The *CYP450* Multigene Family of *Fontainea* and Insights Into Diterpenoid Synthesis

Shahida Mitu

University of the Sunshine Coast

Steven Ogbourne

University of the Sunshine Coast

Anne Klein

University of the Sunshine Coast

Trong Tran

University of the Sunshine Coast

Paul Reddell

EcoBiotics Ltd

Scott Cummins (✉ scummins@usc.edu.au)

University of the Sunshine Coast

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Abstract

Background: Cytochrome P450s (CYP450s) are enzymes that play critical roles in the biosynthesis of physiologically important compounds across all organisms. Although they have been characterised in a large number of plant species, no information relating to these enzymes are available from the genus *Fontainea* (family Euphorbiaceae). *Fontainea* is significant as the genus includes species that produce medicinally significant epoxy-tigliane natural products, one of which has been approved as an anti-cancer therapeutic.

Results: A metabolome analysis showed that *Fontainea* species possess a chemical profile different from various other plant species. The diversity and expression profiles of *Fontainea* CYP450s were investigated from leaf and root tissue. A total of 103 and 123 full-length *CYP450* genes in *Fontainea picrosperma* and *Fontainea venosa*, respectively (and a further 127/125 partial-length) that were phylogenetically classified into clans, families and subfamilies. The majority of *CYP450* identified are most active within root tissue (66.2% *F. picrosperma*, 65.0% *F. venosa*). *Fontainea CYP450* associated with diterpenoid biosynthesis were classified into 2 subfamilies (CYP71D and CYP726A), of which CYP726A1, CYP726A2 and CYP71D1 appear to be exclusive to *Fontainea* species, and significantly more highly expressed in root tissue compared to leaf tissue.

Conclusion: This study presents a comprehensive overview of the *CYP450* gene family in *Fontainea* that may provide important insights into the biosynthesis of the medicinally significant epoxy-tigliane diterpenes found within the genus.

Background

Diterpenes, also known as diterpenoids or isoprenoids, are a structurally diverse class of small molecules that are widespread throughout the plant kingdom. Diterpenes exhibit many and varied biological activities and consequently there is significant commercial interest in their potential applications as pharmaceuticals, food products, and industrial and agricultural chemicals [1–5]. Tigilanol tiglate (TT), a novel epoxy-tigliane diterpene ester extracted from the fruit of *Fontainea picrosperma* (family Euphorbiaceae) [3, 6], is of particular current interest due to its effectiveness as a local treatment for a range of cancers in humans and companion animals [3, 7–9]. Recently, TT was approved by regulatory authorities in Europe and the USA as a veterinary pharmaceutical for the treatment of non-metastatic canine mast cell tumours. TT cannot be synthesised on a commercial scale and instead is manufactured by purification from the fruit of *F. picrosperma* [10]. The natural biosynthetic pathway leading to the biosynthesis of tigliane esters such as TT is currently unknown, yet given the important role cytochrome P450s (CYP450s) play in the biosynthetic pathways of both primary and secondary metabolite production [11], it is likely that they are critical to the biosynthesis of TT and epoxy-tiglanes more generally.

CYP450s are widely distributed in eukaryotes, where they form a large and diverse class of enzymes consisting of more than 35,000 members [12, 13] and play vital roles in biosynthesis of natural products, degradation of xenobiotics, biosynthesis of steroid hormones, drug metabolism and synthesis of secondary metabolites [11, 12]. They catalyse reactions in biosynthetic pathways of many compounds such as alkaloids, flavonoids, lignans, isoprenoids, phenolics, antioxidants and phenylpropanoid [14, 15]. In most plant species, root tissue has relatively higher levels of CYP450 gene expression compared to leaf tissue [16, 17]. CYP450 have relatively low sequence identity among different organisms (such as plants and animals) [18, 19]. In plants, the CYP450

superfamily is one of the largest gene families of enzyme proteins. For instance, the *CYP450* gene family is the third largest gene family present in *Arabidopsis* [20]. At present, 5100 plant CYP450s have been annotated and clustered into two different categories (A type and non-A-Type) and 11 different clans [21]. The A-type CYP450 enzymes are grouped as the CYP71 clan, whereas the non-A type are subdivided into 10 clans - CYP51, CYP72, CYP74, CYP85, CYP86, CYP97, CYP710, CYP711, CYP727, and CYP746 [14, 15, 19] according to the standard nomenclature system [22]. The CYP71 clan includes more than 50% of all plant CYP450s [23, 24] and phylogenetic analysis of diterpenoid CYP450 members reveals that CYP71 and CYP726 are important for the biosynthesis of diterpenoids in the Euphorbiaceae family [25].

CYP450 in plants are membrane bound [26] that primarily localize to the endoplasmic reticulum, chloroplast or mitochondria and other secretory organelles [20]. The molecular mass of plant CYP450s range from 45 to 62 kDa with an average of 55 kDa in molecular mass [27]. They have four conserved key domains: a heme-binding domain, I-helix, K-helix and PERF/W domain [27]. The heme-binding signature motif has 10 conserved residues, among which cysteine is highly conserved, while the heme-iron motif has a binding site for oxygen, which is a requirement for transferring electrons to their substrate [28].

To date, there are no reports of *CYP450* genes in *Fontainea*, yet the next-generation sequencing (NGS) approach provides the ideal tool towards their elucidation in this genus. *Fontainea picrosperma* and *Fontainea venosa* are closely related species that presumably produce similar arrays of natural products, including epoxy-tigliane diterpenes. In this study, we report the general metabolomic profiles of these two *Fontainea* species, and compared to non-*Fontainea* species. Towards better understanding these similarities and differences, we used NGS transcriptomics to elucidate the *Fontainea CYP450* family and their relative gene expression in leaf and root tissue, with particular focus on those predicted to be involved in diterpenoid synthesis.

Results

Metabolomic analysis and CYP450 identification

Metabolomic analysis of leaf tissue from *F. picrosperma*, *F. venosa* and 4 other non-*Fontainea* plant species provided a total of 49,098 mass spectral ions extracted from the LC-MS dataset. A partial least square-discriminant analysis (PLS-DA) was performed to analyse the chemodiversity among samples. The PLS-DA model with three components accounting for 20.8%, 25.0% and 15.8% of the total variance showed that *F. picrosperma* and *F. venosa* were notably separated from other species (Fig. 1A). This untargeted metabolomic analysis indicated that *Fontainea* species were considerably more closely related from a chemical perspective compared to two Euphorbiaceae (*Manihot esculenta*, *Ricinus communis*) and two non-Euphorbiaceae (*Arabidopsis thaliana*, *Solanum lycopersicum*) plants.

To investigate if chemodiversity is correlated with CYP450 diversity, the *F. picrosperma* and *F. venosa* CYP450 were initially identified through NGS, *de novo* reference assembly and gene ontology. Transcriptome libraries were constructed from combined root and leaf tissue for both *F. picrosperma* and *F. venosa* plants using Illumina HiSeq 2500 trimmed reads (150 - 200 bp). In total, 12 Gb and 30 Gb raw reads were generated for *F. picrosperma* and *F. venosa* libraries, respectively, which were assembled into 192,639 (N50 1,450 bp) and 246,608 (N50 1,248 bp) contigs. From these reference *Fontainea* transcriptomes, we identified 103 and 123 full-length *CYP450* genes (and 127 and 125 partial-length) in *F. picrosperma* and *F. venosa*, respectively, all of

which contained the conserved cytochrome P450 domain. The 4 different recognised CYP450 motif/regions (I-helix, K-helix, PERF and heme-binding) are presented with noted conservation (Fig. 1B). A comparative CYP450 sequence identity analysis of the same 6 species used in metabolomic analysis demonstrated that *Fontainea* share considerably more CYP450 homologs (48% of CYP450) compared to other species of Euphorbiaceae and non- Euphorbiaceae (Fig. 1C). The non- Euphorbiaceae species of *A. thaliana* and *S. lycopersicum* shared no CYP450 homologs with *F. picrosperma* or *F. venosa*.

