the cumulative elaboration of both EOs and headspaces sampled by HS-SPME of both *Artemisia umbelliformis* chemotypes. Capital letters: EO GC analysis, lower-case letters: HS-SPME GC analysis.

Figure 3 - HPLC-DAD-UV profiles at 210 nm of *Artemisia umbelliformis* chemotypes Au1 and Au2.

Figure 4 - PCR products derived from all possible combinations of Au1 and Au2 specific primers designed on the coding and non-transcribing regions of the plant 5S-rRNA gene. Lane 1, a single fragment of approximately 224 bp is produced by Au1. Lane 2, a single fragment of about 327 bp is produced by Au2. Lane 3, a single fragment of 204 bp in length amplified using the primer AuF in combination with the primer 5S-P2 in the chemotype Au1. Lane 4, a single fragment of 307 bp in length using the primer AuF in combination with the primer 5S-P2 in the chemotype Au2. Lane 5, a single fragments of 231 bp with primers 5S-P1 and Au2R1 in the chemotype Au2. Lane 6, a single fragments of 275 bp with primers 5S-P1 and Au2R2 in the chemotype Au2. Lane 7, a single fragments of 64 bp with primers Au2F1 and Au2R1 in the chemotype Au2. Lane 8, a single fragments of 108 bp with primers Au2F1 and Au2R1 in the chemotype Au2. Lane 9, a single fragments of 213 bp with primers AuF and Au2R1 in the chemotype Au2. Lane 10, a single fragments of and 255 bp with primers AuF and Au2R2 in the chemotype Au2. Lane 11, purified PCR products obtained by using 5S-P1 and 5S-P2 primers

digested with Rsa I give two major fragments of 123 bp and 82 bp respectively, and a minor fragment of 19 bp not visible in the gel because out of the resolution capacity of the instrument in the chemotype Au1. Lane 12, RsaI cleaved Au2 5S-rRNA spacer region gives a major fragment of 283 bp and two minor fragments of 25 bp (barely visible in the gel) and 19 bp (not visible in the gel). Lane 13, PCR products from the chemotype Au1 not digested by Taq I. Lane 14, purified PCR products from the chemotype Au2 digested using Taq I producing two fragments of 197 and 130 bp.

Figure 5 - Aligments of the nucleotide sequences of 5S-rRNA gene spacer region of *Artemisia umbelliformis* chemotypes Au1 and Au2. Universal primer sequences are indicated in squared solid boxes. *Artemisia umbelliformis* forward primers are indicated in bold. Identical sequences are indicated by (*). Gaps (-) are introduced for the best alignment. RsaI site is evidenced in the squared large dot box, whereas the TaqI site is evidenced in the squared small dots box. Forward and reverse specific primers of the Au2 chemotype are indicated.

Figure 6 - Position of the universal primers (5S-P1 and 5S-P2) flanking the spacer region of 5S-rRNA gene and specific *Artemisia umbelliformis* forward (AuF) and *A. umbelliformis* chemotype Au2 forward (Au2F1) and reverse (AuR1 and AuR2) specific primers used for PCR amplification of the 5S-rRNA spacer region.

Table 1 - Components Characterising the Essential Oils of *Artemisia umbelliformis* Chemotypes Au1 and Au2.

