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Evidence for clonality, breeding system, genetic diversity and genetic structure in large and small populations of *Melaleuca deanei* (Myrtaceae)

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Supplementary Information

A1 Cross Species Amplification of Microsatellites

Approximately 20 mg of silica-dried leaf tissue was ground to a powder in liquid nitrogen following which total DNA was extracted using DNeasy MiniPlant kits (QIAGEN Pty Ltd Doncaster, Vic) according to the manufacturer's instructions. The concentrations of the resulting DNA were measured with a Qubit fluorometer (Invitrogen) and ranged from 4ng/μL to 42ng/μL.

A2 Primer Screening

Thirty four of the primer pairs developed for *M. alternifolia* by Rossetto *et al*. (1999a) were tested to determine if they would prime amplification of DNA from *M. deanei.* For this part of the study, DNA from three sites (Table A1) were screened alongside DNA from *M. alternifolia* (extracted from two plants from the Hawkesbury campus plantings) acting as a positive control. A range of PCR conditions were tested on each primer pair, including increasing and decreasing the primer, Taq and DNA concentrations; examining annealing temperature using a temperature gradient (55–66ºC with increments of 1º C); and altering the cycle number and length. This resulted in the following general protocol: DNA amplified in 12.5 μL reactions mixtures comprising: 0.125 μL Taq DNA polymerase (New England BioLabs); 1.25 μL 10 × Thermopol Buffer (New England BioLabs); 0.25 μL dNTPs; 1 μL forward primer; 1 μL reverse primer, 1 μL DNA, 9.85 μL nuclease free water. Nuclease free water was substituted for DNA in samples used as negative controls and *M. alternifolia* DNA substituted in samples used as positive controls. Polymerase chain reactions (PCR) were performed in a Peltier thermocycler (BioRad Dyad DNA Engine) modified around the following cycling conditions: initial denaturation, 94ºC for 5 min; 30 cycles of denaturation, 94ºC for 30 s; annealing at 55–66ºC for 30 s; extension at 72ºC for 1 min; final extension, 72ºC 5 min. Amplicons were separated in 2% agarose gels, visualised using ethidium bromide (1 μg/mL) or GelRed (Biotium) (0.5 μg/mL) with their size determined by inclusion of a lane containing a 100 bp ladder (BioLine Hyperladder V BIO-33031). The gels were made with electrophoresis running buffer (1 × TBE), and electrophoresis occurred at room temperature at 90 V. Gels were photographed under UV light and a GelDoc (BioRad).

Table A1. Sites from which DNA was trialled during primer screening

Site	GPS
1. Marrua Track, North Turramurra	33 41 29 S, 151 08 41 E
2. Menai Conservation Park	34 00 41 S 151 00 56 E
3. Victoria Road, Wedderburn	34 07 03 S 150 50 11 E

A3 Fluorescent Labelling

The forward primers of potentially useful primer pairs were then modified to include an M13(-21) tail sequence at their 5' end (Schuelke 2000) (labelled sequence-specific forward primers). Reactions were repeated in singleplex with the M13-labelled sequence-specific forward primer, a reverse primer and a fluorescently labelled universal primer (6-carboxyfluorescein (FAM) (M13-FAM Primer probe, Life Technologies catalogue number 450007)), as per Blacket *et al*. (2012).

PCRs were re-optimised to a 12 μL reaction volume comprising: 0.25 μL of BioLine My*Taq* DNA polymerase (BioLine BIO-21108); 2.5 μL My*Taq* buffer; 0.125 μL 1/10 M13(-21)-tailed forward primer (i.e., ¼ of reverse amount); 0.5 μL reverse primer; 0.5 μL FAM-labeled M13(-21) primer; 6 μL water; 1 μL DNA. A water control was used in PCR with each mastermix to test for contamination.

Final PCR reaction conditions were as follows: initial denaturation, 95ºC for 1 min; 35 cycles of denaturation, 94ºC for 15 s; annealing (variable and determined during primer screening) 15 s; extension, 72º C for 10s; 8 cycles of denaturation, 94ºC for 15 s; annealing, 53ºC for 15 s; extension, 72ºC for 10 s; final extension, 72º C 5 min. Amplicons were visualised on gels (Fig. A1).