F. picrosperma CYP450 genes were classified into 10 clans, within which there were 37 families and 45 subfamilies (Table 1). In *F. venosa*, CYP450 genes were classified into 9 clans, containing 37 families and 67 subfamilies (Table 2). The general characteristics of *Fontainea* full-length CYP450 proteins were also investigated, including the amino acid (aa) length, molecular weight, isoelectric point (pI) and presence of secretory signal peptide. Length varied from 300-618 aa in *F. picrosperma* and 301-632 aa in *F. venosa*, with molecular weights ranging from ~27-71 kDa. The pI values ranged from 5.22 - 9.8 in *F. picrosperma* and 5.29- 9.91 in *F. venosa*. We also calculated their instability index (II) and found that 18 *F. picrosperma* and 52 *F. venosa* CYP450 were stable (stability factor < 40). The GRAVY values were negative for all CYP450 proteins, indicating them to be hydrophilic. In *F. picrosperma*, 68 CYP450 were predicted to have a secondary pathway signal peptide, whereas only 5 sequences (FpCYP74B2, FpCYP707A2, FpCYP707A3, FpCYP707A4 and FpCYP88A1) contained chloroplast-targeting peptides. In *F. venosa*, 78 CYP450 had pathway signal peptides and 3 (FvCYP707A1, FvCYP707A2 and FvCYP707A4) had chloroplast-targeting signal peptides. There were no CYP450 with mitochondrial-targeting signal peptides.

Phylogenetic and putative functional analysis of CYP450

A phylogenetic analysis containing 1042 CYP450s from 6 species (*F. picrosperma*, *F. venosa*, *R. communis*, *M. esculenta*, *A. thaliana* and *S. lycopersicum*) confirmed that the majority of *F. picrosperma* and *F. venosa* CYP450 do not show substantial relatedness to CYP450 from species outside of the Euphorbiaceae family (Additional Fig. 1). There were 72 CYP450 genes exclusive to *Fontainea* (identity >92%). A *Fontainea*-specific CYP450 phylogeny showed that in *F. picrosperma*, 16 CYP85 were assigned into 7 families that form a single clade, while the CYP72 clan contained 17 genes assigned to 3 families (Fig. 2A). In *F. venosa*, 14 genes were assigned into 7 families that formed a single clade for CYP85. In the CYP72 clade, 17 CYP450 clustered into 4 families. A single CYP450 was represented in clan CYP710 (FpCYP710A1, FvCYP710A1) and CYP51 (FpCYP51G, FvCYP51G), which are phylogenetically most related to CYP85.

The majority of *F. picrosperma* CYP450 belong to the CYP71 clan (57 genes; 57.68%), followed by the CYP72 and CYP85 clans, which is also known as A-type (Fig. 2B). The CYP71 clan is responsible for alkaloid, sesquiterpenoid, cyclic terpenoid and flavonoid biosynthesis. The majority of *F. venosa* CYP450 also belong to the CYP71 clan (68 genes; 55.28%), followed by the CYP72 and CYP85 clans (Fig. 2B). The non-A type encompass the remaining 46 CYP450, which belong to 9 CYP450 clans and 21 families in *F. picrosperma*. In *F. venosa*, there were 55 non-A type CYP450, which belong to 8 clans and 19 families. Of note, representative CYP450 from CYP711 were absent from *F. venosa*, while CYP97 were more well represented in *F. venosa* compared to *F. picrosperma*.

Comparative expression of CYP450 genes between *Fontainea* species

Overall *CYP450* expression patterns demonstrated that, in both species, root tissue had higher activity (66.2% *F. picrosperma*, 65.0% *F. venosa*), compared to leaf tissue (Fig. 3A). An expression profile heatmap of the 72 homologous *CYP450* genes, common to both species of *Fontainea*, showed that the majority of genes were more active in root tissue (44.4%) compared to leaf tissue (25.0%) (Fig. 3B). In *F. picrosperma*, there were 6 *CYP450* genes exclusively expressed in leaf tissue (CYP82C7, CYP81T1, CYP89A3, CYP73A1, CYP94A1 and CYP727B1) and 6 exclusive to the root tissue (CYP90D1-D2, CYP78A5, CYP71A1-A2, CYP707A4). Also in *F. picrosperma*, 8 *CYP450* were significantly more highly expressed in root tissue, 6 genes were more highly expressed in leaf issue. In *F. venosa*, 8 exclusive to leaf tissue (CYP90D1-D2, CYP734A1, CYP78A5, CYP71A1-A2, CYP707A4 and CYP716E1) and 4 *CYP450* genes were exclusively expressed in root tissue (CYP82C7, CYP89A3, CYP73A1 and CYP81T1) (Fig. 3B). Only 2 *CYP450* genes were significantly more highly expressed in leaf tissue and one gene significantly more highly expressed in root tissue.

***CYP450* genes involved in diterpenoid metabolism**

Phylogenetic analysis of the *Fontainea* *CYP450* classified within clans CYP71D and CYP726A were relatively closely related to those found in other Euphorbiaceae species (*Jatropha curcus*, *Euphorbia peplus*, *Euphorbia latex* and *R. communis*), confirming their position within diterpenoid *CYP450* subfamilies (Fig. 4A). This was additionally supported by conserved motif analysis. Of the 4 *F. picrosperma* CYP726A, FpCYP726A4 was most divergent. A single CYP726A was identified in *F. venosa* (FvCYP726A1), which formed a clade with FpCYP726A1-A2. Three of the *Fontainea* diterpenoid *CYP450* were present in both *F. picrosperma* and *F. venosa* but not in other Euphorbiaceae.

To confirm tissue expression of the common *Fontainea* diterpenoid *CYP450* genes CYP726A1, CYP726A2 and CYP71D1, 12 biological samples of *F. picrosperma* were quantitatively analysed by mapping RNA-seq to the reference transcriptome. These data confirmed that gene expression was significantly higher in root tissue compared to leaf tissue (Fig. 4B). The housekeeping genes, glyceraldehyde-3-P dehydrogenase (GAPC) and elongation factor 1-alpha (EF1α) showed consistency in both leaf and root tissues and higher expression in root tissues compared to leaf tissue [29].

Discussion

Cytochrome P450s are evolutionarily conserved enzymes that are involved in the catalysis of numerous reactions, required for growth, development, defence [30] and secondary metabolism [31]. Prior to this study, no *CYP450* genes had been identified, let alone characterized, in any species of the genus *Fontainea*. This is significant as *CYP450* genes are likely to be important for future understanding of the biosynthetic pathways that produce medicinally significant diterpene esters, such as TT, which are unique to *Fontainea*. Towards that aim, we have identified and classified putative full-length *CYP450* encoding genes in two species of *Fontainea*, *F. picrosperma* and *F. venosa*. Phylogenetic analysis allowed us to identify groups of genes for further evaluation. Moreover, their expression profiles in leaf and root tissues were investigated, with a particular focus on the *CYP450* genes linked with diterpenoid biosynthesis, potentially involved in the production of TT.