				Au1		**Au2**	
Compounds	**MEGA5**[a]	**MEGA5**[b]	**CW**	**Mean**	**Range**	**Mean**	**Range**
α-Thujene	930	929	1038	0.05	tr-0.10	0.13	tr-0.57
α-Pinene	936	936	1031	0.26	tr-0.40	0.60	0.33-1.20
Camphene	954	951	953	0.06	tr-0.30	0.43	tr-1.10
Sabinene	975	975	1132	0.35	0.09-1.40	0.15	tr-0.36
β-Pinene	979	978	1117	1.74	0.10-2.30	5.12	1.73-11.50
β-Myrcene	991	993	1174	0.18	tr-0.30	0.68	0.10-2.90
α-Terpinene	1017	1017	1189	0.18	tr-0.30	0.34	0.10-0.55
p-Cymene	1025	1026	1280	0.27	0.10-0.40	0.21	0.10-0.70
1,8-Cineole	1031	1033	1213	4.86	0.30-9.50	7.58	4.05-14.00
γ-Terpinene	1060	1061	1257	0.35	0.10-0.50	0.75	0.43-1.00
cis-Sabinene hydrate	1070	1069	1268	1.17	0.30-1.50	7.16	2.70-20.08
α-Terpinolene	1089	1089	1292	0.10	tr-0.10	0.21	0.10-0.30
trans-Sabinene hydrate	1098	1097	1488	0.65	0.30-1.00	2.19	1.30-3.35
α-Thujone	1102	1108	1420	36.99	29.70-51.90	0.37	tr-2.00
β-Thujone	1114	1118	1446	9.11	4.60-19.53	0.27	tr-0.65
Sabinol	1142	1141	1708	0.90	0.28-4.10	0.20	0.10-0.50
Camphor	1143	1145	1516	0.15	tr-1.54	0.75	tr-1.70
Borneol	1169	1168	1712	1.14	tr-3.90	11.58	0.09-19.43
Terpinen-4-ol	1177	1177	1619	1.21	0.40-1.48	4.75	3.30-8.15
α-Terpineol	1189	1190	1714	0.29	tr-0.50	1.08	0.40-2.20
Bornyl acetate	1289	1286	1378	0.05	tr-0.10	0.51	tr-1.97
α-Terpinyl acetate	1349	1351	1494	0.58	tr-1.10	2.30	0.70-4.19
Sabinyl isobutirrate	1416[c]	1416	1494	1.35	1.00-3.83	0.01	tr-0.09
β-Caryophyllene	1419	1418	1594	0.40	tr-0.56	2.24	1.00-4.60
Sabinyl isovalerianate	1503[c]	1503	1577	3.94	3.10-7.13	0.16	tr-0.50
Sabinyl valerianate	1516[c]	1516	1605	2.69	1.30-7.28	0.14	tr-0.30
Caryophyllene oxide	1583	1581	1965	1.89	0.57-3.30	3.61	2.15-5.40
Neryl isovalerianate	1584	1585	1679	4.11	1.08-6.90	4.72	1.40-10.00

a: LRI *from Adams library (18)y;*
b: experimental LRI
c: LRI from standards synthesized in the authors' laboratory (2)
Mean and range values are expressed as percent areas normalized vs. nonane as internal standard.

