Terpene synthase genes in *Melaleuca alternifolia*: comparative analysis of lineage-specific subfamily variation within Myrtaceae

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Findings

Thirty-seven candidate terpene synthase (TPS) genes were identified from a genome sequence of *Melaleuca alternifolia* (Australian tea tree), representing the six TPS subfamilies found in angiosperms. Compared to other well-characterised members of Myrtaceae, *M. alternifolia* possessed fewer TPS genes overall, but was proportionally over-represented in the TPS-b1, a subfamily of cyclic monoterpene synthases primarily involved in plant defence against pathogens. Proportionally high numbers of antimicrobial genes may have resulted from a lineage-specific expansion in *Melaleuca* in response to semi-aquatic origins.

Key words: Tea tree, *Eucalyptus*, monoterpene, *Corymbia* ~~terpinolene~~

Abstract

Terpenes are a multifarious group of secondary compounds present throughout the living world that function primarily in defence, or otherwise in regulating interactions between an organism and its environment. Terpene synthases (TPS) are a mid-sized gene family, whose diversity and makeup reflects a plant's ecological requirements and unique adaptive history. Here we catalogue TPS in *Melaleuca alternifolia* and examine lineage-specific expansion in TPS relative to other sequenced Myrtaceae. Overall, far fewer (37) putative TPS genes were identified in *M. alternifolia* compared with *Eucalyptus grandis* (113) and *E. globulus* (106). The number of genes in clade TPS-b1 (12), which produce cyclic monoterpenes, was proportionally larger in *M. alternifolia* than in any other well-characterised plant, and relative to *E. grandis*, the isoprene/ocimene-producing TPS-b2 clade in *M. alternifolia* tended to be proportionally smaller. This suggested there may be lineage-specific subfamily change in *Melaleuca* relative to other sequenced Myrtaceae, perhaps as a consequence of its semi-aquatic evolutionary history.

Introduction

Terpenes are volatile, often aromatic hydrocarbon-based natural compounds produced by plants, fungi, bacteria and some insects, some of which play a role in primary metabolism but many of which are secondary metabolites (Chen et al. 2011). They are found in the essential oils, resins and other tissues of plants, and are believed to increase fitness in a variety of complex ways, including deterring or attracting insects and other herbivorous or pollinating organisms, resisting fungal or bacterial infection (phytoalexins), or by acting as allelochemicals (Külheim et al. 2015). Isoprenes, for example, appear to alleviate heat stress (Behnke et al. 2007), perhaps by stabilising plant membranes or acting as antioxidants (Penuelas et al. 2005); ocimenes have been implicated in defence against insect herbivory (Navia-Giné et al. 2009; Shimoda et al. 2012). The biosynthetic pathways of terpenes are well-understood, and genes for terpene synthases (TPSs – enzymes that catalyse the terminal step of terpene structural modification from 5-carbon isoprene subunits) have already been well-described in plants such as *Arabidopsis* (Herde et al. 2008) and *Eucalyptus* (Keszei et al. 2010).

TPS in plants typically exist as a mid-sized gene family (Chen et al. 2011) but can range in number from 1 in *Physcomitrella patens* (a bryophyte) to 113 in *Eucalyptus grandis*, with larger gene families tending to found in some woody perennials because of the key role of terpenes in defence over their long lifespans (Chen et al. 2011; Kulheim et al. 2015). Studies of the genome organisation of TPS show patterns of clustering into subfamilies at locations in the genome (e.g. Tuskan et al. 2006; Kulheim et al. 2015). This mechanism of gene family evolution is consistent with rounds of gene duplication (Cannon et al. 2004), whereby sections of chromosomes are duplicated in uneven crossing over events or by the action of transposable elements. Gene

duplication is an important source of genetic variation, and duplications account for a large proportion of genes in eukaryotic genomes (Pierce, 2012). When a single gene is duplicated and inserted close to the original, it is termed a local or tandem duplication (TD; Cannon et al., 2004).

As with other gene families involved in adaptive responses, expansion or contraction in gene family size for TPS is thought to occur in response to the nature of the stress (i.e. biotic or abiotic) which appears to influence the magnitude of expansion (Hanada et al. 2008). Lespinet et al. (2002) report that lineage-specific expansions of gene families resulting from retained TDs are very frequently expansions of genes involved in stress response, but it is not clear which type of stress has a stronger relationship with TDs. As an expansion in one orthologous group (OG) in response to an adaptive force acting on one species is often mirrored by a contraction of the same OG in a related but geographically separate species, lineage-specific gene family size variation leaves different genomic signatures for different adaptive histories (Blanc and Wolfe 2004).

A prominent feature of TPS enzymes is that they yield multiple products, with as many as 52 different terpenes being reported from one enzyme (Steele et al. 1998). The Myrtaceae family is notable among the plant families of southern hemisphere origins for its number of essential oil-rich taxa, and the abundance of TPS genes in some species (Külheim et al. 2015; Webb et al. 2014).

Several eucalypts including *Eucalyptus* sp. and *Corymbia citriodora*, as well as *Melaleuca* sp. are grown commercially for terpene-rich essential oil. Among the *Melaleuca*, *Melaleuca alternifolia* (Maiden and Betche) Cheel is the most important for essential oil production because of the proven antimicrobial activity of a major constituent, terpinen-4-ol (Baker 1999; Southwell 2003; Morcia et al. 2012). Because of its commercial importance, it is arguably the best-studied

of any Myrtaceae in terms of terpene chemistry, biochemistry and genetics. Attempts have been made to identify genes underlying biosynthesis of commercially important terpene components and assign function (Shelton et al. 2002, 2004a, 2004b; Keszei et al. 2010b, unreviewed RIRDC report; Webb et al. 2013; Webb et al. 2014) and regulation of oil yield (Webb et al. 2013), but as yet only a single candidate TPS has been reported for this species (Shelton et al. 2004a; Sharkey et al. 2005).

Here we catalogue the TPS genes identified in a draft genome sequence of *Melaleuca alternifolia*. We conduct comparative analysis of the TPS gene family with other sequenced Myrtaceae, including the reference Myrtaceae, *Eucalyptus grandis* (Grattapaglia et al. 2012; Myburg et al. 2014; Kulheim et al. 2015). We find there are comparatively few TPS in *M. alternifolia* relative to other woody perennials, but there is a tendency toward over-representation of the TPS-b1 clade of cyclic monoterpene synthases, and under-representation of the TPS-b2 clade, a subfamily of isoprene/ocimene synthase gene class, relative to other sequenced Myrtaceae.