We report 103 and 123 full-length *CYP450* genes from *F. picrosperma* and *F. venosa*, respectively, that were classified into clans, which cumulatively consisted of 37 families and 67 subfamilies that fit into conformed plant-derived functions, most prominently with diterpenoid, flavonoid and other functions. An ortholog

comparison showed that CYP450 genes of *Fontainea* species are largely unique when compared to other plant species of both Euphorbiaceae and non-Euphorbiaceae. In support of our metabolomics analysis, the *S. lycopersicum* and *A. thaliana* CYP450 showed low overall similarity to *Fontainea* species. This may be attributed to the unique biosynthesis of diterpenoid derivatives that are phorbol ester-specific, found in *Fontainea* and other members of the Euphorbiaceae family.

The total number of *Fontainea* CYP450 identified in this study was consistent with that found in other plant transcriptomics CYP450 research, including the total number of full-length sequences, clans, families and subfamilies [16, 32, 33]. For example, transcriptomic studies allowed the elucidation of 151 full-length CYP450 genes in *Lonicera japonica* [32], 118 full-length in *Taxus chinensis* [33] and 116 full-length in *Salvia miltiorrhiza* [16]. However, in *S. miltiorrhiza*, the tissues used for transcriptomics included leaves, roots and flowers, while in *L. japonica*, flower and buds were used. Our study identified 127 and 125 partial-length CYP450 gene from *F. picrosperma* and *F. venosa*, respectively. To obtain the full-length sequence, additional RNA-seq from the stem, flower and fruit would be helpful, as well as from different stages of development. In addition, this could be complemented by genome sequencing.

If a genome is available for a species, it does provide an alternate mechanism for CYP450 gene identification, through genome-wide interrogation. In *A. thaliana*, this approach identified 246 genes that clustered into 9 clans and 47 families [21, 34], while a much larger number were identified from the soybean (*G. max*) and rice (*Japonica*) genomes, containing 332 and 355 CYP450 genes, respectively [35]. Far fewer were present in the legume (*Medicago truncatula*), where 151 putative CYP450 genes were identified, including 135 novel CYP450 [36]. We expect that once a genome is available for *Fontainea*, a more complete list of full-length CYP450 genes will be established.

Our results using these predictive protein characterisation analyses (i.e. molecular weight, cell localization, function) were in line with prior studies of CYP450 proteins. CYP450s are typically anchored on the surface of the endoplasmic reticulum [37] and some may target to the plastids or mitochondria [38]. It is common that animal CYPs are anchored to mitochondria, but there is no report of any plant CYP with mitochondrial localization [36] except maize, where 3 CYPs have been reported (*Zea mays*) [39]. In our study of *F. picrosperma* or *F. venosa*, no deduced CYP450 proteins were predicted to have mitochondrial targeting peptides. CYP74A1, CYP74B1 and CYP74B2 in *F. picrosperma* and *F. venosa* and CYP726A4 and CYP71B12 in *F. picrosperma* were found in the chloroplast. In other plants, such as *Triticum araraticum*, *Z. mays* all members of CYP74 and CYP701 were targeted to chloroplast [39].

The diversity of different CYP450s between *F. picrosperma* and *F. venosa*, and other species, likely contributes to the observed differences in their chemical profiles. In all plant species that have been researched to date, the largest CYP450 clan is CYP71 [22]. The families and subfamilies within the clan have diverged remarkably during plant evolution, many of which are known to be involved in secondary metabolite biosynthesis of flavonoids and alkaloids [40]. Similarly, the CYP71 family is the largest CYP450 clan in *Fontainea* (see Fig. 2). On the contrary, two CYP711 representatives were identified from *F. picrosperma*, but were absent in *F. venosa*, although CYP711 have been described in other plant species [24]. In our phylogenetic tree, the CYP74 clan is adjacent to CYP711 family, suggesting that *Fontainea* CYP711 may also function within the metabolism of oxylipins and strigolactone signals [24], as strigolactones have been identified as branching inhibition

hormones in plants, and several CYP711 have been experimentally confirmed as strigolactones biosynthetic enzymes [41, 42]. We additionally found that *F. venosa* had more *CYP97* genes compared to *F. picrosperma*; the CYP97 clan is involved in the hydroxylation of carotenoids [43]. Carotenoids are a group of widely distributed pigments derived from the ubiquitous isoprenoid biosynthetic pathway and play diverse roles in plant primary and secondary metabolism. Carotenoids contain two pigments, carotene and lutein, which absorb and transfer energy to protect chlorophyll [34]. We speculate that this may partially explain why *F. venosa* have darker leaves compared to *F. picrosperma*.

We found that *Fontainea* (*F. picrosperma* and *F. venosa*) *CYP450* genes were more actively expressed in root tissue compared to leaf tissue (see Fig. 3). Among those significantly more highly expressed in the root have been associated with fertility reduction (CYP78A), UV stress tolerance (CYP84A), gibberellin metabolism (CYP714A) and jasmonic acid metabolism (CYP74A) [44–47]. Those significantly more highly expressed in the leaf include those previously associated with catalysing successive oxidation steps of the plant hormone jasmonoyl-isoleucine for catabolic turnover (CYP94), expression of ABA 8'-hydroxylase and affects ABA levels to control seed dormancy (CYP707A), hydroxylation of carotenoids (CYP97), biosynthesis of castasterone in the brassinosteroid biosynthetic pathway (CYP85A) and glucosinolate metabolism (CYP83) [37, 43, 48–50]. Some species variation existed in CYP450 homolog tissue expression (see Fig. 3B). This may be explained by the different growth and developmental stage of plants from which the tissue was sampled, as CYP450s are involved in the regulation of plant hormone metabolism, growth and development and hormones are involved in formation and development of flowers, leaves, stems and fruits [51].

Diterpenoids are one of the most widespread classes of secondary metabolites in higher plants, which are synthesized from basic isoprene units (C_5H_8) and further modified by various oxidoreductases, acyltransferases, dehydrogenases and glucosyltransferases [52]. CYP450-dependent oxidative modification is essential for the biosynthesis of diterpenes [52]. There are countless products formed in plants, among them diterpenoids are one of the most diverse groups, consisting of more than 12,000 metabolites [53] that have proven to be valuable as therapeutic drugs. According to previous research, CYP71D and CYP726A subfamilies are key CYP450s involved in diterpenoid biosynthesis [25, 53] and most of these diterpenoids can only be found in plants [54]. Our phylogenetic analysis of diterpenoid CYP71 clan members revealed that *Fontainea* have representatives within two different diterpenoid subfamilies, namely CYP71D and CYP726A.

F. picrosperma diterpenoid *CYP450* genes are significantly more highly expressed in root tissue compared to leaf tissue. The expression of genes can be affected by the developmental stage of plants, environmental conditions, seasonal and diurnal effects as well as biotic and abiotic stress [55]. Therefore, future research should explore the expression of the identified *CYP450* genes under these different scenarios and in additional tissues. In other Euphorbiaceae, diterpenoid genes were found to be co-regulated in rhizome and hairy roots [17] or highly expressed in root tissue compared to leaf and flower [16]. Nonetheless, *Fontainea* CYP71D and CYP726A genes are excellent candidates for involvement in diterpenoid biosynthesis pathways, in particular, the biosynthesis of epoxy-tigliane diterpene esters, which are only found in species of *Fontainea*, although further experiments are required to confirm this hypothesis. Their identification allows for experimental analysis of their function, for example, *in vitro* expression of the proteins followed by activity detection, or by knock-in and knock-out can be completed. This may be followed by activity detection *in vivo*, depending on the availability of a robust experimental system. Also, the analysis of high TT producing *F. picrosperma*, compared

with low producers, will provide guidance about CYP450 (and other genes) that potentially regulate TT production.