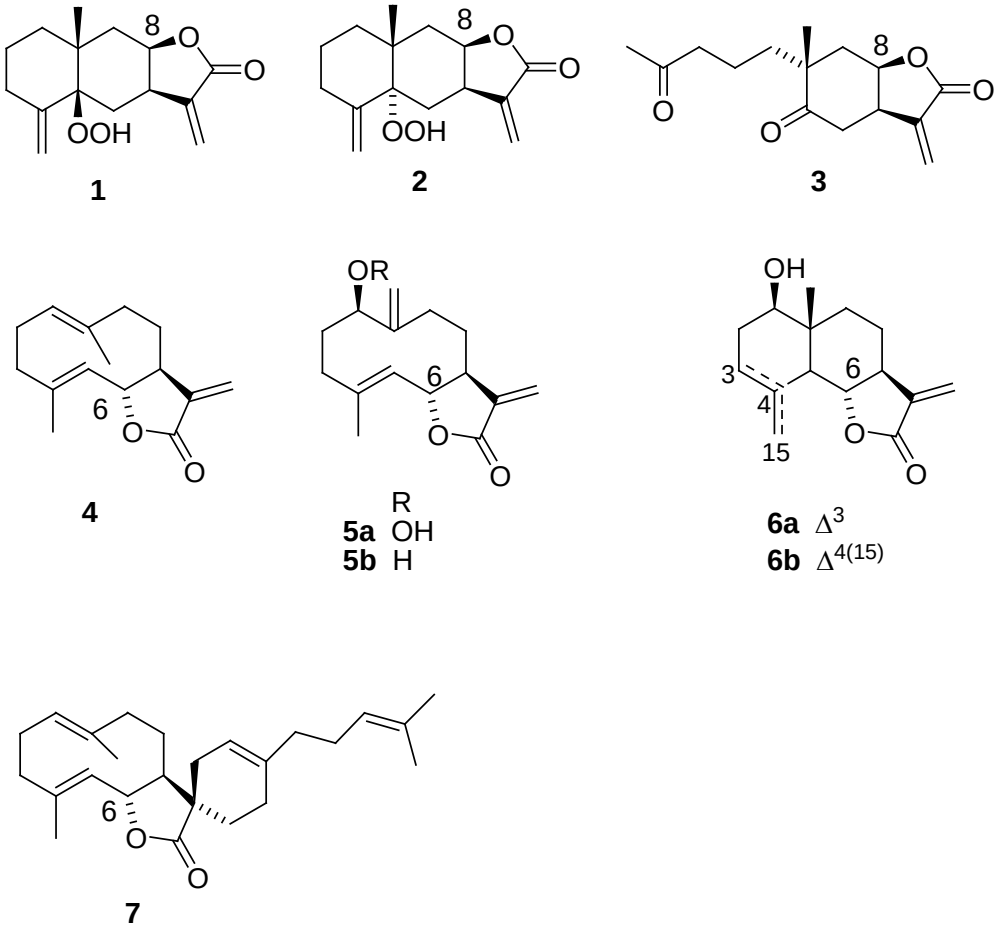
Table 2 – Mean and Range Amount of α- + β- thujone Expressed as g/100g of Essential Oil in the Investigated Samples of *Artemisia umbelliformis* Chemotypes Au1 and Au2.

	n	Range	Mean	SD
Chemotype AU1	26	18.0-57.3	36.8	14.7
Chemotype AU2	10	0.2-0.4	0.3	0.1

α-thujone ($Y= 0.3105x + 3.311$, $R^2 = 0.9995$)

β-thujone ($Y = 0.2896x + 0.1380$; $R^2 = 0.9994$)

Figure 1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OR
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5a OH
5b H
OH
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6a Δ3
6b Δ4(15)
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7

