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Genetic control of monoterpene composition in the essential oil of *Melaleuca alternifolia* (Cheel)

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Abstract Previous studies into the population structure of *Melaleuca alternifolia* by both isozyme and microsatellite analysis revealed little evidence for genetic structuring within genetic provenances. In contrast, analysis of the oil composition within these same regions showed distinct clustering of chemotypes within the provenances suggesting either that chemotype was not under genetic control, or that there is strong environmental selection for plant chemotypes. To investigate the level of genetic control of monoterpene composition in the essential oil of *M. alternifolia*, individuals representing the three extreme chemotypes of high terpinen-4-ol, high 1,8-cineole and high terpinolene were crossed with an individual with the commercially desirable high terpinen-4-ol oil profile. The progeny resulting from these crosses displayed oil profiles that were intermediate to that of the parent. Further analysis of the survey of oil chemotypes within the natural population also suggests that these intermediate chemotypes may arise naturally between regions containing high proportions of the extreme chemotypes. These results imply that there is a level of genetic structure for chemotype determination within the genetic provenance that is undetected by isozyme and microsatellite analysis. This information could play a vital role in the selection of appropriate genetic material to be used in future essential oil selection and breeding programs.

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Introduction

Melaleuca alternifolia is an evergreen Australian native tree that, along with other *Melaleuca*, *Leptospermum* and *Callistemon*, is commonly referred to as the Tea Tree. *M. alternifolia* is the primary source of the commercially available antiseptic, Tea Tree oil, commonly used in cosmetics and pharmaceuticals. The antiseptic nature of Tea Tree oil is primarily attributed to the presence of the oxygenated monoterpene, terpinen-4-ol, but also includes other monoterpenes including 1,8-cineole, α-terpinene, γ-terpinene and terpinolene (Fig. 1). The International Standard (International Standards Organisation 1996) requires that commercial Tea Tree oil has a minimum terpinen-4-ol content of 30% and a maximum 1,8-cineole content of 15%. However, market desires are for an oil with the highest terpinen-4-ol and the lowest 1,8-cineole content possible. For this reason, there has been a strong incentive to establish plantations containing only plants of the high terpinen-4-ol chemotype.

Initial studies into the relative composition of monoterpenes in the *M. alternifolia* essential oil identified three main chemotypes consisting of a high terpinen-4-ol chemotype, a high 1,8-cineole chemotype and a high terpinolene chemotype (Penfold et al. 1948). Since then, further studies have revealed the existence of up to six individual chemotypes consisting of the original three chemotypes but also including three additional high 1,8-cineole chemotypes with varying levels of terpinen-4-ol and terpinolene (Homer et al. 2000). Considerable variations in the yield and composition of the essential oil from individuals in plantations and natural stands indicate that there is a potential for increasing plantation productivity through selective breeding. As a method to facilitate such breeding programs genetic studies were conducted using both isozymes (Butcher et al. 1992) and microsatellites (Rossetto et al. 1999) to define the genet-

ic structure and diversity within the natural population of Tea Tree. Information obtained from the microsatellite analysis revealed the existence of two geographically isolated genetic provenances. The existence of geographical boundaries and the predominant occurrence of the high terpinolene chemotype in a single genetic provenance supports this division. The remaining five chemotypes cluster in specific geographic regions within a single genetic provenance with fewer geographical boundaries. However, within the two provenances there was little sub-structuring of the population at the genetic level, implying relatively high levels of gene flow (Rossetto et al. 1999).

There are two possible explanations for the differences observed between the microsatellite data and the chemotype distribution data. Either variation in chemotype is not under strong genetic control or, alternatively, it is under strong genetic control but selection at the level of the chemotype is sufficiently strong to counteract the effects of gene flow. Clustering of chemotypes in small geographical regions is not unique to *M. alternifolia* and has been observed in other natural populations of the Myrtaceae (Egerton-Warburton et al. 1998) but the level of genetic influence on the chemotype in this population has yet to be examined. The present study was undertaken to assess the genetic influence on the oil composition of *M. alternifolia*. To facilitate this the composition of the essential oil was analysed in crosses made between individuals of different chemotype and geographical origin, and grown under uniform conditions.