Conclusions

This research represents an important first step in understanding the role of *CYP450* genes involved in the biosynthesis of diterpene esters found only in species of *Fontainea*. A metabolome analysis showed that *Fontainea* species possess a chemical profile different from other plant species. This could at least partially be explained by the diversity of unique *CYP450* found in *Fontainea*. Further intra-genus chemical variation could also be due to variation in *CYP450* between the two *Fontainea* species investigated, including the diterpenoid *CYP450* genes. Our study showed that the majority of *Fontainea CYP450* genes identified are more active in the root tissue compare to leaf tissue. The root-derived *F. picrosperma* diterpenoid *CYP450* genes identified in this study (i.e. CYP71D1, CYP726A1 and CYP726A2) are strong candidates as key enzymes in the biosynthesis of medicinally significant diterpene esters of the epoxy-tigliane class.

Materials And Methods

Metabolome analysis

Fresh mature leaves of *F. picrosperma*, *F. venosa*, *M. esculenta*, *R. communis*, *S. lycopersicum* and *A. thaliana* were collected from at least 2 individual plants. The leaves were cleaned and placed over silica-gel in zip-lock bags for 2 weeks in dark at room temperature. From 0.1 g of dried ground leaves, 3 mL of methanol was added. Ultrasound extraction was carried out for 1 min at room temperature prior to filtration onto a pre-weighed 20 mL borosilicate glass scintillation vial. The residue was extracted again with 3 mL of methanol in the same conditions. The methanol in the filtrate was evaporated using a Genevac centrifugal EZ-2 evaporator. The dried residue was then reconstituted in methanol to prepare a solution at 1 mg/mL and stored at -20°C in dark. Separation was performed on an ExionLC uHPLC system (Shimadzu) equipped with a Kinetex C₁₈ column (2.1 x 150 mm, 100 Å). The column compartment was maintained at 40°C and the auto-sampler was kept at 15°C. The mobile phase was composed of water with 0.1% formic acid (v/v) (solvent A) and acetonitrile with 0.1% formic acid (v/v) (solvent B), and was run at a flow rate of 0.5 mL/min. The linear gradient started at 2% solvent B for 0.1 min, increased to 100% solvent B for 14.5 min and then was kept at this level for 1.4 min. Starting conditions were achieved in 0.5 min and re-equilibrated for 3.5 min, resulting in a total uHPLC run time of 20 min. The injection volume was 5 µL. The X500R QTOF mass spectrometer (Sciex) equipped with an ESI source in a positive mode was controlled by the Sciex OS software. The curtain gas, gas 1, gas 2 were set at 25, 40 and 50 psi, respectively. The spray voltage, declustering potential and collision energy were applied at 5500, 100 and 35 V, respectively. Spectral data was recorded in the mass range of *m/z* 100-1500 Da. The MS ions were extracted from LC-MS data using MZmine 2 (version 2.53) with setting parameters including retention time 0.6-17.5 min, retention time tolerance of 0.1 min, mass range *m/z* 100-1,500 Da, mass tolerance of 0.02 Da, a noise threshold of 100. The pre-processed peak table data matrix was submitted onto a web-based service MetaboAnalyst (<https://www.metaboanalyst.ca/>), for metabolomic data analysis.

Plant tissue collection for transcriptomics

F. picrosperma and *F. venosa* seedlings were provided by EcoBiotics Ltd and plants were grown in the University of the Sunshine Coast (Sippy Downs) greenhouse. Plants were grown in independent pots and kept in the greenhouse at ambient temperature and humidity according to Mitu et al [29]. The plants were used from 2 - 4 years old and healthy, fully expanded leaves and actively growing root tips, including the apical meristem and root caps were dissected (single leaf from each plant) and (1 cm² root) from plants of each species and preserved following the procedure described by Mitu et al. [29].

RNA isolation

Total RNA was isolated from ~100 mg of leaf and root tissue of 12 individual *F. picrosperma* and 3 individual *F. venosa* using the Qiagen mini plant kit (Hilden, Germany), according to the manufacturer's protocol. The initial yield and purity of RNA were measured using a Nanodrop spectrophotometer 2000c (Thermo Scientific, Waltham, MA, USA) at 260 and 280 nm and agarose gel electrophoresis. An Agilent Bioanalyzer 2100 (Agilent Technologies, USA) was used to analyse the RNA integrity number (RIN). High-quality total RNA (RIN >7) was provided to Novogene (Beijing, China) for cDNA synthesis (cDNA Rapid Library Preparation Kit, Roche, Mannheim, Germany) and paired-end Illumina HiSeq 2500 sequencing (Illumina, San Diego, CA, USA).

De novo assembly and functional annotation

Two reference transcriptome libraries were prepared from two individual plant samples of *F. picrosperma* and *F. venosa* leaf and root tissue. Quality of raw reads of each library were checked separately using FastQC [56] and Trimmomatic [57]. Trimmed reads of the different RNA-seq libraries for *F. picrosperma* and *F. venosa* were merged separately prior to assembly using Trinity [58], which applies a *de novo* reconstruction method. Quality of the assembly was assessed using the built-in Trinity Perl script to generate an N50 value. Alignment coverage rate was calculated using the program Bowtie [59] with a cut-off set at 70%. Following assembly, Transdecoder was used to predict open reading frames (ORFs) with default parameter of minimum 100 amino acids ORF length. Sequence datasets can be found in the NCBI (NCBI; www.ncbi.nlm.nih.gov), Sequence Read Archive (SRA) database under the accession number PRJNA687112. The assembled protein sequences were used as queries against NR protein database (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (KEGG Ortholog database) and GO (Gene Ontology) to identify putative protein functions using the BLAST algorithm with an E-value cut-off of 10⁻⁵ [60].

Classification and characterization of *Fontainea* CYP450 genes

An integrated HMM-search and InterProScan-verification approach was applied to identify the putative *CYP450* gene families in *Fontainea* species. The CYP450 family HMM model was used through HMMER3 [61]. The filtered sequences were further blasted using NCBI (<https://www.ncbi.nlm.nih.gov/>) with the cut-off E-value of 10⁻⁵. Sequences annotated as CYP450 members were collected. The full-length CYP450 proteins were identified manually using nucleotide sequences in ExPASy [62] based on Chen et al. [16] and after annotation those sequences that did not match with any plant species were discarded. After filtering, the coding sequences of the resultant subjects were retrieved. Finally, results from the two methods were integrated and corrected manually. A BLAST search of *F. picrosperma* and *F. venosa* CYP450 genes against 814 previously

identified *CYP450* genes from 4 different plant species (*A. thaliana*, *R. communis*, *M. esculenta*, *S. lycopersicum*) was performed for *CYP450* gene classification.

All full-length *CYP450* genes were named according to the standard *CYP450* nomenclature [22]. Briefly, 40%, 55% and 97% sequence identities were used as cut-offs for family, subfamily and allelic variants, respectively. According to a previous study [51], functions of *CYP450* clans were identified. We also calculated their instability index (II) using ExPASy tool (<https://web.expasy.org/protparam/>). Theoretical iso-electric points (PI) and molecular weight (kDa) were used to assess the physicochemical properties of putative *CYP450*s for each full-length *CYP450* protein, as predicted by the ExPASy tool (<http://www.expasy.org/tools/>). The GRAVY values were calculated using GRAVY calculator (<http://www.gravy-calculator.de/>). The cellular locations were predicted using the TargetP1.1 server with specificity >0.95 (<http://www.cbs.dtu.dk/services/TargetP/>). Furthermore, *CYP450* motifs were confirmed by Multiple Expectation Maximization for Motif Elicitation (MEME) program with the motif number set to 10 and all other parameters were default (<http://alternate.memesuite.org/>) [63]. *CYP450* protein sequences were collected from *F. picrosperma*, *F. venosa* and other species that have appropriate genomic data available: *A. thaliana*, *R. communis*, *M. esculenta*, *S. lycopersicum*. *F. picrosperma*. A pairwise BLASTp comparison was performed using Geneious R11 11.0.2 with default parameters.