Figure 2

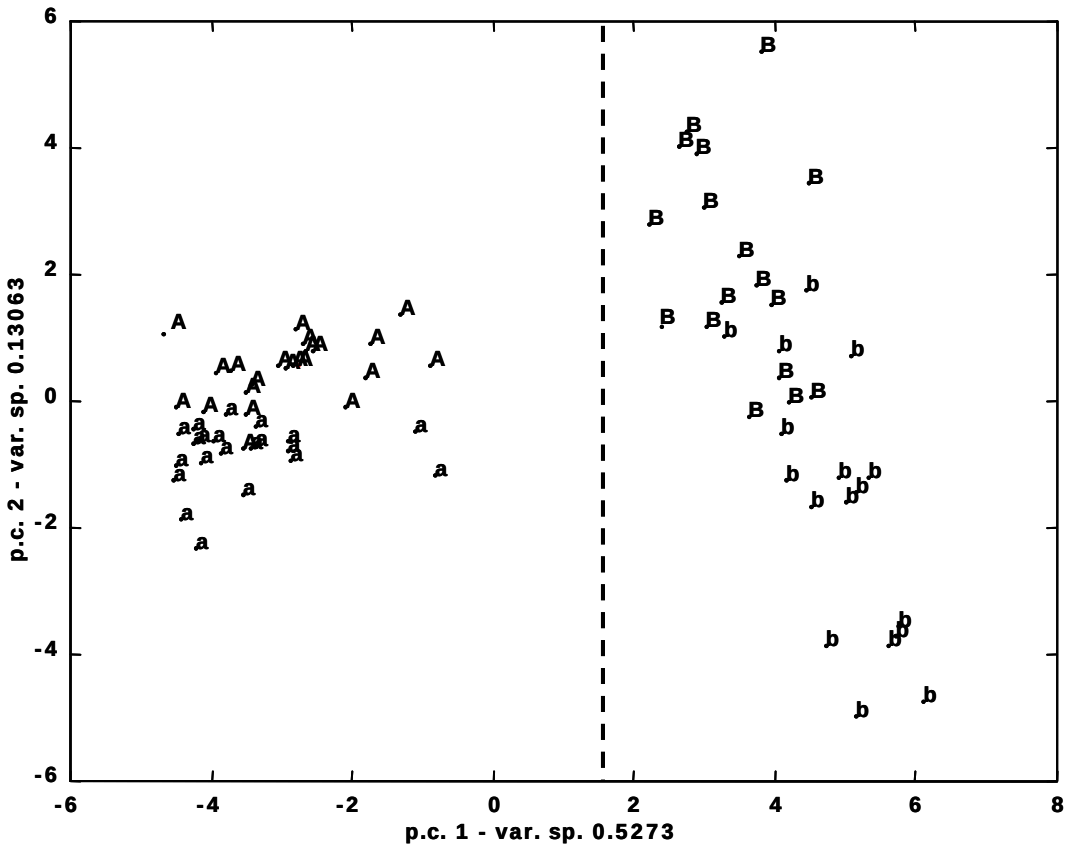
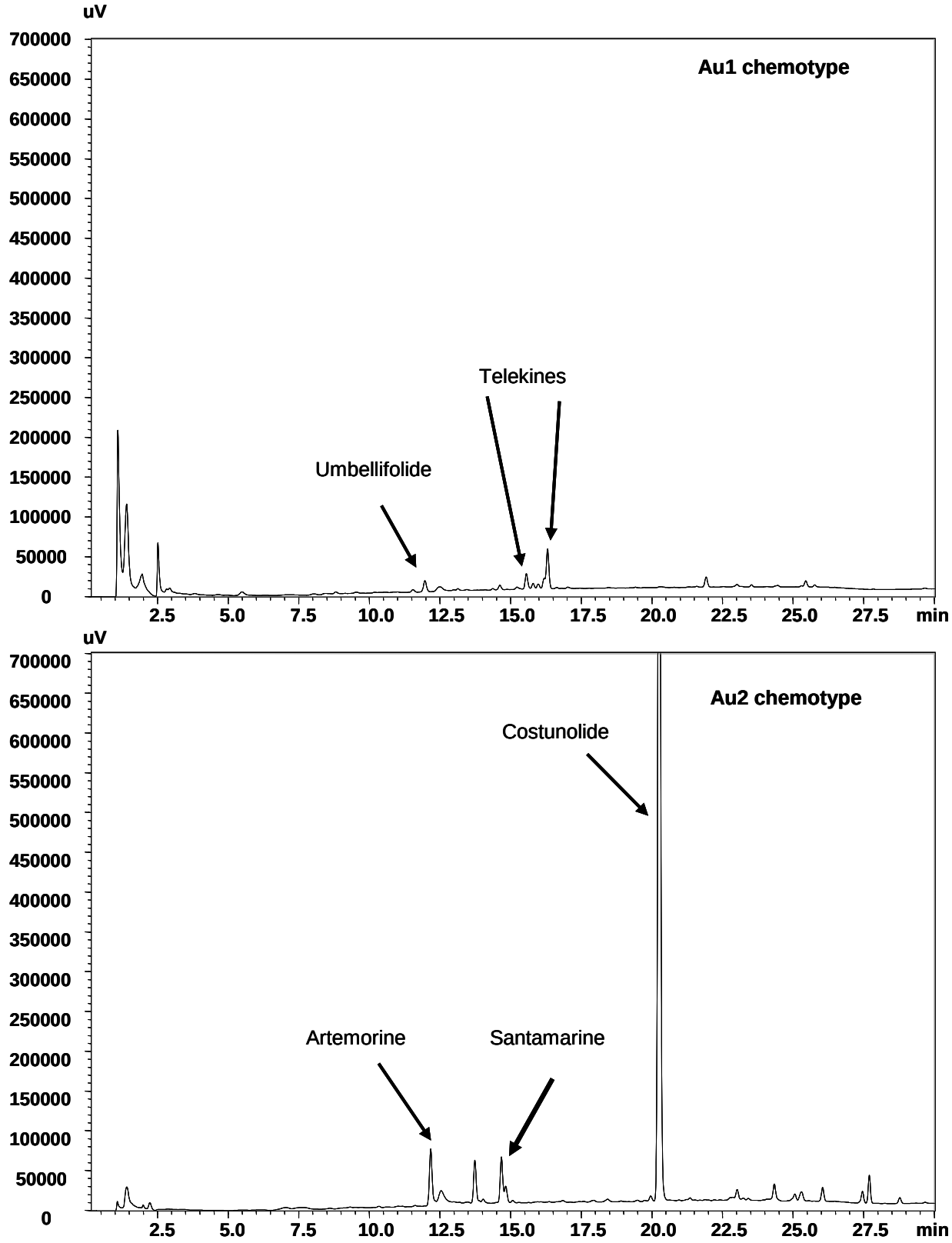