Materials and Methods

Genome sequencing

A draft genomic sequence for the reference genotype SCU01 of *M. alternifolia* was generated using short read Illumina sequence data (See Online Resource 1 for details of Results and Methodology). This individual has Chemotype 4 terpene chemistry (high 1,8-cineole and intermediate terpinen-4-ol) and was clonally replicated and archived in a germplasm resource collection located at the Lismore campus of SCU (Shepherd et al. 2015).

Sequencing was performed on a Hiseq 2000 (Illumina) at the Australian Genome Research Facility. In brief, a total of 100 Gb of high quality paired-end 100 bp long sequence reads were generated by an Illumina Hiseq to give approximately 141 X genome coverage based on a cytological estimate of 710 Mb (See Online Resource 2). Raw sequencing reads were trimmed to remove low-quality bases and adaptor sequences. Reads in FASTQ format were first checked for quality using FASTQC (Andrews 2015), followed by removal of adapter sequences, poly-N stretches and low quality (Phred score < 20) reads using the BBDuck module of the BBMap software package (version 34_90; http://sourceforge.net/projects/bbmap). A draft assembly of *M. alternifolia* was constructed using the CLC de novo assembler (CLC Bio, Aarhus, Denmark). The draft genome comprised a total of 221,396 contigs with total length of 356 Mb and an N50 of 8,778 bp.

Gene annotation with the Maker pipeline version v2.31.8 (Cantarel et al. 2008) produced 33,184 draft gene models with an annotation edit distance of >0.35. Analysis of single copy gene coverage using the BUSCO method (Simão et al. 2015) predicted 90% of single copy genes

(80% complete, 10% fragmented) captured in this set of contigs (data not shown). To check Maker's efficacy, tBLASTn was used against the *M. alternifolia* genome assembly to explore the presence of TPS genes outside of Maker gene models (amino acid queries from Kulheim et al. 2015. See Online Resource 3). Two query sequences (TPSb line 1 & TPSf line 2) returned no hits. Hits to all other queries (116 in total) were associated (overlapping or contained within) with gene models predicted by Maker (see Online Resource 4 for tabulated results). This suggests that the pipeline, which used protein sequence evidence from *E. grandis, C. citriodora and Vitus* sp. to draw gene model predictions, is at least as effective as a straight homology search, having search parameters relaxed enough to allow for some missing consensus sequences and using multiple lines of evidence.

Mining the genome

Methods in a 2015 study by Külheim et al. of TPS genes in *E. grandis* served as a template. Using known conserved protein regions of 6 TPS subfamilies as BLAST queries (CoGeBLAST and NCBI BLAST+, using default parameters), searches were performed on the *Melaleuca* v1 genome assembly.

To establish whether the conserved domains (CDs) used for mining the *E. grandis* genome were suitable for locating TPS genes and confidently assigning subfamilies in *M. alternifolia*, one CD from each subfamily was BLASTed to both genomes, and the highest e-values for each search recorded. E-values for both species were indeed comparable in

significance (for tabulated data see Online Resource 5), indicating that queries used to mine the well-studied *E. grandis* reference Myrtaceae genome are applicable to *M. alternifolia*.

To gather a broad pool of candidates, a cutoff e-value of 1e-08 was used to select the highest hits for each subfamily query (TPS-a, -b, -c, -e, -f and -g) to the *M. alternifolia* assembly. This cutoff was established when it became apparent that any hits with e-values less significant than 1e-10 invariably appeared in multiple search results, indicating that the subfamily-specific sensitivity of searches tapered off below that point. 1e-08 was chosen as a conservative value in the event that some e-values of relevant gene models happened to fall below 1e-10.

The pool of candidate gene models returned by these searches was sorted by subfamily and then structurally analysed using GEvo (https://genomevolution. org/coge/Gevo.pl; Lyons & Freeling 2008) to ascertain exon number (which varies depending upon subfamily; Külheim et al. 2015), and FeatView (https://genomevolution.org/coge/FeatView.pl) to ascertain number and placement of stop codons. Models were given a ranking according to a modified version of Külheim's system, which is as follows: 1 = full length, no premature stop codons; 2 = full length, up to 2 stop codons; 3 = full length, no stop codon; 4 = pseudogenes, more than 2 stop codons; 5 = partial gene. (Ultimately, all classes of gene were included in the phylogeny, as incomplete genes could have been truncated simply by being part of a very short contig.)

Using ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and PCLR release 0.9 (http://www.andrewschein.com/cgi-bin/pclr/pclr.cgi), models were analysed to detect the presence of chloroplast transit peptide sequences (cTPs) (Emanuelsson et al. 1999). As all but the

sesquiterpenes (C15) are produced in the chloroplast (Külheim et al. 2015), most TPS genes should contain a cTP.

Phylogeny

In order to replicate as closely as possible Külheim's phylogeny methods, a test run was performed using only the 113 *E. grandis* TPS amino acid sequences published with the 2015 paper. Using PhyML 3.0 (http://phylogeny.lirmm.fr; Dereeper et al. 2008) a ClustalW alignment was constructed from the 113 sequences. Gblocks curation was skipped, as the analysis returned by a curated pipeline did not satisfactorily resolve some subfamilies (for example, TPS-e appeared as a clade flanked either side by TPS-c genes).

As per Külheim et al., the Jones-Taylor-Thornton amino acid substitution model was used to create a maximum-likelihood phylogenetic tree file (.tree) with 100 bootstrapped replicates, and the resulting file was imported to FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/; Rambaut 2014) for visualisation and editing. The tree (Online Resource 6) was manually rooted from the node at which types I and III (i.e. subfamilies c, e, f and a, b, g) diverge.

As the phylogenetic tree that resulted from using the above settings showed very high structural similarity to that of Külheim et al. (2015), the same settings were applied using the set of 113 E. grandis TPS genes plus the 37 *M. alternifolia* candidate gene models identified using BLAST, as well as the coding sequence for the putative monoterpene synthase transcript obtained by Shelton et al. (2004; GenBank accession AY279379.1) The alignment for this phylogeny can be found in Online Resource 7. A tree was constructed as outlined above; Figure

1 is one maximum-likelihood tree, which shows average numbers of amino acid substitutions per branch as branch length relative to the scale.

Results

Putative TPS genes and subfamily proportions

Thirty-seven candidate TPS genes with high similarity to conserved TPS regions were identified in the *M. alternifolia* genome (Table 1; Figure 2; all gene models are listed in Online Resource 8; .fasta files of 37 amino acid sequences are attached as Online Resource 9).