Phylogenetic analysis of *CYP450*

One hundred and three (103) full-length genes in *F. picrosperma* and 123 genes in *F. venosa* were used for phylogenetic representation in *Fontainea* species. Sequence alignment was performed using Geneious 11.02 software performing ClustalW alignment. The phylogenetic tree was constructed using FastTree and maximum likelihood (ML) algorithm. The statistical bootstrap support of each branch was assessed by re-sampling the amino acid positions 1,000 times. The maximum likelihood phylogenetic tree and evolutionary analyses were carried out using iTOL web server (<https://itol.embl.de/>) [64]. For conserved domain identification, multiple sequence alignment of full-length *Fontainea* species protein sequences were carried out using ClustalX program using default parameters [65]. The alignment file was submitted to Web Logo generator software for generating the logo of conserved domains available at (<http://weblogo.berkeley.edu/>) [66].

Relative *CYP450* gene expression

Total RNA was extracted from leaf and root tissue of 3 different *F. picrosperma* and *F. venosa* plants using an RNAeasy plant extraction kit from QIAGEN® (Hilden, Germany) according to Mitu et al. [29]. The expression levels of *CYP450* genes were calculated using the CLC genomic 11.01 software package following default parameters. Raw counts for RNA-sequencing data of *F. picrosperma* and *F. venosa* genes were normalized to Transcripts Per Million (TPM). Levels of expression were represented as the log2 ratio of transcript abundance between leaf and root tissues. Next, we generated a z-score for sequencing depth normalized reads counts. Expression of each enzyme in leaf and root tissue was analysed by normal clustering. Relative expression profiles of *CYP450* genes were presented in the form of a heatmap, which was constructed using z-score with Clustvis (<https://biit.cs.ut.ee/clustvis/>) [67], using default parameters and a hierarchical clustering analysis to assess biological sample relatedness. We also identified homolog sequences in both *Fontainea* species (percentage of identity 92%). Another heatmap was constructed with z-score of average TPM value of 3 different plant of each species. To determine the statistically significant differences between leaf and root

tissue of homolog sequences, we used Microsoft Excel software 2013 to conduct Student's *t*-test. Values are reported as average z-score from three different plant of *Fontainea* species. Significant differences: $p < 0.05$.

Phylogenetic and quantitative analysis of diterpenoid *CYP450*

Previously identified diterpenoid *CYP450s* genes from members of the Euphorbiaceae family were acquired from NCBI (NCBI; www.ncbi.nlm.nih.gov), then used to identify *Fontainea* diterpenoid *CYP450s* genes, by homology. Identified genes were used to construct a phylogeny tree using Geneious 11.02 software following ClustalW alignment. The phylogenetic tree was constructed using the maximum likelihood method, as described above. For quantitative analysis, RNA-seq from leaf and root tissue from 12 *F. picrosperma* individuals were used. The high-quality cleaned reads were aligned to the *F. picrosperma* reference transcriptomes using CLC genomic workbench 11.01 following default settings. To identify their gene expression values, TPMs value was calculated, as described above and a bar graph was created with their average TPM value. Diterpenoid genes showing statistically significant differential expression between root and leaf tissue of *F. picrosperma* were determined by Student's *t*-test using Microsoft Excel software 2013. Values were reported as average TPM value from 12 different plant of *F. picrosperma*. Significant differences: $p < 0.05$ and $p < 0.01$.

Abbreviations

CYP450: Cytochrome P450

EF1 α : Elongation factor 1-alpha

kDa: kilodalton

GAPC: Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic

GRAVY: Grand average of hydropathy value

TT: Tigilanol tiglate

TPM: Transcripts Per Million

Declarations

Availability of data and materials

The raw sequence data from this study have been deposited in the publicly accessible NCBI Sequence Read Archive (SRA) database as accession number PRJNA687112. All data generated and used in this article is included as Additional Figures 1-3 and Additional Files 1-2.

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Author Contributions

SAM performed the experiments, analysed the data, wrote the paper, prepared figures and/or tables. AHK helped in transcriptome analysis and TDT conducted the metabolomics analysis. The project was conceptualized by SMO, PWR and SFC. All authors contributed to drafting the manuscript and approval of the submitted version.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

SAM, AHK, SFC and TDT declare no competing interests. PWR is a shareholder and Executive Director of EcoBiotics Ltd and QBiotics Group. SMO is a shareholder and Non-Executive Director of QBiotics Group.

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Tables

Table 1.

List of full-length *Fontainea picrosperma* CYP450s identified in this study. Cellular location of the protein predicted using the TargetP program. 'C': chloroplast; 'S': secreted; '*': unknown and '-' not secreted.

Gene name	Length	Type	Family	Subfamily	Instability index (ii)	Clan	PI	Mol. Wt (kDa)	Loc
FpCYP727B1	360	Non-A	CYP727	CYP727B	33.54	CYP727	8.26	40.4	-
FpCYP97C1	552	Non-A	CYP97	CYP97C	33.6	CYP97	7.57	61.92	*
FpCYP94A1	499	Non-A	CYP94	CYP94A	42.39	CYP86	6.79	56.97	S
FpCYP86A1	545	Non-A	CYP86	CYP86A	45.8		8.4	62.07	S
FpCYP86A2*	528	Non-A	CYP86	CYP86A	46.42		7.55	60.38	S
FpCYP96A1	334	Non-A	CYP96	CYP96A	48.86		8.75	39.16	S
FpCYP711A1*	393	Non-A	CYP711	CYP711A	32.09		6.1	44.82	S
FpCYP711A2*	529	Non-A	CYP711	CYP711A	35.1	CYP72	8.72	60.64	S
FpCYP714A1	535	Non-A	CYP714	CYP714A	41.82		9.14	61.25	S
FpCYP714A2	426	Non-A	CYP714	CYP714A	37.63		9.12	48.55	*
FpCYP714A3	353	Non-A	CYP714	CYP714A	40.39		8.68	40.66	-
FpCYP714A4*	529	Non-A	CYP714	CYP714A	39.97		9.08	60.3	S
FpCYP709F*	519	Non-A	CYP709	CYP709F	32.92		7.93	59.96	S
FpCYP709F1*	423	Non-A	CYP709	CYP709F	41.98		8.93	48.35	-
FpCYP709F2*	523	Non-A	CYP709	CYP709F	41.16		9.44	59.31	S
FpCYP734A1	520	Non-A	CYP734	CYP734A	44.48		8.81	59.34	S
FpCYP734A3*	510	Non-A	CYP734	CYP734A	35.42		8.29	58.52	S
FpCYP721A1	368	Non-A	CYP721	CYP721A	44.48		9.28	42.38	S