A4 Development of the Multiplex Panels

Primers that amplified clear, reproducible bands in singleplex at similar optimal annealing temperatures and with size differences of at least 60 base pairs and variation (polymorphism) among the three test sites were then identified and trialled in true multiplex reactions (single reaction PCR with two primers and a single fluorescent colour

tag amplified in the same reaction). Some alterations of *Taq* and primer concentrations and annealing temperatures were again required per general guidelines available in Henegariu *et al.* (1997). Pseudomultiplexing was also employed where the products of four true multiplex amplifications each utilising a different coloured fluorescent tag were combined into a single sample for fragment analysis (terms *sensu* Guichoux *et al*. 2011). Additional, fluorescently-labelled, M13(-21) primer probes for binding with the forward primers were used for this (M13-VIC–fluorescent green, M13-PET–fluorescent red and M13-NED–fluorescent yellow).

My*Taq* Red Mix (BIO 25043) was used for final reaction cocktails. This altered the reaction cocktail mix, as the mix incorporates the buffer. Final PCR amplifications were performed in 12 μL reaction mixtures each containing 8 μL of BioLine MyT*aq Red* DNA polymerase Mix (BioLine BIO-25043). To amplify one microsatellite sequence, the following mixture was used: 0.2 μL of 10 μM forward primer with an M13(-21) sequence appended at its 5'end, 0.8 μL 10 μM reverse primer, 0.8 μL of 10 μM oligofluorescent dye-labelled (FAM/PET/NED/VIC) M13(-21) primer, 1 μL DNA and 1.2 μL of DNA-free water. For multiplex amplifications, additional primer pairs were added and the amount of water adjusted appropriately.

The different *Taq* necessitated a different thermocycler protocol. PCR reactions were run on a Peltier thermocycler (BioRad Dyad DNA Engine) under the following cycling conditions: 95^{o}C *Taq* activation for1 min, followed by 28 cycles of denaturation 95^{o}C for 15 s, optimal annealing temperature (ranging from 57^{o}C to 61^{o}C, see Table A3) for 15 s, 72^{o}C for 10 s, followed by 9 cycles of 53^{o}C for 15 s, 72^{o}C for 10 s and a final extension step at 72^{o}C for 10 min prior to cooling to 0^{o}C.

Amplicons were visualised following separation by capillary electrophoresis in an ABI 3500 capillary fragment analyser (Applied Biosystems 3500 Genetic Analyser). The analyser was equipped with an 8 channel, 50 cm capillary system which was loaded with POP-7 polymer and calibrated with a DS-33/G5 Matrix Standard Kit. GeneScan 600 LIZ v2.0 was used in each sample as the sizing standard. The samples were run under default settings according to the manufacturer's instructions.

Amplifications were deemed to be successful if they produced clear products within 100 bp of the corresponding fragment from *M. alternifolia* (Rossetto *et al.* 2000) and displayed a typical microsatellite profile. Samples were run at least three times with each

primer combination using DNA from the three different sites (Table A1) to check the reproducibility of peaks.

A number of serial dilutions were prepared initially and assayed to obtain optimal sample dilution for fluorescent signal between 200 and 6000 rfu (reference fluorescent units or the y axis on the electropherogram) (Prince 2015).

A5 Scoring Microsatellites

PCR products were visualised using GeneMapper Version 5 (2012 Life Technologies), one of the most popular and reliable software packages for fragment analysis (Prince 2015). Microsatellite peaks were identified from baseline background noise using a minimal allele intensity (minimum peak height) cut-off of 400 rfu and sized by comparing their electrophoretic migrations with fragments in the LIZ size standard (Fig. A2). Heterozygotes were called where the intensity of the second peak (y axis height) exceeded 50% of the first. True allele size calls were recorded using decimal numbers and then binned, i.e., converted from real value DNA fragment sizes to discrete units (bin width = 1.2) with an integer label assigned. Manual binning of allele sizes within a small range of raw fragment lengths is routinely employed to reduce errors in allele calling that result from differences in allele mobility under differing temperatures or separation matrices when samples are run on different days (Amos *et al*. 2007). Microsatellite shape and stutter bands were helpful in distinguishing true alleles from artefacts (See Guichoux *et al*. 2011).