Fourteen genes clustered with subfamily TPS-a, which produce sesquiterpenes (C15); twelve with TPS-b1, which produce cyclic monoterpenes (C10, e.g. sabinene hydrate and 1,8-cineole); two with TPS-b2, which produce isoprenes and ocimenes (C5, C10); one with TPS-c, which produce diterpenes (C20); one with TPS-e, which produce mono-, sesqui- and diterpenes; three with TPS-f, which also produce mono-, sesqui- and diterpenes; and four with TPS-g, which predominantly produce acyclic mono-, sesqui- and diterpenes.

Of all well-studied plants represented in Table 1 and Figure 2, *M. alternifolia* has the highest number of TPS-b1 genes as a proportion of the total number of TPS genes: 32.4%, compared with *Populus trichocarpa*, the next-highest at 31.2%. TPS gene subfamily proportions do not differ significantly between *M. alternifolia* and *E. grandis* (χ^2= 1.74; χcrit= 12.59; p= 0.05), although tea tree has a proportionally larger set of TPS-b1 (cyclic monoterpene) genes and a smaller set of TPS-b2 (isoprene/ocimene) genes. However, differences in subfamily proportions between *M. alternifolia* and both *P. trichocarpa* (a well-characterised woody dicot) and *A.*

thaliana (a well-characterised herbaceous annual) were significant: χ^2= 26.85 and 36.08, respectively.

Transit peptides

Only five *M. alternifolia* genes from TPS subfamilies -a (1 gene), -b1 (2), -b2 (1) and -g (1), were predicted by ChloroP 1.1 to contain cTPs. For context, the 113 *E. grandis* genes from Külheim et al. (2015) were run through ChloroP 1.1, which found six genes from subfamilies –a (4), -b (1) and –e (1) with cTPs. (cTP-containing genes from both species are listed in Online Resource 10.) TPS-a genes with a predicted transit peptide were compared between *M. alternifolia* and *E. grandis* (the sole predicted cTP-containing TPS-b gene from *E. grandis*, Eucgr.K00875.1.v2.0, was found to be a very small, incomplete gene model, leaving only TPS-a in common between the two species). Sequence identity between these TPS-a genes was between 70.1% (MelG016248 to Eucgr.H04978) and 82.3% (MelG016248 to Eucgr.F03396).

Interestingly, results from analysis of the same 37 gene models using PCLR r0.9 returned the same 5 models as predicted by ChloroP (see Online Resource 11), with no others predicted to contain chloroplast transit peptides.

Sequence identity between predicted cTP-containing TPS-a genes from both species did not greatly exceed that between TPS-a genes not predicted to contain a cTP (70.1 - 82.3% for genes with predicted cTPs, compared to 65.6 - 79.1% for those without, calculated by comparing 6 randomly-selected non-cTP *E. grandis* TPS-a genes with MelG016248, the only *M. alternifolia* TPS-a gene predicted to contain a cTP). A BLAST search of the *Eucalyptus grandis* BRASUZ1

(Phytozome unmasked v2) genome assembly using the amino acid sequence of MelG016248 did return hits to 4 of 6 *E. grandis* predicted cTP-containing TPS-a genes (Eucgr.D01103, Eucgr.E00419, Eucgr.F03396, and Eucgr.H04978). However, these hits ranged from HSP #88 to #23, with many other genes returning higher scores, making it unlikely that these cTP-predicted genes from both species are orthologues.

Phylogeny

A foundation for comparative analysis was established by replicating the Külheim et al. 2015 phylogenetic tree for *E. grandis*. Our tree had a high degree of resemblance with that of Külheim et al. 2015 (see Online Resource 12 for .tree format file), with all subfamilies resolving into clades of identical size and structure.

Inclusion of the 37 *M. alternifolia* candidates, however, induced some repositioning of clades (Figure 1; see Online Resource 13 for .tree file). For example, resolution was lost in the splitting within Type I subfamilies, with TPS-f appearing as a sister group to both -c and -e (in the E. grandis phylogeny, -c split off first, followed by -e and then -f). Whereas, in the tree containing only *E. grandis* genes, TPS-g was a sister to the greater -b group (bootstrap at g/b node = 0.53), and the phylogeny that includes both species showed -g as a sister to -a. The inclusion of a set of genes from a different (albeit closely-related) species therefore reduced certainty in the branching order of TPS subfamily clades.

The TPS-b1 gene MelG017535 showed very high divergence (as represented by branch length in Figure 1) from the other genes in its clade. When an alignment and phylogeny were produced using only the 37 *M. alternifolia* genes (tree not included in this report), MelG017535

showed a similarly high divergence from other TPS-b1 genes. The gene has 6 exons – 1 fewer than the usual 7 observed by Külheim et al.

Finally, the *M. alternifolia* mRNA sequenced and classified as a putative monoterpene synthase persistently clustered not with the TPS-b1 cyclic monoterpene subfamily, as originally proposed by Shelton et al. (2004), but with the TPS-b2 isoprene/ocimene subfamily (ISPS). In addition, this mRNA sequence had 100% sequence identity to one gene model in the *M. alternifolia* assembly, MelG010433.

Discussion

Putative TPS genes and subfamily proportions

Given the BUSCO gene coverage estimate of 90%, it is probable that there are slightly more (41) than 37 TPS genes in the *M. alternifolia* genome than inferred. Refinements to the genome assembly using data derived from further sequencing may bear this out. However, in sequencing a genome as highly heterozygous as *M. alternifolia* there is a chance that both alleles from one locus may be incorrectly assigned to different loci, which would appear to increase the number of paralogues on the assembly.

From the much lower number of putative TPS genes found in *M. alternifolia* compared to *E. grandis* (37 versus 113), results imply that evolutionary forces have acted differentially upon the two lineages since they diverged. Although there are far fewer TPS genes in *M. alternifolia* overall, all subfamilies were nonetheless represented. TPS-c is conserved in land plants and is thought to represent the base of the TPS tree, originating as a diterpene synthase producing gibberellin (regulatory plant hormone) precursors (Yamaguchi 2008). TPS-e and -f – conserved in vascular plants – are also linked to hormone production, sharing a common progenitor gene coding for an ent-kaurene synthase, also a gibberellin precursor (Chen et al. 2011). In contrast, TPS-a, -b and -g are angiosperm-specific, and their products (mono-, sesqui- and diterpenes) have been characterised as playing ecological rather than primary metabolic or regulatory roles (Chen et al. 2011). A salient question is whether this low number of ‘ecological’ TPS genes in

M. alternifolia compared to *E. grandis* represents a reduction, or the retention of an ancestral state.

Orthologous pairing has been observed in most of the TPS genes in *E. grandis*, with large genomic clusters consisting of both functional and pseudogenes (Külheim et al. 2015) pointing to a proliferation of gene duplication events. Thornhill et al. (2015) report an estimated divergence of the genera *Melaleuca* and *Eucalyptus* at ~68 million years ago, and that the closest sister tribe to the Melaleucaceae, the monotypic Osbornieae (divergence ~56 million years ago), is the only member of Myrtaceae to occur in a mangrove growth form and habitat. This suggests the existence of a basal estuarine or riparian progenitor of these tribes between 68 and 56 million years ago.