FpCYP721A2	499	Non-A	CYP721	CYP721A	39.12		9.24	57.06	S
FpCYP721A3	429	Non-A	CYP721	CYP721A	45.73		8.45	49.11	S
FpCYP721A5	334	Non-A	CYP721	CYP721A	40.28		8.68	38.73	S
FpCYP721A6*	512	Non-A	CYP721	CYP721A	40.94		9.2	58.25	S
FpCYP721A7*	332	Non-A	CYP721	CYP721A	43.55		7.07	38.84	-
FpCYP72A1	440	Non-A	CYP72	CYP72A	42.58		9.2	50.01	S
FpCYP72A2*	405	Non-A	CYP72	CYP72A	39.66		9.58	46.29	S
FpCYP51G	485	Non-A	CYP51	CYP51G	36.21	CYP51	6.96	55.62	*
FpCYP710A1	504	Non-A	CYP710	CYP710A	41.32	CYP710	9.13	57.69	S
FpCYP74A1	521	Non-A	CYP74	CYP74B	51.19	CYP74	8.74	58.38	C
FpCYP74B1	496	Non-A	CYP74	CYP74B	51.66		8.55	55.67	C
FpCYP74B2	492	Non-A	CYP74	CYP74B	51.86		8.13	55.25	C
FpCYP707A2	440	Non-A	CYP707	CYP707A	40.71	CYP85	9.12	50.5	S
FpCYP707A3	470	Non-A	CYP707	CYP707A	43.31		9.23	53.69	S
FpCYP707A4	489	Non-A	CYP707	CYP707A	41.31		9.04	56.05	*
FpCYP88A1	479	Non-A	CYP88	CYP88A	40.55		9.06	54.7	S
FpCYP88A2	482	Non-A	CYP88	CYP88A	40.16		8.65	55.99	S
FpCYP88A3	489	Non-A	CYP88	CYP88A	44.99		9.15	56.9	S
FpCYP88A4*	446	Non-A	CYP88	CYP88A	44.33		8.92	51.07	S
FpCYP733A1*	324	Non-A	CYP733	CYP733A	34.08		8.52	36.53	S
FpCYP733A2*	476	Non-A	CYP733	CYP733A	39.44		9.11	54.74	S

FpCYP733A3*	414	Non-A	CYP733	CYP733A	40.75		9.28	47.7	*
FpCYP728D1*	326	Non-A	CYP728	CYP728D	40.59		9.75	37.57	*
FpCYP728D2	476	Non-A	CYP728	CYP728D	42.7		9.43	54.78	*
FpCYP716E1	410	Non-A	CYP716	CYP716E	35.24		9.07	46.71	*
FpCYP90D1	514	Non-A	CYP90	CYP90D	50.04		9.57	58.06	*
FpCYP90B1	494	Non-A	CYP90	CYP90B	46.45		8.87	56.62	S
FpCYP85A1	464	Non-A	CYP85	CYP85A	40.53		9.25	53.25	S
FpCYP93B1	512	A	CYP93	CYP93B	30.25	CYP71	8.72	59.62	S
FpCYP93D1	514	A	CYP93	CYP93D	35.84		6.52	57.99	S
FpCYP712C1	534	A	CYP712	CYP712C	36.06		8.21	60.27	*
FpCYP82C2*	362	A	CYP82	CYP82C	38.11		9.02	41.89	*
FpCYP82C3*	528	A	CYP82	CYP82C	36.1		8.66	60.29	*
FpCYP82J1*	520	A	CYP82	CYP82J	35.58		6.97	58.78	S
FpCYP82C5	320	A	CYP82	CYP82C	23.68		5.64	35.19	*
FpCYP82D1*	306	A	CYP82	CYP82D	34.39		9.8	27.7	S
FpCYP82D3*	407	A	CYP82	CYP82D	41.37		6.24	46.64	*
FpCYP82C6*	519	A	CYP82	CYP82C	38.41		8.1	59.04	S
FpCYP82C7	310	A	CYP82	CYP82C	47.75		9.33	35	*
FpCYP82C8	510	A	CYP82	CYP82C	43.49		8.51	57.69	*
FpCYP76D1	517	A	CYP76	CYP76D	50.71		8.75	58.09	S
FpCYP81T1	308	A	CYP81	CYP81T	42.27		9.52	35.93	S
FpCYP81T2	496	A	CYP81	CYP81K	43.06		8.73	56.37	S
FpCYP81K1	505	A	CYP81	CYP81K	46.38		7.15	57.23	S
FpCYP81K2	336	A	CYP81	CYP81K	52.28		9.17	38.76	S
FpCYP76D2	498	A	CYP76	CYP76D	49.8		8.94	57.4	S
FpCYP76D3*	316	A	CYP76	CYP76D	50.45		7.63	36.54	S
FpCYP76D4*	499	A	CYP76	CYP76D	52.65		8.26	57.6	S

FpCYP81S1	507	A	CYP81	CYP81S	53.39	8.17	58.4	S
FpCYP706C1	517	A	CYP706	CYP706C	37.2	7.21	57.32	S
FpCYP76C1*	310	A	CYP76	CYP76C	39.16	5.22	35.76	*
FpCYP76C2	495	A	CYP76	CYP76C	37.54	6.62	56.57	S
FpCYP76C3	304	A	CYP76	CYP76C	45.99	8.81	34.15	S
FpCYP76C4	309	A	CYP76	CYP76C	42.45	7.58	34.74	S
FpCYP76C5*	373	A	CYP76	CYP76C	42.04	9.27	42.75	S
FpCYP76D5*	300	A	CYP76	CYP76D	40.34	9.02	27.22	S
FpCYP76D6*	504	A	CYP76	CYP76D	42.06	7.92	57.97	S
FpCYP92A1	444	A	CYP92	CYP92D	28.16	5.84	50.71	*
FpCYP84A1*	405	A	CYP84	CYP84A	43.34	7.06	46.46	S
FpCYP84A2	513	A	CYP84	CYP84A	45.24	6.36	58.83	S
FpCYP71A1	498	A	CYP71	CYP71A	37.17	8.52	56.03	*
FpCYP71A2	431	A	CYP71	CYP71A	35.72	6.37	49.77	S
FpCYP71A3	498	A	CYP71	CYP71A	37.17	8.52	56.03	*
FpCYP83	496	A	CYP83		31.08	8.92	56.1	S
FpCYP71B3*	464	A	CYP71	CYP71B	46.97	9	53.29	*
FpCYP71B4*	513	A	CYP71	CYP71B	45.36	8.59	58.78	S
FpCYP71B5*	311	A	CYP71	CYP71B	40.63	9.14	35.62	S
FpCYP71B6	329	A	CYP71	CYP71B	46.63	9.45	37.42	S
FpCYP71D1	497	A	CYP71	CYP71D	40.04	8.51	56.89	S
FpCYP71B10*	519	A	CYP71	CYP71B	40.55	8.57	59.1	S
FpCYP726A1	501	A	CYP726	CYP726A	38.85	8.66	56.38	S
FpCYP726A2	498	A	CYP726	CYP726A	38.84	8.72	56.11	S
FpCYP726A3*	512	A	CYP726	CYP726A	39.99	8.58	58.16	S
FpCYP726A4*	614	A	CYP726	CYP726A	41.58	6.56	68.87	C
FpCYP71D3*	395	A	CYP71	CYP71D	46.46	9.26	44.82	S
FpCYP71B12*	342	A	CYP71	CYP71B	36.6	9.12	37.94	C
FpCYP78A3	528	A	CYP78	CYP78A	28.71	9.11	59.95	*
FpCYP78A4	509	A	CYP78	CYP78A	39.21	8.89	58.15	S
FpCYP78A5	535	A	CYP78	CYP78A	39.98	6.3	60.07	*

FpCYP73A1	505	A	CYP73	CYP73A	44.78	9.1	58.36	S
FpCYP701A1	408	A	CYP701	CYP701A	42.79	6.71	46.77	*
FpCYP77A1	502	A	CYP77	CYP77A	39.35	8.96	57.61	S
FpCYP89A1	508	A	CYP89	CYP89A	52.37	8.51	58.45	S
FpCYP89A2	517	A	CYP89	CYP89A	52.46	7.69	59.06	S
FpCYP89A3	534	A	CYP89	CYP89A	60.59	8.91	61.38	*

Table 2.