Figure 3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Au1 chemotype
Telekines
Umbellifolide
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
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min
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Au2 chemotype
Costunolide
Artemorine
Santamarine

Figure 4

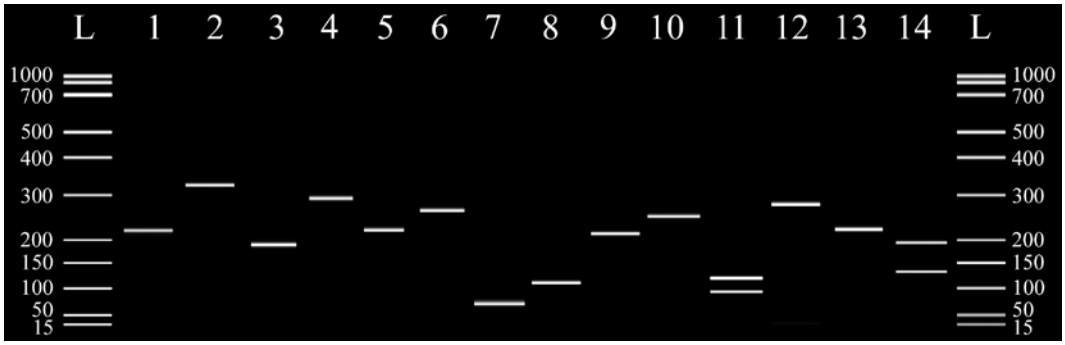
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Figure 5