A6 Cross Species Amplification of Microsatellites

DNA yields adequate for PCR ranged from 4.3 ng to 44 ng. Twenty three of the 34 primers screened amplified clear, reproducible bands from *M. deanei* and *M. alternifolia* controls in simplex. Eleven were rejected because of: (1) multiple fragments or non-specific amplification of other loci (too variable/too much noise): 009, 010, 023 and 133; (2) did not amplify consistently/blanks re-occurring: 081 and 131; (3) weak, no stutter: 084, 127, 130; or (4) blank, null alleles: 001 and 015 (Table A2). A further four (008, 025, 041 and 143) were rejected because of monomorphism or a failure to show polymorphism in samples from the test sites (Table A2) as they are genetically uninformative. Amplicon

size ranges were similar to those allele sizes found in the species for which the primers were originally developed (Table A3).

Interestingly, SSR marker 071, which was reported monomorphic by Rossetto in *M. alternifolia*, was found to be polymorphic and useful in *M. deanei*. In all cases, better results were obtained at annealing temperatures 4–10°C higher than those published by Rossetto *et al.* (1999a) for PCR with *M. alternifolia,* the species for which these primers were originally developed. The lower temperatures initially trialled with *M. deanei* resulted in multiple bands on agarose gels indicating amplification of non-specific DNA fragments and typical of PCR in which annealing temperatures are too low (Rychlik *et al*. 1990).

Table A2. Results at each step of the screening process on thirty four microsatellite primer pairs developed for *M. alternifolia* and tested in *M. deanei.*

Primer pair from *M. alternifolia* tried	Amplified in simplex in *M. deanei* close to published length of *M. alternifolia*	Amplified with M13 tag on forward primer and universal M13 fluoro tail	Variation across three trial sites	Consistent/ reliable in multiplex with a clear microsatellite profile
001	No			
003	Yes	No		
007	Yes	Yes	Yes	Yes
008	Yes	Yes	No	
009	No			
010	No			
013	Yes	Yes	Yes	Yes
014	Yes	Yes	Yes	Yes
015	No			
016	Yes	Yes	Yes	Yes
023	No			
025	Yes	Yes	No	
031	Yes	No		
039	Yes	Yes	Yes	No
041	Yes	Yes	No	

044	Yes	Yes	Yes	No
048	Yes	Yes	Yes	Yes
052	Yes	Yes	Yes	Yes
071	Yes	Yes	Yes	Yes
078	Yes	Yes	Yes	No
081	No			
084	No			
098	Yes	Yes	Yes	Yes
123	Yes	Yes	Yes	Yes
124	Yes	No		
125	Yes	Yes	Yes	No
127	Yes	Yes	Yes	Yes
130	No			
131	No			
133	No			
134	Yes	Yes	No	
136	Yes	Yes	Yes	Yes
137	Yes	Yes	Yes	Yes
145	Yes	Yes	Yes	Yes