List of full-length *Fontainea venosa* CYP450s identified in this study. Cellular location of the protein predicted using the TargetP program. 'C': chloroplast; 'S': secreted; '*': unknown and '-' not secreted.

Gene name	Length	Type	Family	Subfamily	Instability index (ii)	Clan	PI	Mol. Wt (kDa)	Loc
FvCYP97B1*	391	Non-A	CYP97	CYP97B	46.12	CYP97	6.63	44.46	-
FvCYP97B2*	587	Non-A	CYP97	CYP97B	43.71		8.04	66.16	*
FvCYP97A*	632	Non-A	CYP97	CYP97A	43.34		6.1	70.76	*
FvCYP97A1*	419	Non-A	CYP97	CYP97A	46.66		5.29	47.55	*
FvCYP97A2*	441	Non-A	CYP97	CYP97A	41.18		6.2	50.47	*
FvCYP97C1	566	Non-A	CYP97	CYP97C	32.16		8.24	63.23	*
FvCYP704A1*	520	Non-A	CYP704	CYP704A	41.27	CYP86	6.32	60.1	S
FvCYP704A2*	457	Non-A	CYP704	CYP704A	41.94		5.43	53.17	S
FvCYP704A3*	390	Non-A	CYP704	CYP704A	41.62		8.48	44.12	*
FvCYP704A4*	516	Non-A	CYP704	CYP704A	39.07		8.18	59.67	S
FvCYP94D1*	503	Non-A	CYP94	CYP94D	38		7	58.19	S
FvCYP94A1	499	Non-A	CYP94	CYP94A	40.16		8.18	56.37	S
FvCYP94C1*	452	Non-A	CYP94	CYP94C	47.09	CYP86	9.42	51.82	S
FvCYP94B1*	514	Non-A	CYP94	CYP94B	42.88		8.95	59.52	S
FvCYP86A1	545	Non-A	CYP86	CYP86A	45.31		8.65	62.02	S
FvCYP86B1*	550	Non-A	CYP86	CYP86B	41.66		8.74	63.41	-
FvCYP96A1	333	Non-A	CYP96	CYP96A	49.51		8.26	39.24	S
FvCYP727B1	550	Non-A	CYP72	CYP727B	33.13	CYP727	8.7	62.33	-

FvCYP727B2	548	Non-A	CYP72	CYP727B	32.14		8.24	62.36	-
FvCYP714E1*	382	Non-A	CYP714	CYP714E	38.03	CYP72	9.32	58.57	S
FvCYP714E2*	515	Non-A	CYP714	CYP714E	35.26		9.2	43.72	S
FvCYP714A1	549	Non-A	CYP714	CYP714A	39.3		9.28	62.67	S
FvCYP714A5*	512	Non-A	CYP714	CYP714A	36.34		9.36	58.8	S
FvCYP734A1	520	Non-A	CYP734	CYP734A	45.3		8.49	59.34	S
FvCYP734A2	349	Non-A	CYP734	CYP734A	46.25		6.02	39.75	*
FvCYP734A4*	360	Non-A	CYP734	CYP734A	38.02		8.74	40.24	*
FvCYP734A5*	526	Non-A	CYP734	CYP734A	41.86		9.59	60.09	S
FvCYP721A1	499	Non-A	CYP721	CYP721A	43.06		9.14	57.1	S
FvCYP721A3	512	Non-A	CYP721	CYP721A	42.85		9.14	58.1	S
FvCYP721A4	403	Non-A	CYP721	CYP721A	45.12		9.17	46.32	S
FvCYP721A5	335	Non-A	CYP721	CYP721A	42.39		8.48	38.76	*
FvCYP721A8*	369	Non-A	CYP72	CYP721A	48.81		9.61	42.9	S
FvCYP721A9*	311	Non-A	CYP72	CYP721A	51.09		7.7	36.23	S
FvCYP72D1*	519	Non-A	CYP72	CYP72D	32.21		8.02	59.24	S
FvCYP72D2*	355	Non-A	CYP72	CYP72D	38.3		5.53	40.77	-
FvCYP72A1	505	Non-A	CYP72	CYP72A	45.33		9.21	58.87	S
FvCYP51G	485	Non-A	CYP51	CYP51G	36.63	CYP51	7.72	55.74	S
FvCYP710A1	411	Non-A	CYP710	CYP710A	41.13	CYP710	8.68	46.98	*
FvCYP74A1	517	Non-A	CYP74	CYP74A	48.45	CYP74	8.07	57.45	C

FvCYP74B1	496	Non-A	CYP74	CYP74B	51.46		8.91	55.81	C
FvCYP74B2	492	Non-A	CYP74	CYP74B	50.35		7.63	55.2	C
FvCYP707A1*	347	Non-A	CYP707	CYP707A	30.45	CYP85	9.27	39.89	S
FvCYP707A2	553	Non-A	CYP707	CYP707A	43.04		9.23	63.52	*
FvCYP707A4	463	Non-A	CYP707	CYP707A	40.45		9.05	52.43	S
FvCYP88A1	479	Non-A	CYP88	CYP88A	40.52		9.09	54.67	S
FvCYP88A3	490	Non-A	CYP88	CYP88A	41.57		9.05	56.76	S
FvCYP85A1	464	Non-A	CYP85	CYP85A	41.83		9.23	53.3	S
FvCYP722A1*	476	Non-A	CYP722	CYP722A	41.48		9.13	54.67	S
FvCYP728D2	478	Non-A	CYP728	CYP728D	41.32		9.46	54.05	*
FvCYP716E1	410	Non-A	CYP716	CYP716E	38.77		8.92	47.87	*
FvCYP716C1*	403	Non-A	CYP716	CYP716C	44.65		8.92	46.21	*
FvCYP90A1*	473	Non-A	CYP90	CYP90A	41.04		9.18	54.75	*
FvCYP90D1	547	Non-A	CYP90	CYP90D	47.54		9.55	63.9	*
FvCYP90D2	360	Non-A	CYP90	CYP90D	40.74		9.09	41.2	*
FvCYP90B1	494	Non-A	CYP90	CYP90B	47.54		8.86	56.73	S
FvCYP79D1*	524	A	CYP79	CYP79D	37.27	CYP71	9.22	59.35	S
FvCYP79D2*	526	A	CYP79	CYP79D	37.18		5.49	35.34	S
FvCYP93B1	512	A	CYP93	CYP93B	30.31		8.61	58.18	S
FvCYP93D1	514	A	CYP93	CYP93D	36.8		6.12	57.95	S
FvCYP93A1*	515	A	CYP93	CYP93A	35.37		6.39	58.08	S
FvCYP712C1	503	A	CYP712	CYP712C	30.73		7.98	56.85	S
FvCYP712E1*	349	A	CYP712	CYP712E	42.4		5.58	40.66	*