Materials and methods

Plant material

Oil composition was assayed in 110 individuals from the Main Camp Plantation, (data not shown). From this selection two individuals were selected to be representative of the high terpinen-4-ol chemotype, chemotype 1 (Homer et al. 2000). One individual, PP1, was chosen to be the maternal parent in all crosses while the second individual, G1, was the paternal parent for the chemotype 1 cross. The paternal parents for the 1,8-cineole (Grafton) and the terpinolene (Lyra) crosses were selected as representative individuals of chemotypes 5 and 2 respectively from an independent survey of the oil composition of *M. alternifolia* in natural stands. The resultant progeny were grown in a greenhouse until approximately 1.2 m in height. Mature leaf material was harvested at this stage for further analysis.

Oil composition and analysis

Leaf oil composition was analysed on a Hewlett-Packard 6890 Gas Chromatograph (GC) coupled to a Hewlett-Packard 7694 Headspace sampler. The GC was fitted with a SGE 50 m BP-1, 0.5-μm column, Flame Ionisation Detector (FID) and operated under the following conditions: Helium carrier gas; head pressure 10.3 psi, split injection 20:1; injector temperature 200 °C; detector temperature 250 °C. The temperature program for analysis was as follows: initial temperature 60 °C (held for 1 min) ramping at 6 °C/min to 280 °C (held for 2 min). The headspace sampler was equipped with a 1-ml injector loop held at 110 °C and a transfer line also held at 110 °C and vials were incubated at 97 °C for 55 min prior to sampling. For each plant analyzed, approximately 0.1 g of dry leaf material was added to a 10-ml headspace vial with 1 ml of distilled water. The vials were sealed and placed in the headspace carousel in batches of 40. Two blanks were run with each batch as reference standards at the beginning and end of each batch. The reference solution consisted of 2,000 mg of *M. alternifolia* oil (Batch No 6081, Main Camp Plantation, Rappville, NSW) and 50 ml of polysorbate 20 made up to a total volume of 1 l. Each standard consisted of a 1-ml sample of this reference solution added to a 10-ml headspace vial.

Compounds were identified by analysis of Tea Tree oil standard (Batch No. 6081) by both GC-Mass Spectroscopy (GC-MS) and Headspace-GC (HS-GC). The relative amount of each compound was calculated as a percentage of the total oil-using area under the peak. The oil yield of samples was determined using total area figures obtained by HS-GC. These figures were calibrated to estimate steam distillation yields expressed as percent dry weight using the calibration formulae described by Homer et al. (2000).

Results and discussion

Chemotypes of *M. alternifolia* are determined by the relative quantity of the three main components of the essential oil, 1,8-cineole, terpinen-4-ol and terpinolene (Penfold et al. 1948; Butcher et al. 1994; Homer et al. 2000). The chemotypes were first identified as ‘Type’ and Varieties A–D. Since then Homer et al. (2000) has shown the existence of a sixth distinct chemotype in this species which collectively have been designated Chemotypes 1–6 (Table 1). Within these six chemotypes, there are corresponding chemotypes in which terpinen-4-ol,

Table 1 Composition of the predominant monoterpenes, based on the steam distilled, or the calculated steam distilled, percentage of *M. alternifolia* chemotypes

Chemotype	1 ‘Type’	2, ‘Var D’	3, ‘Var C’	4, ‘Var A’	5, ‘Var B’	6
Compound						
Terpinen-4-ol (adjusted)	34–54	<5	16–19	16–20	<6	<4
Terpinen-4-ol (Homer et al.)	34–54	<5	16–19	16–20	<6	<4
Terpinen-4-ol (Butcher et al.)	na-42	1–2	15–20	na	na	na
1,8-Cineole (adjusted)	0–8	5–26	18–28	24–44	55–72	47–64
1,8-Cineole (Homer et al.)	0–8	10–26	18–28	24–44	55–72	47–64
1,8-Cineole (Butcher et al.)	0–11	17–34	30–36	36–48	65–71	na
Terpinolene (adjusted)	1–5	48–74	18–27	0–2	0–2	6–15
Terpinolene (Homer et al.)	1–5	48–69	18–27	0–2	0–2	6–15
Terpinolene (Butcher et al.)	na	28–57	10–18	na	na	na