Sharkey et al. (2013) functionally characterised an isoprene synthase gene from *E. globulus* (EglobTPS106; GenBank AB266390.1) that is almost identical (99.6%) to the *E. grandis* gene EgranTPS084 (Eucgr.K00881; GenBank XM_010037321), the single *E. grandis* TPS-b2 gene that fulfils the criteria for isoprene synthases outlined in the 2013 Sharkey paper. The remaining 8 TPS-b2 genes are putative ocimene synthases (or of unknown function). In *M. alternifolia*, 2 putative TPS-b2 genes were identified by this study, one of which, MelG010433, appears to code for the mRNA transcript described by Shelton et al. (2004) and functionally characterised as an ISPS by Sharkey et al. (2005). The other *M. alternifolia* TPS-b2 gene, MelG013034, lacks the isoprene synthase-specific amino acids and may be considered a putative

ocimene synthase until it is functionally characterised. Thus, a breakdown of TPS-b2 for *E. grandis* is 1 isoprene, 8 ocimene, whereas for *M. alternifolia* it is 1 isoprene, 1 ocimene.

Transcripts encoding ocimene synthases accumulate in leaves in response to insect herbivory (Navia-Giné et al. 2009). (E)-β-ocimene appears to play a role in attracting the insect predators of herbivorous spider mites (Shimoda et al. 2012), which occur in Australia (Wilson et al. 1996). That *M. alternifolia* possesses only a single putative ocimene synthase gene, compared to *E. grandis*' 8, suggests that tea tree has either evolved other strategies to deter herbivores, or that pressures imposed by herbivory differ in magnitude or variety from those undergone by the eucalypts.

In addition, the eucalypts appear to have a proportionally smaller TPS-b1 subfamily than *M. alternifolia*. TPS subfamily proportions observed in *Corymbia citriodora* subsp. *variegata* tend to mirror *Eucalyptus* sp. ratios: a proportionally larger TPS-b2 relative to TPS-b1 (cyclic monoterpene synthases). This suggests proportionally higher representation of the TPS-b2 may be a feature of the eucalypt group more broadly, reflecting either their higher degree of relatedness or their more similar ecological history.

Conversely, the subfamily TPS-b1 is proportionally larger in *M. alternifolia* than in any representative plant (dicot, monocot or moss) in Figure 2, suggesting that duplicate retention or lineage-specific gene family expansion in this subfamily has been an important adaptation in tea tree. Cyclic monoterpenes have been shown to increase membrane permeability of fungal hyphae, effectively inhibiting growth of fungal plant pathogens (Tao et al. 2014). They have also been shown to inhibit the action of bacterial polygalacturonase enzymes, which phytopathogenic

bacteria use to break down the pectin of plant cell walls (Rasoul et al. 2012). Keszei et al. (2010b, unreviewed RIRDC report) hypothesise that the ancestral form of the TPS-b1 enzyme for both *Melaleuca* and *Eucalyptus* was one responsible for cineole biosynthesis. 1,8-cineole has been shown to inhibit the growth of gram-positive and -negative bacteria, and yeasts (Silva et al. 2011).

Given the warm, subtropical habitat of tea tree's evolution, it is unsurprising that an arsenal of antimicrobial secondary metabolites such as cyclic monoterpenes should have been selected for. That at least two of the TPS-b1 genes appear to be the result of tandem duplication raises the possibility that biotic stress may have stimulated the expansion of this TPS subfamily. Barlow (1988) suggested that both *Melaleuca* and *Eucalyptus* may both have had their origins at rainforest margins, from whence they differentiated – *Melaleuca* as a seasonally-drowned habitat specialist, and *Eucalyptus* as a coloniser of low-nutrient, seasonally drier soils.

Transit peptides

The 113 *E. grandis* TPS genes identified by Külheim et al. (2015) are putatively functional based on RNA expression data from seven tissue types. As listed in Table 1, *E. grandis* has at least 38 genes that do not encode cytosol-destined sesquiterpene synthases but do encode plastid-destined TPS enzymes of other classes (from subfamilies -b1, -b2 and -c). Thus, we should expect at least 38 *E. grandis* genes with predicted cTPs. That ChloroP 1.1 predicted only 6 of these indicates that such an analysis as applied to *M. alternifolia* may be erroneous. Therefore, the cTP data returned by ChloroP 1.1 analysis should be regarded with caution. However, that both ChloroP and PCLR predicted cTPs in the same 5 *M. alternifolia* gene models despite the programs'

differing systems of prediction (neural network versus principal component analysis, respectively) adds another line of evidence to the putatively functional status of these 5 genes.

In a review of plastid transit peptides, Bruce (2001) noted that their "extreme diversity in sequence and evolution" means that they are still poorly-characterised. It remains possible that the ChloroP 1.1 and PCLR r0.9 software were unable to detect many of the cTPs of TPS genes in *M. alternifolia* and *E. grandis*.

Phylogeny

Minor differences in some bootstrap values between the model phylogeny of Külheim et al. (2015) and the one in this study may have been the result of unreleased manual adjustments to the alignment performed by the authors of the 2015 study, or simply from slight variation in the 100 bootstrapped replicas used to construct the final consensus tree. Additionally, joint confidence (i.e. overall confidence incorporating the bootstrap values of all nodes) in large trees is inescapably low (Soltis and Soltis 2003). In any case, the phylogenetic trees produced in this study possess nodes with bootstrap values of <80% in similar numbers to the trees of Külheim et al., which illustrates fundamental uncertainties in the relationships between TPS subfamilies. It is tempting to view a phylogeny with high bootstrap values as being directly reflective of the actual relationships between loci. However, as Felsenstein (1985) notes, "Bootstrapping provides us with a confidence interval within which is contained not the true phylogeny, but the phylogeny that would be estimated upon repeated sampling of many characters from the underlying pool of characters." In other words, a bootstrap value indicates only that the analysis returned the same

result many times. From this we must be careful of confidently inferring actual evolutionary relationships.