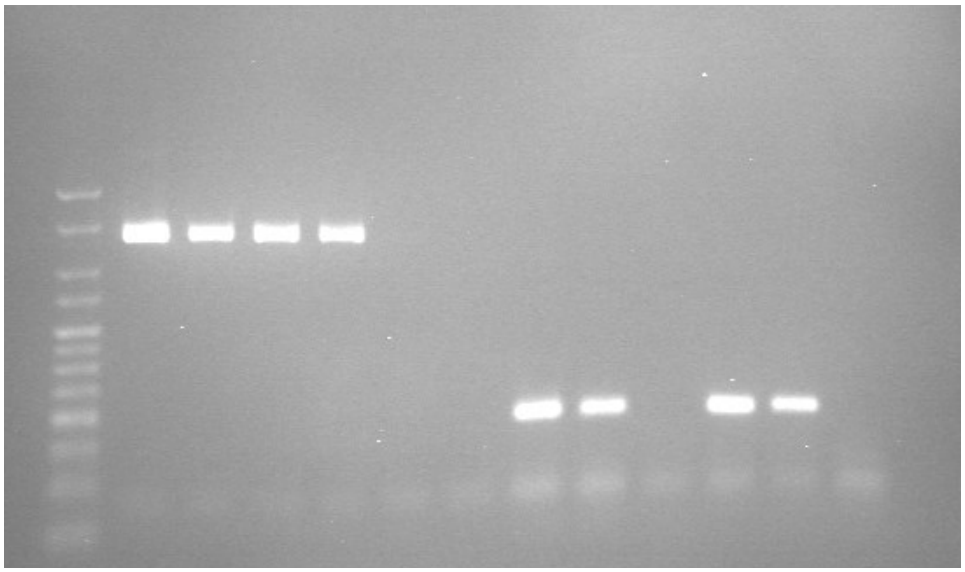

Fig. A1. Simplex PCR of primer pairs, 071 and 078 on agarose gel. The forward primer of each has an M13 tail is FAM-labelled, tested using DNA from M. alternifolia as a positive control in lanes 1 and 7, from three M. deanei sites in lanes 2–4 and 8–10 and non-template (water) negative controls in lanes 6 and 11.

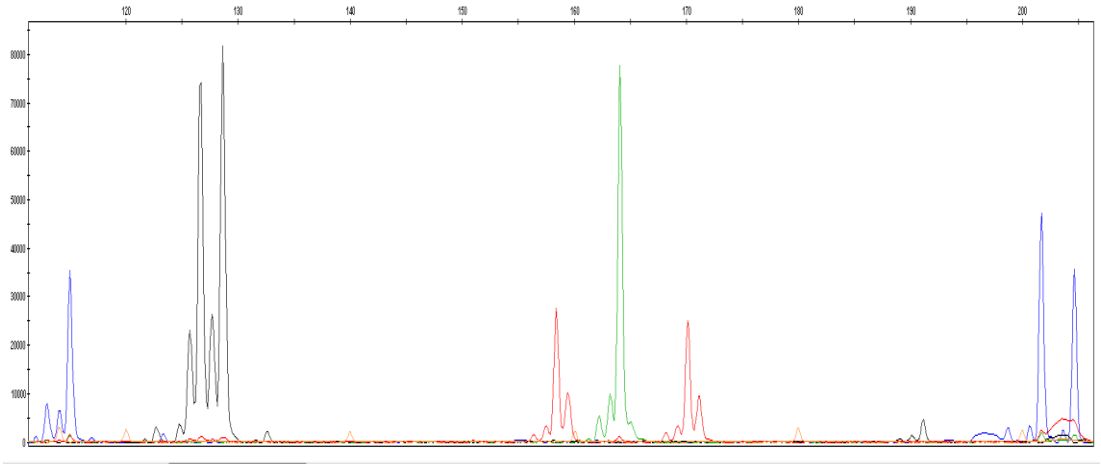

Fig. A2. Electropherogram of a multiplex sample showing detection of biallelic microsatellite fragments tagged with four different fluorescent dyes. Sample plant Showground, St. Ives, 'plant' 2.

Table A3. Final multiplex panels colour coded by fluorescent tagging. Blue = FAM, red = PET, yellow = NED and green = VIC.