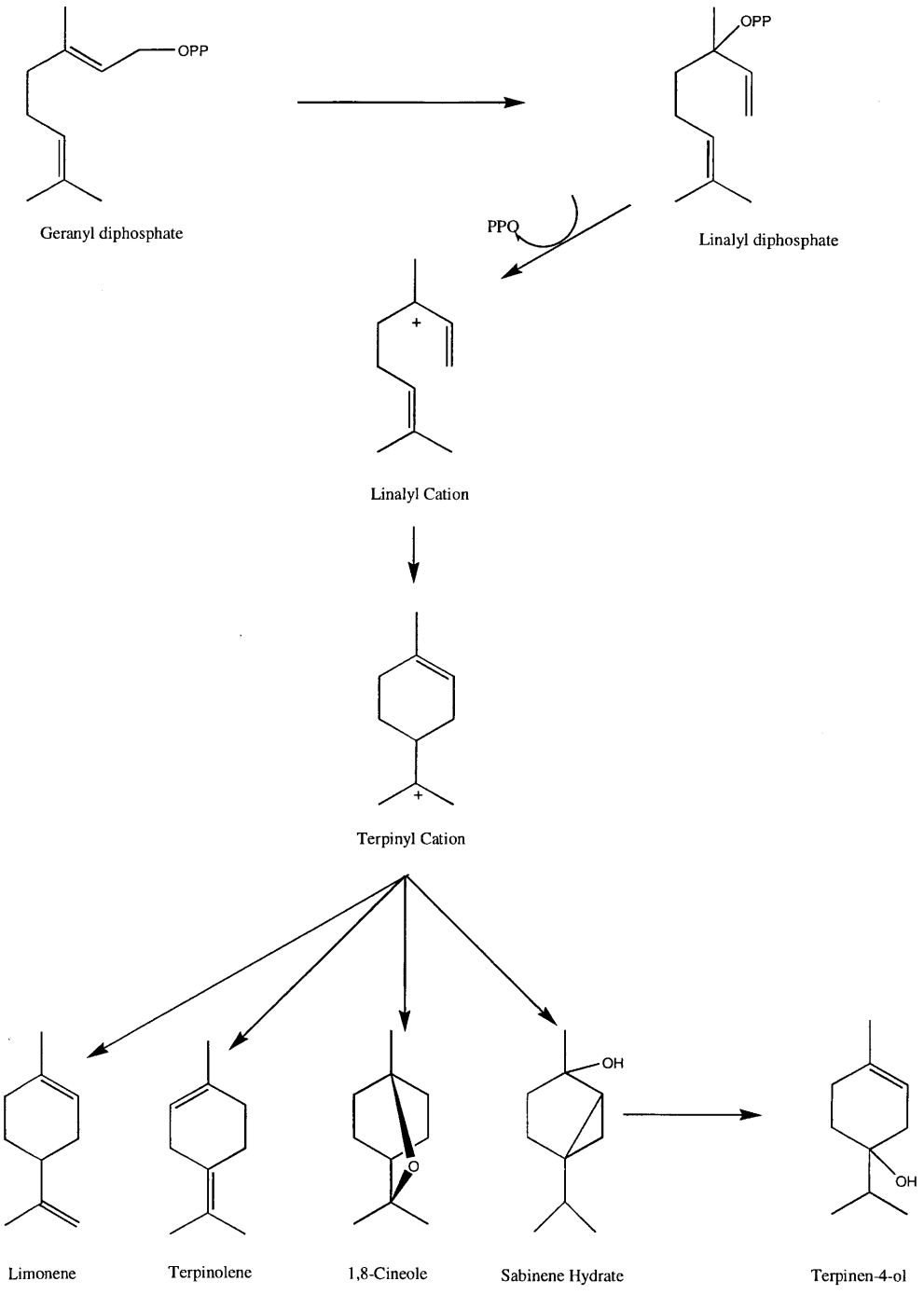

Fig. 1 Proposed biosynthetic pathway of monoterpenes frequently present in the essential oil of *Melaleuca* and *Eucalyptus*

1,8-cineole and terpinolene dominate individually. The remaining three chemotypes are dominated by 1,8-cineole but vary according to their relative terpinen-4-ol and terpinolene content. Butcher et al. (1994) showed that terpinen-4-ol levels were associated with the levels of α-pinene, α-terpinene, p-cymene and γ-terpinene, whilst 1,8-cineole levels were associated with limonene and α-terpineol levels, whereas terpinolene levels appear to be associated with 1,8-cineole, limonene and γ-terpinene levels.

The formation of multiple products from a single monoterpene synthase is not uncommon and has been displayed in many monoterpene synthases isolated from a diverse variety of plants including *Mentha spicata* (Colby et al. 1993), *Abies grandis* (Bohlmann et al. 1997, 1999), *Salvia officinalis* (Wise et al. 1998) and *Arabidopsis thaliana* (Bohlmann et al. 2000). Recent isolation and recombinant expression of 1,8-cineole synthase from *S. officinalis* (Wise et al. 1998) has shown that this enzyme, whilst predominantly catalysing the formation of 1,8-cineole, also catalyses the formation of another oxygenated monoterpene, α-terpineol, as well as a mixture of olefins. Isolation of a terpinolene synthase from *A. grandis* (Bohlmann et al. 1997), which predomi-