Confidence in the finer grouping of individual loci was much higher than for the broader relationships between TPS clades, both in the phylogenetic tree produced by Külheim et al. (2015) and the two trees produced for this study (with and without *M. alternifolia* genes). However, the inferred relationships between TPS subfamilies mostly mirror those found by Chen et al. (2011) in a phylogeny of putative full-length TPS genes from 7 sequenced plant genomes and representative characterised gymnosperm TPS sequences. Slight differences lie in the splitting of Type I (TPS-c, -e and -f) clade, and in the order of branching within Type III (TPS-a, -b and -g). For the purposes of assigning TPS subfamilies to gene models, however, the phylogeny produced in this study was deemed adequate.

The 2011 study by Chen et al. characterised clades TPS-a, -b and -g as encoding enzymes involved in ecological interactions rather than primary metabolism or hormonal regulation. These three subfamilies, which show considerable divergence in sequence to the other TPS clades, contain the highest number of putative TPS genes in *M. alternifolia* (14, 14 and 4 genes, respectively) and together make up 32 of the 37 genes identified in this study. The remaining 5 genes from TPS-c, -e and dicot-specific TPS-f (1,1 and 3 genes) are, based on the characterisation of Chen et al., likely to encode enzymes that produce plant hormone precursors.

The long branch of TPS-b1 gene MelG017535 suggests high divergence from the other genes in that clade. However, its lack of a 7^{th} exon compared to other TPS-b1 gene models could be due to the inclusion of an intron, or fusion with another gene. If the striking difference in

sequence and single lost exon are not artefacts of sequencing or errors in gene model prediction, this gene, once verified, warrants further investigation as a potential new subtype of TPS-b1.

Gene model MelG010433, which is identical in sequence to the mRNA studied and classified as a TPS-b1 monoterpene synthase gene by Shelton et al. (2004), showed a tendency to cluster with TPS-b2 rather than TPS-b1 genes. This is supported by Sharkey et al. (2005), who functionally characterised this transcript as an ISPS, and by Keszei et al. (2010), who also concluded that the sequence codes for an ISPS in TPS-b2.

Conclusion

This study provides crucial baseline estimates for TPS gene numbers and subfamilies in *M. alternifolia*. This information will be important in further elucidation of the tea tree's evolutionary history, the broader study of gene family evolution, and in understanding in greater detail the ecological functions of terpenes in the family Myrtaceae.

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Table and Figure Captions

Table 1 Number of TPS genes in 12 plant species, broken down by TPS subfamily/class of terpene product. *M. alternifolia* has less than 1/2 of the number of TPS genes of three other Myrtaceae species, *E. grandis*, *E. globulus* and *C. citriodora* subsp. *variegata*, but still has representatives from all subfamilies found in Myrtaceae. Adapted from Chen et al. (2011) and Külheim et al. (2015). Methods for *C. citriodora* subsp. *variegata* data provided in Online Resource 14.

Terpene	**type**	***M. alternifolia***	***E. grandis***	***E. globulus***	***C. citriodora*** subsp. variegata	***V. vinifera***	***P. trichocarpa***	***A. thaliana***	***S. lycopersicum***	***S. bicolor***	***O. sativa***	***S. moellendorffii***	***P. patens***
TPS-a	sesqui	14	52	45	52	29	13	23	12	15	19	0	0
TPS-b1	mono	12	27	28	15	8	10	6	8	2	0	0	0
TPS-b2	isoprene/ ocimene	2	9	10	6	2	2	0	0	0	0	0	0
TPS-c	di	1	2	2	1	2	2	1	2	1	3	3	2
TPS-e	mono, sesqui, di	1	3	2	1	1	2	1	5	3	9	3	0
TPS-f	mono, sesqui, di	3	7	9	5	0	1	1	0	0	0	0	0
TPS-g	mono, sesqui, di	4	13	10	9	15	2	1	2	3	1	0	0
TPS-h	di	0	0	0	0	0	0	0	0	0	0	8	0
TOTAL		**37**	**113**	**106**	**89**	**57**	**32**	**33**	**29**	**24**	**32**	**14**	**2**

Figure 1 Phylogeny of 37 *M. alternifolia* putative TPS genes with 113 *E. grandis* TPS genes from Külheim et al. (2015) and 1 *M. alternifolia* putative monoterpene synthase from Shelton et al. (2004). *M. alternifolia* genes are indicated by a black dot. Scale = average number of amino acid substitutions per branch *(JPEG produced using Figtree v1.4.2. And GIMP)*