Panel 1 Locus	Primer sequence 5'-3'	Published length	Amplicon size range obtained	Temp optima (ºC)	No. of alleles in trials
136	F: GCTTCTAGGATTATGGATTTCTCAG R: GTCACTTTCACTTTCAGTTGGAA	169	152-170	59	5
48	F: TGAGGCAATGCACAGTCTCAGT R TCTCAACCCACTAAAGCGGAGT	337	330-380	59	6
145	F: CCTCCTCCGTTAATCGCTGC R: ACCCGTCCGCAGAATCGCAA	202	190-211	60	5
7	F: AAACCCTAGAAAGAAACTCCCT R: GGTCTGGAAGCACCCATTT	129	95-121	60	8
123	F: CCCCTAGCATCTCGCCACTGTTAT R: TCGACGGTTGACTCTGGTTGTAGTT	173	155-169	58	6
71	F: CGTCCCTTCAAACCGTCCTCAA R: AGCATCCTCAGCCACCACACAA	359	365-386	58	7
14	F: GCCTGCATCAGACATGATATAGA R: CCATGAGACTCTTTTGCGATT	132	120-126	59	3
Panel 2 Locus	**Primer sequence 5'-3'**	**Published length**	**Amplicon size range obtained**	**Temp optima (ºC)**	**No. of alleles in trials**
13	F: CCGAATCATCTAAAAGGAGTCT R: CCAAGTTGACACACTTACTTCAA	128	116-140	61	8
98	F: TTGACGCATAAGATGCAA R: TGAGGTGGCTTCATATTTGT	200	169-195	57	4
16	F: AGAGATCAATCAACCGTCTTG R: CCCATCTTTTCCCTCATTTAC	144	121-159	60	7
52	F: CTGCCGCATTAGGGAATCATT R: CAGAACGCCAGTTGATGAAAGC	225	329-350	60	5
137	F: CAAGTGACTCTGACGACGTTGG R: CAGTAGGAGAGGGGATCAATTTG	205	188-208	58	4

A further eleven of the 23 primers that were selected in simplex trials were screened out owing to a failure to incorporate the M13 tail and fluorescent tagging; inconsistency or unreliability in multiplex trials or size similarity of amplicons meant they could not be efficiently included in two panels (Table A2). This left 12 markers in 2 multiplex panels (Table A3).

By careful screening and extensive trials, a suitable primer set and two multiplex panels of microsatellite markers that were originally developed for *M. alternifolia* were established for *M. deanei* during this study. Null alleles and non-amplifications resulted in two of these loci being discarded from the final marker set for all site comparisons. The null alleles and non-amplifications may have resulted from polymorphisms in the annealing regions as variation or mutation in the nucleotide sequence of microsatellite flanking regions may prevent primers from binding to template DNA during amplification. This is more likely in species that have diverged from the population from which the primers were designed. Other causes include technical problems with the amplification, such as the preferential amplification of short alleles due to inconsistent DNA template quality or quantity or slippage during the PCR amplification (Chapuis & Estoup 2006).

Rossetto *et al*. (1999a) assayed five of these microsatellite loci in *M. alternifolia* across its distributional range and reported an average of 19.6 alleles per loci with a range from 7–31. I obtained a narrower range from a subset of these microsatellites in *M. deanei* of 3–14 alleles or an average of 7.7 alleles per locus. Consistent with Rossetto *et al* (1999b) I also detected greater numbers of alleles in the dimer dinucleotide repeat loci than the trimers (Table 3).

Table A4. Pairwise Nei value comparisons between 8 populations of *M. deanei* estimated from 10 microsatellite loci

	St. Ives	Galston	Brooklyn	Dural	Menai	Spillway	ND	WBurn
St. Ives	0							
Galston	0.372	0						
Brooklyn	0.244	0.335	0					
Dural	0.536	0.400	0.384	0				
Menai	0.329	0.306	0.273	0.383	0			
Spillway	0.529	0.213	0.367	0.345	0.213	0		
ND	0.423	0.212	0.455	0.406	0.247	0.216	0	
WBurn	0.404	0.239	0.335	0.311	0.183	0.162	0.176	0

Table A5. Pairwise F_{st} comparisons between 8 populations of *M. deanei* estimated from 10 microsatellite loci

	St. Ives	Galston	Brooklyn	Dural	Menai	Spillway	ND	WBurn
St. Ives	0							
Galston	0.101	0						
Brooklyn	0.093	0.097	0					
Dural	0.115	0.069	0.108	0				
Menai	0.108	0.087	0.096	0.105	0			
Spillway	0.147	0.074	0.124	0.088	0.076	0		
ND	0.112	0.063	0.126	0.074	0.081	0.063	0	
WBurn	0.111	0.056	0.102	0.071	0.062	0.053	0.048	0

Table A6. Mean number of alleles per locus (N_a) after rarefaction to a standardised sample size of 15 across all and within each population.