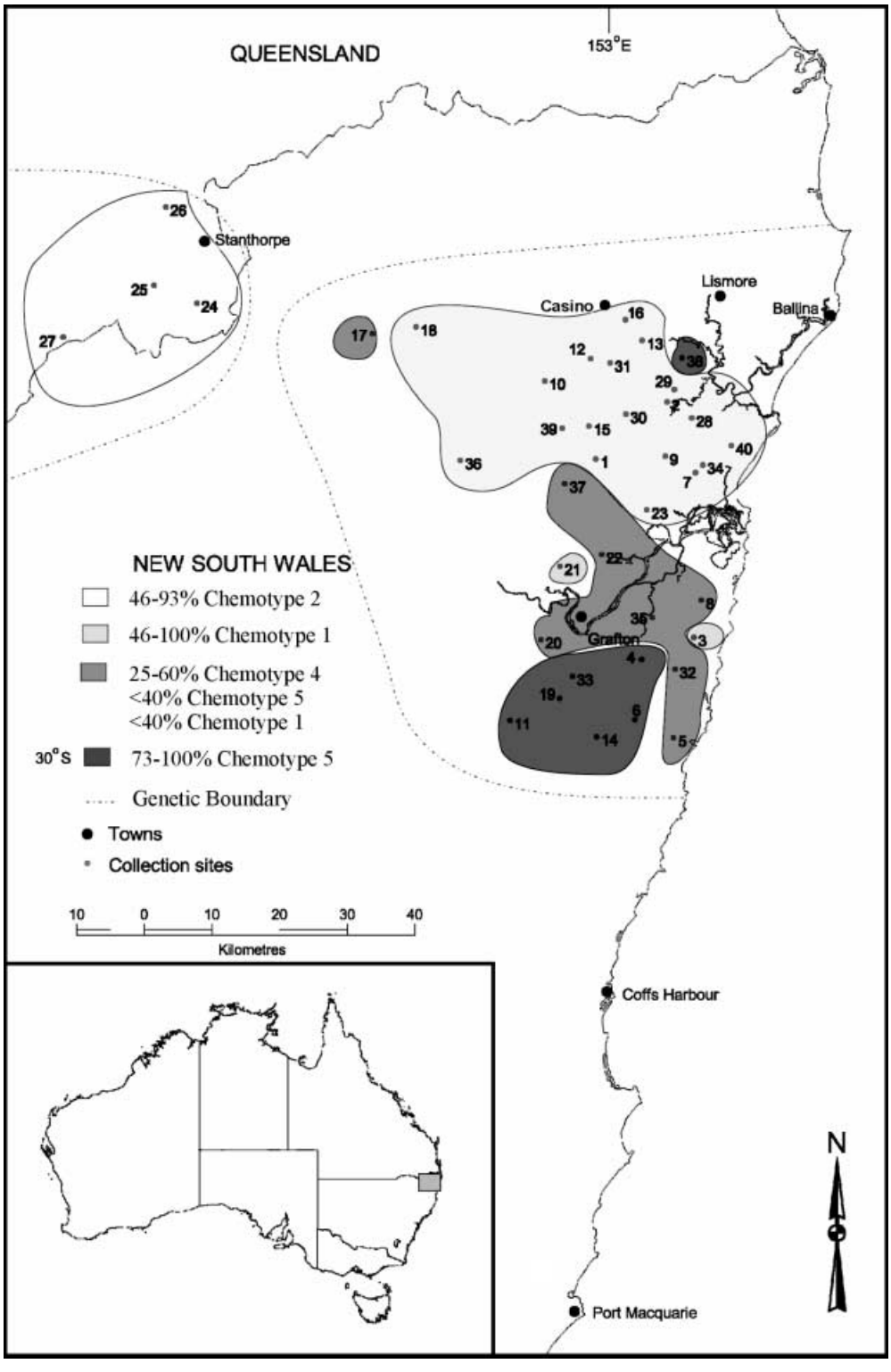

Fig. 2 Genetic provenances and distribution of chemotypes across the natural population of *M. alternifolia*

nantly catalyses the formation of terpinolene, also catalyses the formation of numerous other monoterpenes. This is believed to be due to the promiscuousness of the highly reactive terpinyl cation-intermediate of the cyclisation (Fig. 1). The terpinyl cation-intermediate can then either be de-protonated to form the olefin monoterpenes or quenched by water to form the oxygenated monoterpenes found in Tea Tree oil. This mechanism permits a vast array of monoterpenes to be produced