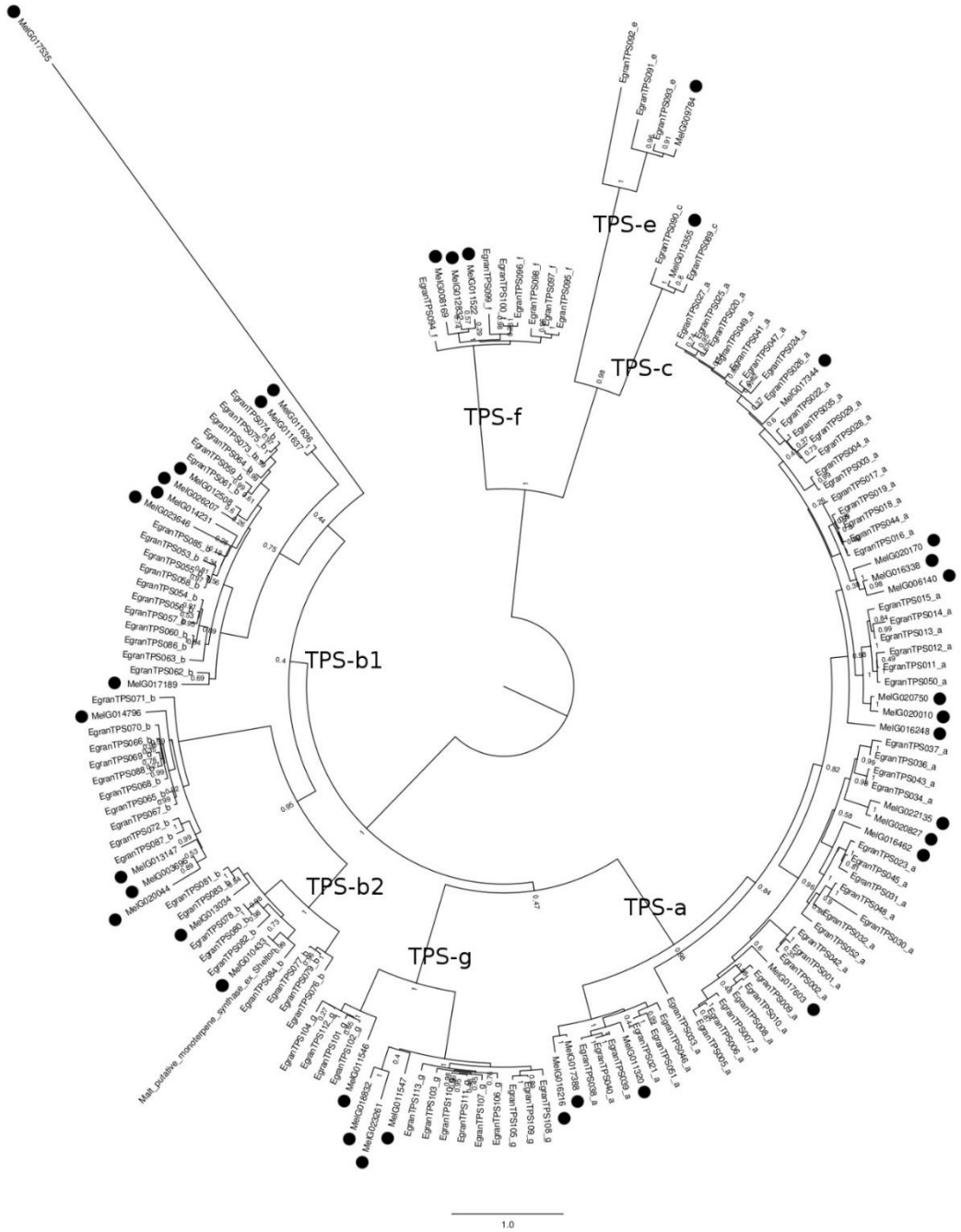
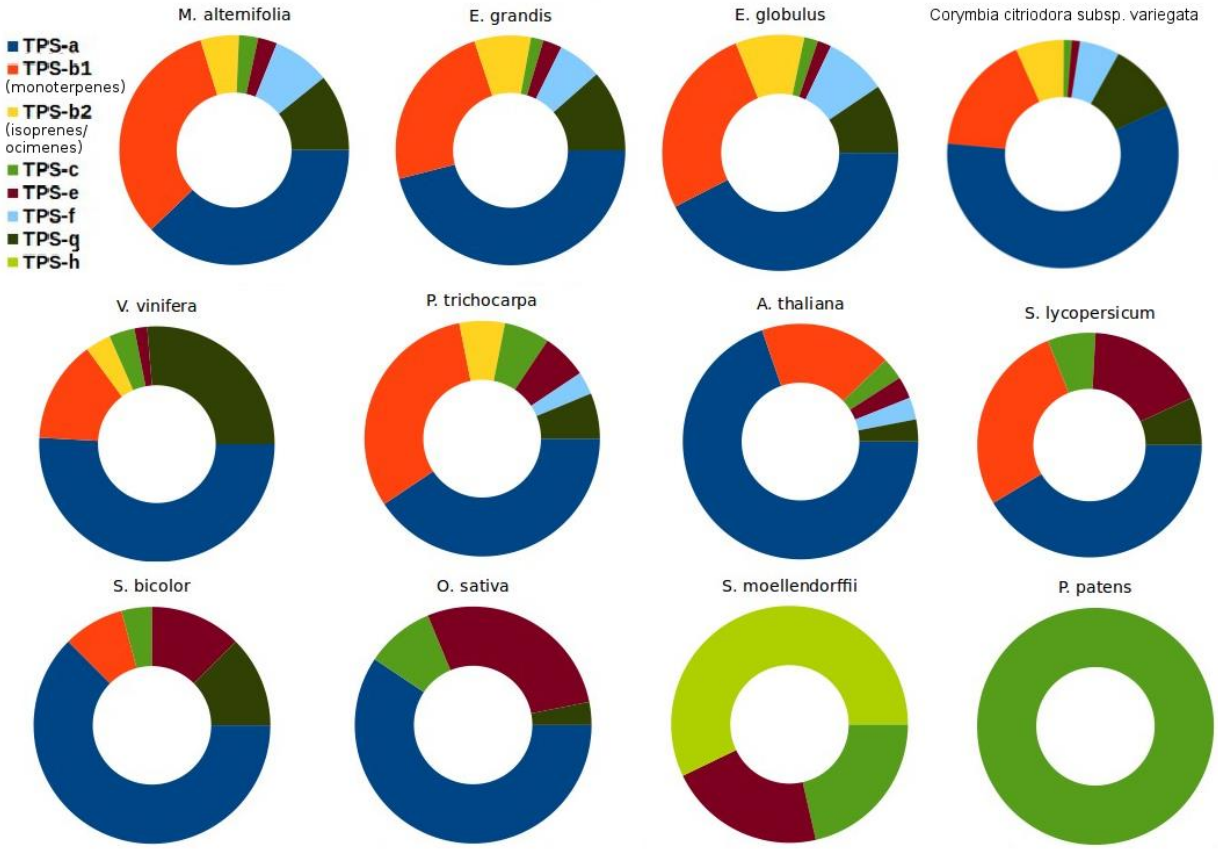


Figure 2 Proportion of TPS gene subfamilies found in 12 plant species as listed in Table 1. *M. alternifolia* contains the highest proportion of TPS-b1 genes. Gene proportions in *M. alternifolia* do not differ significantly from those in *E. grandis* (χ^2= 1.74; χcrit= 12.59; p= 0.05). *(JPEG produced using LibreOffice Calc and GIMP)*.



Online Resources

Online Resource 1 Calvert et al_ESM_1.pdf

Methods and results for generating a draft genome sequence for *M. alternifolia.*

Online Resource 2 Calvert et al_ESM_2.pdf

Flow cytometry methods for genome size estimation in *M. alternifolia.*

Online Resource 3 Calvert et al_ESM_3.pdf

TPS subfamily conserved amino acid sequences used as queries for BLASTing the *M. alternifolia* assembly. Originally published in a supplementary file with Külheim et al., 2015.

Online Resource 4 Calvert et al_ESM_4.pdf

tBLASTn searches of Melaleuca alternifolia (Southern Cross Plant Science unmasked vv1.1) genome using queries listed in Kulheim et al. (2015) (Calvert et al_ESM_3.pdf) to find TPS genes not predicted by MAKER v2.31.8.