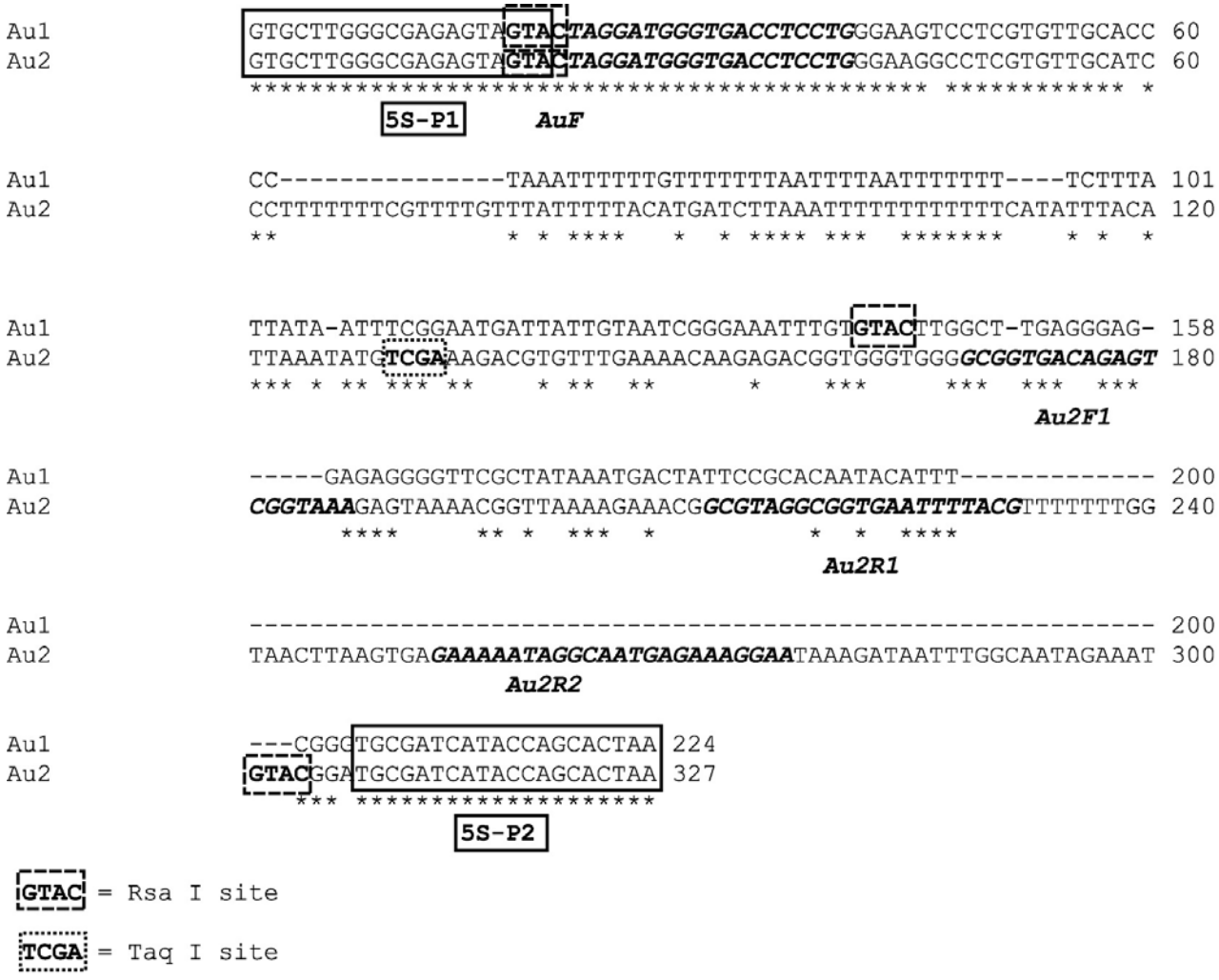
Au1 GTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAAGTCCTCGTGTTGCACC 60
Au2 GTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAAGGCCTCGTGTTGCATC 60
5S-P1
AuF
Au1 CC---------------TAAATTTTTTGTTTTTTTAATTTTAATTTTTTT----TCTTTA 101
Au2 CCTTTTTTTCGTTTTGTTTATTTTTACATGATCTTAAATTTTTTTTTTTTCATATTTACA 120
Au1 TTATA-ATTTCGGAATGATTATTGTAATCGGGAAATTTGTGTACTTGGCT-TGAGGGAG- 158
Au2 TTAAATATGTCGAAAGACGTGTTTGAAAACAAGAGACGGTGGGTGGGGCGGTGACAGAGT 180
Au2F1
Au1 -----GAGAGGGGTTCGCTATAAATGACTATTCCGCACAATACATTT------------ 200
Au2 CGGTAAAGAGTAAAACGGTTAAAAGAAACGGCGTAGGCGGTGAATTTTACGTTTTTTTGG 240
Au2R1
Au1 ---------- 200
Au2 TAACTTAAGTGAGAAAAATAGGCAATGAGAAAGGAATAAAGATAATTTGGCAATAGAAAT 300
Au2R2
Au1 ---CGGGTGCGATCATACCAGCACTAA 224
Au2 GTACGGATGCGATCATACCAGCACTAA 327
5S-P2
GTAC = Rsa I site
TCGA = Taq I site

Figure 6