by a comparatively small number of enzymes. It is therefore likely that the numerous monoterpenes present in Tea Tree oil are the products of relatively few enzymes which synthesize a predominant monoterpene in association with lesser amounts of numerous other monoterpenes. For this

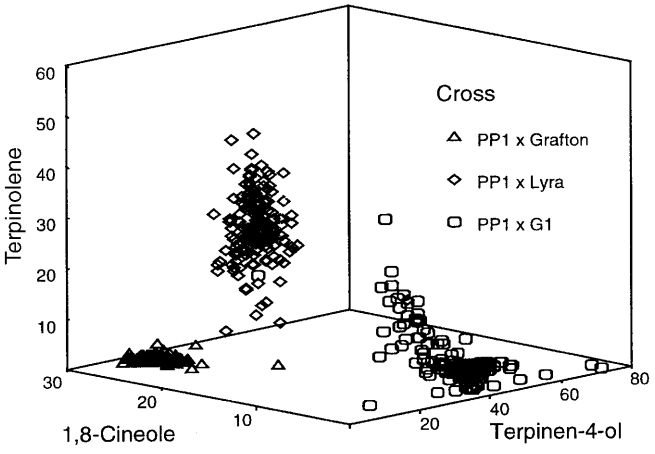

Fig. 3 3D scatter plot of the F1 population displaying the unimodal distribution of each cross based on essential oil monoterpene composition

reason, only the contribution of the three predominant monoterpenes from Tea Tree oil, terpinen-4-ol, 1,8-cineole and terpinolene, were assessed in each individual.