Online Resource 5 Calvert et al_ESM_5.pdf

Top e-values for conserved TPS subfamily domain queries in *M. alternifolia* and *E. grandis.*

Online Resource 6 Calvert et al_ESM_6.pdf

Maximum-likelihood tree produced using the 113 amino acid sequences of *E. grandis* TPS genes (from subfamilies a, b, c, e, f and g) identified by Külheim et al. (2015). Tree is rooted at the branching of Type I and III genes. Phylogeny shows high structural similarity to Külheim et al. down to branch length, which denotes relationship distance. Scale = average number of amino acid substitutions per branch *(JPEG produced using Figtree v1.4.2. And GIMP).*

Online Resource 7 Calvert et al_ESM_7.fasta

FASTA Alignment of 113 E. grandis TPS genes plus the 37 *M. alternifolia* candidate gene models identified using BLAST, as well as the coding sequence for a putative monoterpene synthase transcript obtained by Shelton et al. (2004; GenBank accession AY279379.1). Using

PhyML 3.0 (http://phylogeny.lirmm.fr; Dereeper et al. 2008) with default settings, a ClustalW alignment was constructed from the 113 sequences. Gblocks curation was skipped.

Online Resource 8 Calvert et al_ESM_8.pdf

37 *M. alternifolia* TPS gene models listed by subfamily. Quality class ranking as per Külheim et al. (2015) is as follows: 1 = Full length; no prem stop codons. 2 = Full length; up to 2 stop

codons. 3 = Full length; no stop codon. 4 = Pseudogenes: more than 2 stop codons. 5 = Partial gene.

Online Resource 9 Calvert et al_ESM_9.fasta
Amino acid sequences of thirty-seven candidate TPS genes with high similarity to conserved TPS regions identified in the *M. alternifolia* genome.

Online Resource 10 Calvert et al_ESM_10.pdf
M. alternifolia and *E. grandis* gene models predicted by ChloroP 1.1 to contain chloroplast transit peptides.

Online Resource 11 Calvert et al_ESM_11.pdf
M. alternifolia TPS gene models (37) analysed using PCLR (Schein et al 2001) for presence of chloroplast transit peptides (cTP).

Online Resource 12 Calvert et al_ESM_12.tree
Phylogenetic tree file. Replication of the Külheim et al. 2015 phylogenetic tree for TPS genes in *E. grandis*, used as a foundation for comparative analysis.

Online Resource 13 Calvert et al_ESM_13.tree
Phylogenetic tree file. Replication of the Külheim et al. 2015 phylogenetic tree for TPS genes in *E. grandis*, with inclusion of 37 *M. alternifolia* putative TPS genes and one putative monoterpene synthase gene (Shelton et al. 2004).

Online Resource 14 Calvert et al_ESM_14.pdf
Methods for collection of *C. citriodora* subsp. *variegata* data provided in Table 1.

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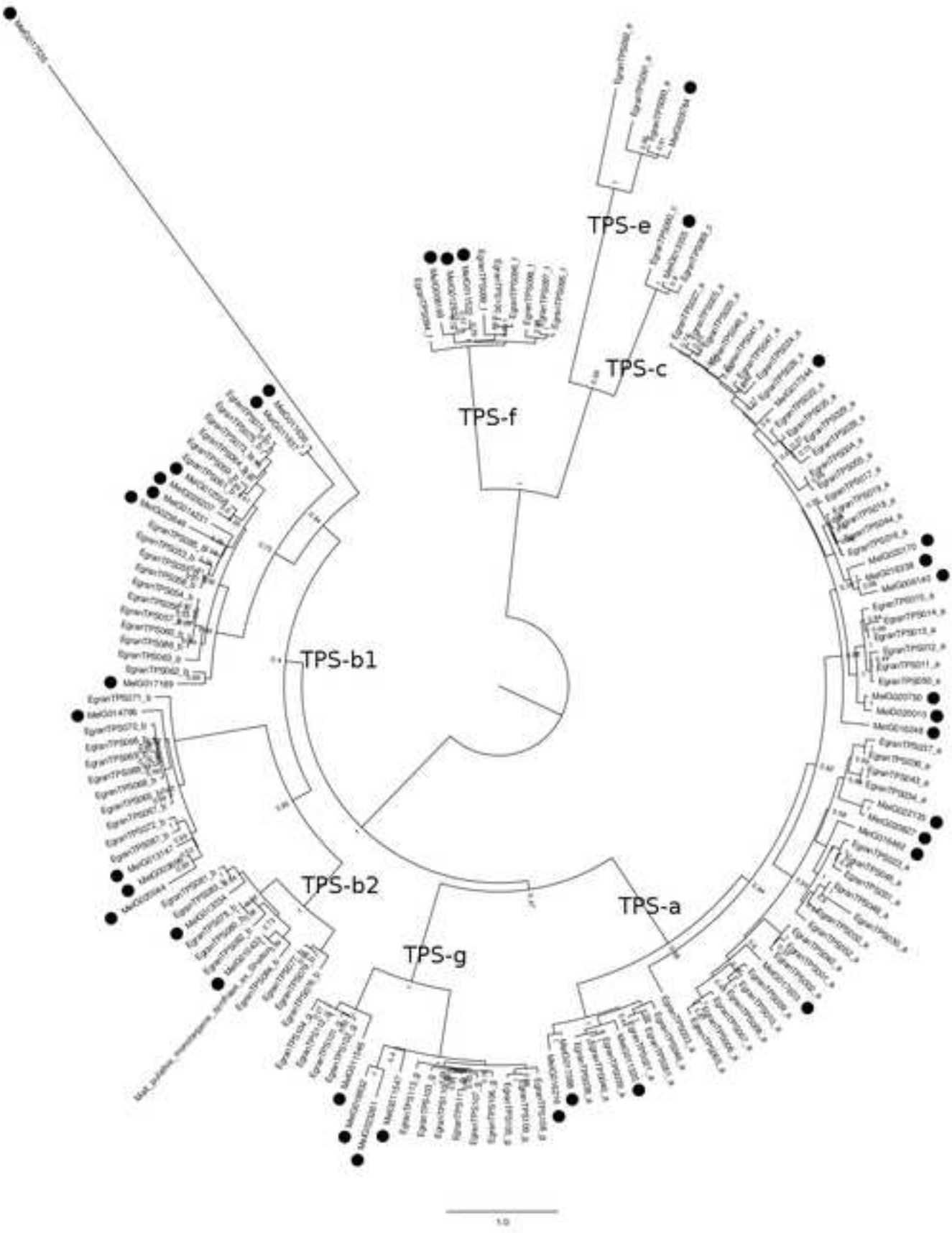