FvCYP82C4*	520	A	CYP82	CYP82C	38.44	8.83	59.86	S
FvCYP82J2*	360	A	CYP82	CYP82J	34.04	9.29	40.5	S
FvCYP82J3*	455	A	CYP82	CYP82J	38.56	6.74	51.89	-
FvCYP82C5	319	A	CYP82	CYP82C	21.55	5.95	35.37	*
FvCYP82D2*	320	A	CYP82	CYP82D	43.94	6.61	36.06	*
FvCYP82C7	514	A	CYP82	CYP82C	49.83	7.61	58.84	S
FvCYP76D1	517	A	CYP76	CYP76D	52.75	8.62	58.98	S
FvCYP81T1	307	A	CYP81	CYP81T	45.32	9.52	35.76	S
FvCYP81T2	496	A	CYP81	CYP81T	47.83	8.85	56.28	S
FvCYP81D1*	501	A	CYP81	CYP81D	47.78	8.9	57.91	*
FvCYP81D2*	514	A	CYP81	CYP81D	45.65	8.6	59.53	S
FvCYP81K1	505	A	CYP81	CYP81K	44.71	8.22	57.46	S
FvCYP81K2	322	A	CYP81	CYP81K	51.8	9.13	37.8	S
FvCYP76D2	498	A	CYP76	CYP76D	48.92	8.39	56.22	S
FvCYP81S1	504	A	CYP81	CYP81S	54.93	8.34	57.07	S
FvCYP81S2*	306	A	CYP81	CYP81S	52.02	9.05	35.19	S
FvCYP706C1	517	A	CYP706	CYP706C	37.72	8.37	57.22	S
FvCYP76H1*	503	A	CYP76	CYP76H	38.56	8.69	57.06	S
FvCYP76I1*	509	A	CYP76	CYP76I	30.36	9.11	58.12	S
FvCYP76C2	495	A	CYP76	CYP76C	41.51	6.88	56.79	S
FvCYP76D8*	493	A	CYP76	CYP76D	41.85	6.16	55	S
FvCYP76D7*	494	A	CYP76	CYP76D	37.17	6.19	55.01	S
FvCYP92A1	330	A	CYP92	CYP92A	26.99	9.22	37.7	*
FvCYP84A2	499	A	CYP84	CYP84A	45.27	6.07	56	*
FvCYP71B11*	526	A	CYP71	CYP71B	38.06	8.53	60.37	S
FvCYP71B2*	342	A	CYP71	CYP71B	45.86	8.64	39.28	S
FvCYP71*	516	A	CYP71		36.06	6.83	59.34	*
FvCYP71AU1*	311	A	CYP71	CYP71AU	29.54	6.97	35.94	S
FvCYP71AU2*	500	A	CYP71	CYP71AU	32.65	6.15	56.48	S
FvCYP71A1	499	A	CYP71	CYP71A	35.27	8.03	57.42	*
FvCYP83B1*	498	A	CYP83	CYP83B	42.49	9.07	57.05	S

FvCYP83B2*	497	A	CYP83	CYP83B	39.52	8.65	57.59	S
FvCYP83	339	A	CYP83		34.22	9.91	38.67	S
FvCYP71C1*	506	A	CYP71	CYP71C	36.92	7.65	57.95	S
FvCYP71C2*	305	A	CYP71	CYP71C	31.29	8.26	34.31	S
FvCYP71AP*	511	A	CYP71	CYP71AP	47.75	5.92	58.97	S
FvCYP71B12*	301	A	CYP71	CYP71B	43.28	9.69	27.62	S
FvCYP71B13*	506	A	CYP71	CYP71B	39.73	9.04	58.87	S
FvCYP71B6	349	A	CYP71	CYP71B	47.68	9.56	40.18	S
FvCYP71D1	498	A	CYP71	CYP71D	40	8.32	56.18	S
FvCYP71B7*	514	A	CYP71	CYP71B	43.43	8.73	58.25	S
FvCYP71B8*	320	A	CYP71	CYP71B	49.73	9.22	36.59	S
FvCYP71B9*	343	A	CYP71	CYP71B	37.29	6.28	38.62	S
FvCYP726A1	501	A	CYP726	CYP726A	37.98	8.8	56.33	*
FvCYP71D2*	503	A	CYP71	CYP71D	43.37	7.71	56.75	S
FvCYP71B11*	548	A	CYP71	CYP71B	38.06	8.53	60.37	*
FvCYP98A1*	508	A	CYP98	CYP98A	39.14	7.77	57.96	S
FvCYP78A1*	538	A	CYP78	CYP78A	29.08	8.38	60.6	S
FvCYP78A2*	468	A	CYP78	CYP78A	39.04	8.93	52.54	-
FvCYP78A3	528	A	CYP78	CYP78A	29.5	8.93	59.96	*
FvCYP78A4	514	A	CYP78	CYP78A	37.91	9.16	58.96	*
FvCYP78A5	534	A	CYP78	CYP78A	38.41	6.26	60.03	S
FvCYP73A1	505	A	CYP73	CYP73A	45.67	9.11	58.38	*
FvCYP701A1	514	A	CYP701	CYP701A	43.82	7.62	58.51	S
FvCYP701A2	408	A	CYP701	CYP701A	45.2	6.15	46.67	*
FvCYP77B1*	505	A	CYP77	CYP77B	40	7.53	57.06	*
FvCYP77A1	502	A	CYP77	CYP77A	37.73	9.29	57.89	S
FvCYP89A1	514	A	CYP89	CYP89A	51.56	8.51	59.23	S
FvCYP89A2	338	A	CYP89	CYP89A	49.41	5.64	39.59	S
FvCYP89A3	520	A	CYP89	CYP89A	54.72	8.78	59.54	*
FvCYP89A4	517	A	CYP89	CYP89A	49.02	8.77	59.14	*

Figures

Figure 1

Comparative metabolome and CYP450 analysis in *Fontainea* and 4 other non-*Fontainea* plant species (A) Partial least square-discriminant analysis (PLS-DA) derived from leaf tissue of six different plants according to their metabolome. (B) A-type and non A-type conserved motifs in *Fontainea* species CYP450 represented by weblogo. Motif names are shown above each logo. (C) Venn diagram showing CYP450 homology matches (>90% identity) across the 6 plant species after pairwise comparison.

Figure 2

Phylogenetic and putative functional analysis of CYP450 in *Fontainea picrosperma* and *Fontainea venosa*, including their clans. (A) Maximum likelihood tree constructed using FastTree in Geneious. Asterisks represent CYP450 unique to each *Fontainea* species (percentage of identity 92%). The branch length represents genetic distance and the value on the branch is the support rate. (B) Distribution of CYP450 in *F. picrosperma* and *F. venosa* based on clans. CYP71, alkaloid, sesquiterpenoids, cyclic terpenoid and flavonoid synthesis; CYP74, synthesis of oxylipin derivatives and allene oxide in the octadecanoid and jasmonate pathways; CYP51, regulates the synthesis of sterols and triterpenes; CYP85, modification of cyclic terpenes and sterols in the brassinosteroid (BR), abscisic acid (ABA) and gibberellin (GA) pathways; CYP97, hydroxylation of carotenoids; CYP72, catabolism of isoprenoid hormones CYP711 are identified as a strigolactones biosynthetic enzyme. The functions of CYP727 are currently unknown.

Figure 3

Fontainea CYP450 expression analysis. (A) Expression profiles of 103 and 123 full-length CYP450 genes in Fontainea picrosperma and Fontainea venosa based on RNA-seq experiment. See Additional File 2 and Additional Fig. 2 for list of genes. (B) Homolog CYP450 average z-score value expression profile in Fontainea species. Red circle, represents significantly different ($p < 0.05$) between root and leaf tissues in *F. picrosperma*. Green circle represents significantly different ($p < 0.05$) between root and leaf tissues in *F. venosa*. See Additional Fig. 3 for significance values. Venn diagrams shown below indicates those CYP450 exclusive and shared between leaf and root tissues.

Figure 4

Phylogenetic analysis of diterpenoid CYP genes in *Fontainea* species. (A) Phylogenetic presentation of *Fontainea* species-specific CYP genes with other Euphorbiaceae and their motif analysis. Circled genes are present in both *Fontainea* species and asterisk genes are species-specific (percentage of identity 92%). (B) Graph showing gene expression of diterpenoid genes across 12 biological replicates of *Fontainea picrosperma* in leaf and root tissue. Significant differences: * $p < 0.05$ and ** $p < 0.01$. TPM, transcripts per million.

Supplementary Files

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