As a method to evaluate the level of genetic control on the essential oil composition of *M. alternifolia*, crosses were made between three distinctly different chemotypes from three different locations and the resulting progeny grown under uniform conditions. A single high terpinen-4-ol, chemotype 1 individual (PP1) was chosen to be the maternal parent for all crosses. The paternal parents consisted of a high terpinen-4-ol, chemotype 1 individual (G1) collected from the same plantation as the maternal parent, a high 1,8-cineole, chemotype 5 individual (Grafton) from the same genetic provenance as the maternal parent, and a high terpinolene, chemotype 2 individual (Lyra) from the other smaller genetic provenance (Fig. 2).

To visualise any possible segregation of chemotypes within the crosses, a 3D scatter plot was created (Fig. 3) with each axis representing terpinene-4-ol, 1,8-cineole or terpinolene as these are the three constituents that determine an individuals chemotype. No evidence of segregation was displayed by any of the crosses in regards to the oil profile. Each crossed population displayed a unimodal distribution of an oil profile intermediate to that of the parents. For this reason the average oil profile of each cluster was taken and assumed to be the typical oil profile for this cross.

Both parents for the chemotype 1, high terpinen-4-ol cross were obtained from the same location in the large genetic provenance. This cross resulted in 215 progeny with oil profiles similar to that of the parents. The average oil profile of this cross was approximately 38% terpinen-4-ol, 3% 1,8-cineole and 5.5 % terpinolene (Table 2).

The cross between the chemotype 1, high terpinen-4-ol, PP1 maternal parent and the chemotype 6, high 1,8-cineole Grafton paternal parent, resulted in 201 F1 progeny. The mean oil profile of these siblings was clearly that of a typical chemotype 4 plant with plants containing approximately 25% 1,8-cineole, 15% terpinen-4-ol and 1.5% terpinolene (Table 2). This oil profile is one that is essentially an intermediate of both parents. The terpinen-4-ol levels are approximately 2-fold lower than that of the high terpinen-4-ol parent. Similarly the 1,8-cineole content of the progeny is approximately 3-fold lower than that of the high 1,8-cineole parent. Further analysis of the data obtained by Homer et al. (2000) revealed that the chemotype 5 plants, those with the highest 1,8-cineole content, predominated in a region flanked by regions containing a relatively high proportion of chemotype 4 individuals (Fig. 1). As the geographic distance increases away from the predominantly chemotype 5 region, the occurrence of chemotype 1 individuals also increases revealing a gradual stratification in chemotypes from high 1,8-cineole chemotype 5 individuals to high terpinen-4-ol, chemotype 1 individuals via intermediate chemotype 4 individuals. A single outlying group of chemotype 5 individuals at the extreme north of the largest genetic provenance also show a similar pattern of stratification. The sites adjacent to this outlying chemotype 5 group, (sites 13 and 16) are also adjacent to an area consisting of >95% chemotype 1 individuals; subsequently, these sites bounded by chemotype 1 and chemotype 5 rich areas contain a relatively high proportion (25%) of the intermediate chemotype 4 individuals. Although not as distinct as in *M. alternifolia*, a similar pattern of chemotypic stratification can be observed in the four chemotypes in the natural population of *Chamelaucium uncinatum*, another member of the Myrtaceae family. In this population, endemic to southwestern Australia, chemotypes high in citronellal and limonene are clustered at the northern and southern extremities of the population respectively. The remaining two chemotypes occur in regions central to and flanking

Table 2 Comparison of the mean calculated steam distilled oil composition of the progeny and parents