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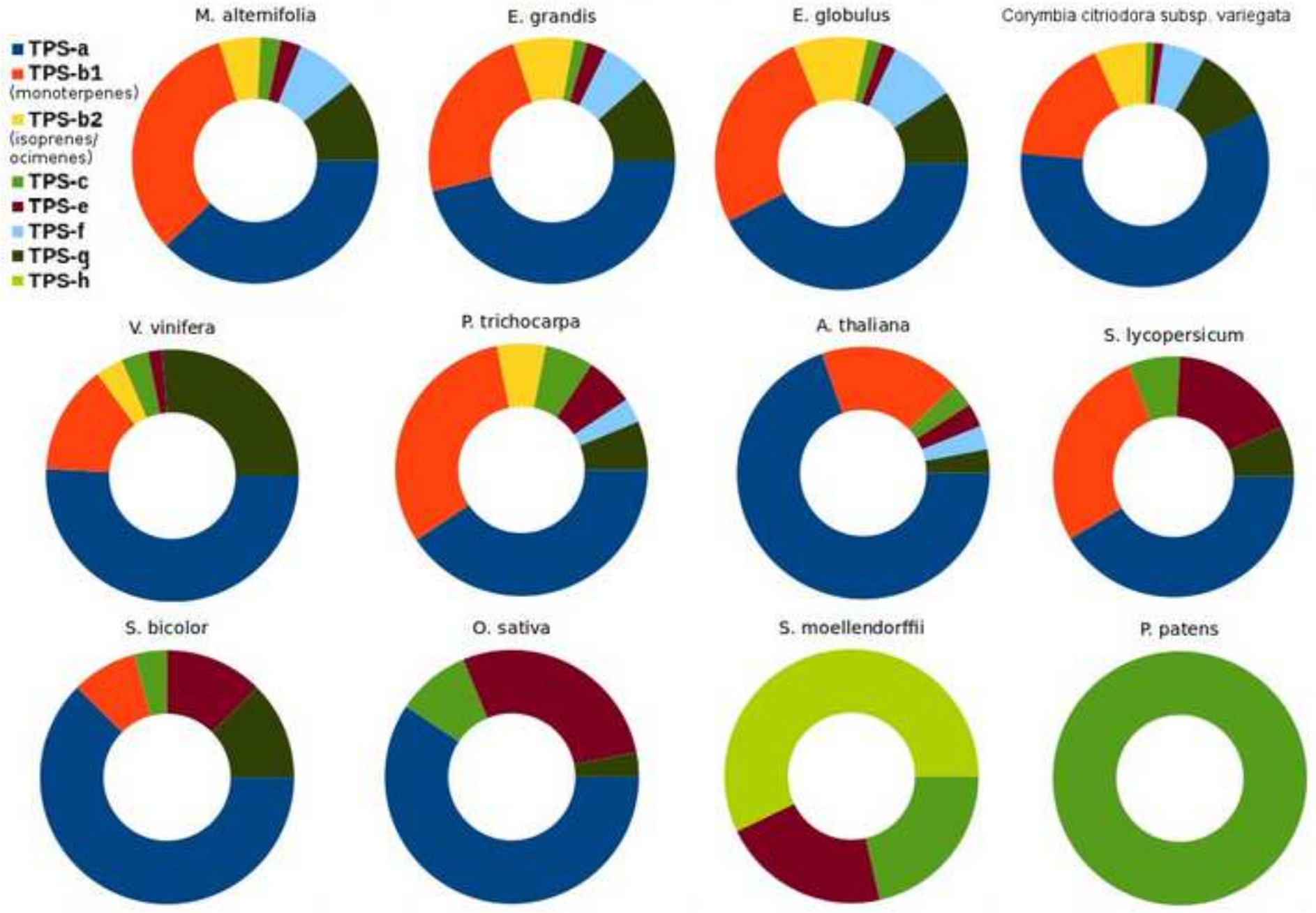

Table

TERPENE SYNTHASE GENES IN MELALEUCA ALTERNIFOLIA: COMPARATIVE ANALYSIS OF LINEAGE-SPECIFIC SUBFAMILY VARIATION WITHIN MYRTACEAE

Plant Systematics and Evolution

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For all tables in spreadsheet format, please refer to file *Calvert et al_Tables.ods*. For Online Resources tables, please see folder *Calvert et al Online Resources*.

Table 1 Number of TPS genes in 12 plant species, broken down by TPS subfamily/class of terpene product. *M. alternifolia* has less than 1/2 of the number of TPS genes of three other Myrtaceae species, *E. grandis*, *E. globulus* and *C. citriodora* subsp. *variegata*, but still has representatives from all subfamilies found in Myrtaceae. Adapted from Chen et al. (2011) and Külheim et al. (2015). Methods for *C. citriodora* subsp. *variegata* data provided in Online Resource 14.

Terpene	type	*M. alternifolia*	*E. grandis*	*E. globulus*	*C. citriodora* subsp. *variegata*	*V. vinifera*	*P. trichocarpa*	*A. thaliana*	*S. lycopersicum*	*S. bicolor*	*O. sativa*	*S. moellendorffii*	*P. patens*
TPS-a	sesqui	14	52	45	52	29	13	23	12	15	19	0	0
TPS-b1	mono	12	27	28	15	8	10	6	8	2	0	0	0
TPS-b2	isoprene/ ocimene	2	9	10	6	2	2	0	0	0	0	0	0
TPS-c	di	1	2	2	1	2	2	1	2	1	3	3	2
TPS-e	mono, sesqui, di	1	3	2	1	1	2	1	5	3	9	3	0
TPS-f	mono, sesqui, di	3	7	9	5	0	1	1	0	0	0	0	0
TPS-g	mono, sesqui, di	4	13	10	9	15	2	1	2	3	1	0	0
TPS-h	di	0	0	0	0	0	0	0	0	0	0	8	0
TOTAL		**37**	**113**	**106**	**89**	**57**	**32**	**33**	**29**	**24**	**32**	**14**	**2**

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New data available on Melaleuca alternifolia genome size estimate. Although value in review paper was best available at the time, methodological refinement has provided improved more reliable and repeatable estimates tested on a wider range of ecotypes for the taxa. The proposed additional author~~s,~~ ~~Tim Rhodes and~~ Bronwyn Barkla contributed to this work.

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The authors declare no conflict of interests

New Author Contributions statement:

Calvert - Carried out all primary analysis and interpretation and drafted and developed the manuscript
Baten - Prepared the platform resource of a Melaleuca genome sequence. Provided bioinformatic support and intellectual content, helping with design and choice of analysis.
Butler - Provided intellectual input into the development of the analytical approach and comparative analyses, aided in revising the manuscript and responding to reviewers comments

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Shepherd - Conceived the project concept and primary design, contributed to the preparation of the original submission, responses to reviewers and revised version.
Barkla – Developed methodology, performed the flow cytometry estimation of genome size for Melaleuca and analysed data.

New Acknowledgement Section:

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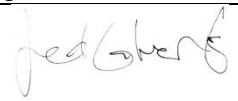
1st author	Jed	Calvert	I agree to the proposed new authorship shown in section 4		Southern Cross University	9 June 2017
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