PP1		G1		Lyra		Grafton	
		Terpinen-4-ol	48.5%	Terpinen-4-ol	1%	Terpinen-4-ol	1%
		1,8-Cineole	1.5%	1,8-Cineole	5.5%	1,8-Cineole	70.5%
		Terpinolene	2.5%	Terpinolene	74%	Terpinolene	<1%
Terpinen-4-ol	44%	Terpinen-4-ol	38% +/– 8.5%	Terpinen-4-ol	12.5% +/– 4%	Terpinen-4-ol	15% +/– 2%
1,8-Cineole	1%	1,8-Cineole	3% +/– 1%	1,8-Cineole	14.5% +/– 2%	1,8-Cineole	26% +/–1.5%
Terpinolene	3%	Terpinolene	5.5% +/– 5.5%	Terpinolene	31.5% +/– 7%	Terpinolene	1.5% +/– 2.5%
Number of Individuals		215		178		201	

the limonene and citronellal chemotypes. One of these two chemotypes is dominated by α-pinene. The fourth chemotype displays an oil profile intermediate to that of the southern, northern and other central chemotypes and is co-dominated by limonene, citronellal and α-pinene (Egerton-Warburton et al. 1998). Although there is no genetic data to confirm gene flow between these chemotypes their proximity to each other (within 700 km) suggests that gene flow is likely to occur.

The cross between the high terpinolene, chemotype 2 parent, Lyra, and the high terpinen-4-ol parent, chemotype 1, PP1 parent, yielded 178 F1 progeny. The average oil composition for these progeny was approximately 12.5% terpinen-4-ol, 14.5% 1,8-cineole and 31.5% terpinolene. This oil profile does not align precisely with any previously defined chemotype but closely resembles chemotype 3, with a profile intermediate to that of the parents. This oil profile differs from the previously defined boundaries for chemotype 3 by being 5% higher in terpinolene content and 3.5% lower in both 1,8-cineole and terpinen-4-ol content. This minor deviation from the prior description of the chemotype 3 oil profile is most likely to have arisen because the Lyra parent is an extreme outlyer with a terpinolene content already 5% higher and a 1,8-cineole content 5% lower than previously defined levels (Table 1). The broadening of the chemotype boundaries from the prior description of the chemotype 3 oil profile is most likely to be an artifact caused by the rarity of this chemotype in the natural population.

Chemotype 3 was displayed in only ten of the 615 individuals sampled by Homer et al. (2000); of these ten individuals, nine were located in the smaller, geographically isolated, genetic provenance. In the smaller genetic provenance, chemotype 3 is relatively abundant, representing approximately 20% of the individuals. This relative abundance may have arisen as a result of similar crosses occurring naturally within the provenance. The rarity of chemotype 3 within the larger genetic provenance, with only one individual in 555 plants sampled, is most likely explained by its limited breeding opportunities restricting genetic combinations to occur, rather than strong phenotypic selection for this chemotype in specific geographic regions.

Noticeable in the Lyra × PP1 cross is the fact that all oil constituents are not in proportions intermediate to that of the parents. The level of 1,8-cineole in the progeny is, on average, approximately 10% higher than that of the parents. The occurrence of compounds in greater abundance in the progeny than in either of the parents is not a new phenomenon in the Myrtaceae family to which *Melaleuca* belongs. Although investigations into the variability of essential oil composition in controlled infra-specific crosses have been limited in number, investigations in inter-specific and inter-generic crosses have been more numerous. Increased proportions of monoterpene constituents in the progeny compared to the parents have been observed before in inter-specific hybrids of other members of the Myrtaceae, such as *Eucalyptus* (Grayling and Brooker 1996). In these cases, as in the case of *M. alternifolia*, the essential oil profile is generally an intermediate of both parents, with the exception of the occasional compound which appears to display a form of hybrid vigour and occurs in quantities greater than that of either parent. This is a stark contrast to some genre of plants, such as *Mentha* which has served as a model plant for terpene genetics, and in which the essential oil composition of the progeny is determined predominantly by the male parent (Patra et al. 2001).

Of particular interest are the inter-specific hybrids of *Eucalyptus grandis* and *Eucalyptus urophylla* that were generated and mapped with respect to the monoterpene composition of their essential oil (Shepherd et al. 1999). In this cross a maternal *E. grandis* individual, common to all progeny, was heterozygous for the QTL of interest while the paternal *E. urophylla* plants were homozygous-null individuals. The relative proportions of the monoterpene in the progeny were determined using the log ratios between the individual constituents of the essential oil. Individual progeny heterozygous for the QTL were strongly linked to those with the highest log ratios and, therefore, higher proportions of limonene. These results imply that substantial influence on the genetics controlling the proportion of limonene in the essential oil can be attributed to the QTL associated with the *E. grandis* maternal parent. These may suggest a parental bias, similar to that of mint (Patra et al. 2001), in determining oil composition in *Eucalyptus*. This is contrary to the observations in *M. alternifolia* but may be due for multiple reasons. Although *Eucalyptus* and *Melaleuca* are closely related, the genetics controlling monoterpene composition may vary between genus or even between species. Another explanation is that the differing outcomes are artifacts of different methods used to evaluate the monoterpene proportions in the essential oil. In the *Eucalyptus* study the essential oil profiles of the parent trees are unknown, inhibiting a direct comparison between the parents and the progeny. This method of QTL analysis also requires the QTL affecting oil composition to be absent or at a very low frequency in one of the parents, and hence does not allow the identification of additive effects. The restricted amount of genetic diversity used in both the *Eucalyptus* and*Melaleuca* studies may represent different genetic combinations possible in both genra. The *Eucalyptus* example may be examining a single gene effect in combination with a null allele, while the *Melaleuca* example may be examining additive gene effects. A definite conclusion may only be reached using a much broader selection of both maternal and paternal parents.

Although the conversion to log ratios allows the proportional data to be analysed using standard statistical methodology, the formation of multiple products by these enzymes may bias the results. The calculation of log ratios between the monoterpenes may, in actuality, be examining the ratio of the different monoterpenes produced by a single enzyme. The formation of more than two products from a single enzyme would result in a strong correlation, as is shown in *Eucalyptus*, between

different log ratios. Without the log ratio data from the maternal parent it is difficult to determine if the log ratio data of the progeny accurately reflects the proportion of the various monoterpenes in the essential oil, or the enzyme specificity of the maternal 'limonene synthase'. While both methods determin the proportions of monoterpenes in the essential oils, caution should be displayed until the unusual property of the production of multiple monoterpenes from a single enzyme is further understood and the statistical bias issues can be resolved.

This present study has been unable to elucidate clearly the precise genetic basis for the presence of chemotypes in *M. alternifolia*. Nevertheless, what is clear from the data is that the chemical profile of the essential oil has a strong genetic basis. The high level of geographic structuring of chemotypes in the field appears to reflect a high level of genetic structuring for the genes controlling the chemotype. By contrast, isozyme data (Butcher et al. 1992) and microsatellite data (Rossetto et al. 1999) have failed to show a high level of genetic structuring or any evidence of isolation by distance in the largest genetic provenance. Microsatellites are presumed to be effectively neutral to selection; therefore, gene flow, even at a limited level, will tend to homogenize allele frequencies across a population (Mills and Allendorf 1996). Therefore, interpretation of the microsatellite data tends to indicate that there is at least some gene flow within the genetic provenances of *M. alternifolia*. The chemotype data therefore indicates that selection for chemotypes at the local level is occurring and is sufficient to counteract the effects of gene flow.

In conclusion, the results of these crosses, combined with further analysis of the distribution of chemotypes in the natural population, suggest that there is a lower level of genetic hierarchy within the genetic provenances that was not detected by either isozyme or microsatellite analysis. This information may have strong implications to future Tea Tree breeding programs if genetic material is to be obtained from the natural population. If the data from the analysis of monoterpene composition is to be extrapolated, it suggests that the largest amount of genetic diversity could be ascertained by selecting individuals with the greatest difference in oil profiles. Until more is understood about the genetics of monoterpene composition and biosynthesis in *M. alternifolia*, it appears that phenotypic selection of genetic material for breeding programs still offers the largest opportunity to manipulate and select plants with superior oil profiles for commercial propagation.

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