Locus	Total alleles	St Ives	Galston	Brooklyn	Dural	Menai	Spillway	N Dam	WBurn
M136	5	2	5	3	5	2	4	3	4
M145	6.3	3	5	4	4	4	4	5	3.9
M007	6	3	6	3	3	4	6.5	5	3
M123	4.7	3	3	4	3	3	2.8	5.1	4
M071	5.6	2	4.7	5	3	4	5.8	5.3	5
M014	3	2	3	3	3	3	2.8	3	3
M013	7.5	3	8.2	6.7	5.9	3	4.9	4.9	5.4
M016	11.5	5	6.3	4	3.9	3	6.8	7.4	6.6
M052	4.9	2	4.7	1	4	3	4.6	4.7	5.5
M098	4.2	2	4	1	4	1	1	4.3	2.7
Mean	5.9	2.7	5	3.5	3.9	3	4.3	4.8	4.3

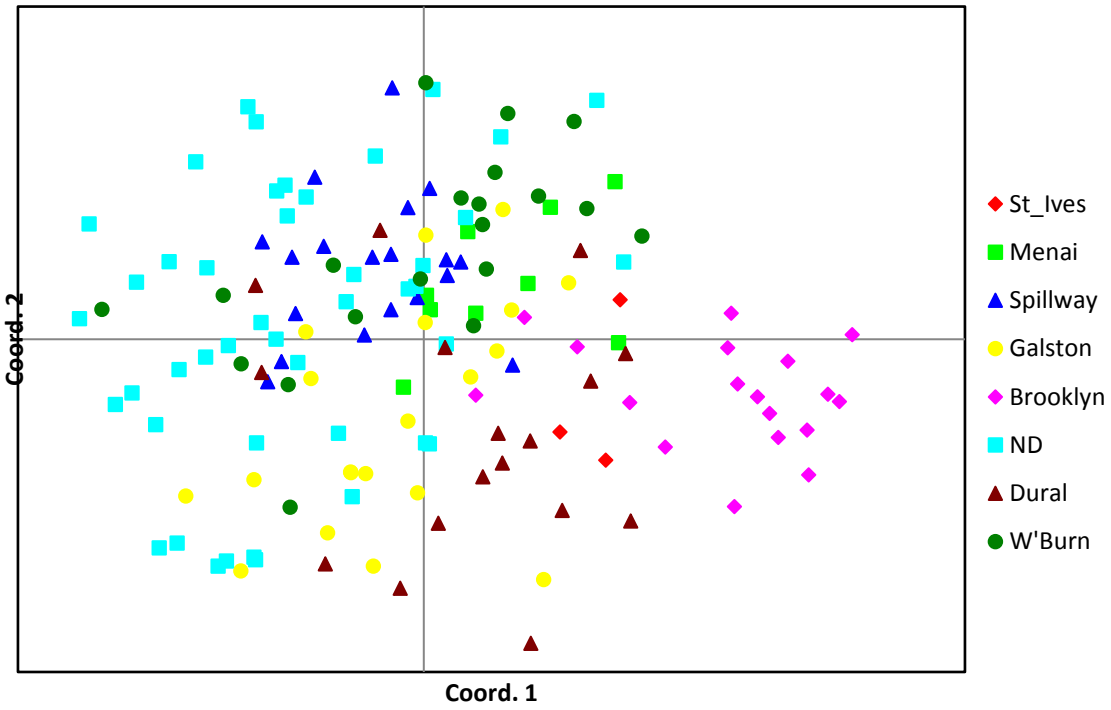

Fig. A3. Principal coordinates analysis illustrating weak grouping of populations using genetic distances among individual SSR genotypes

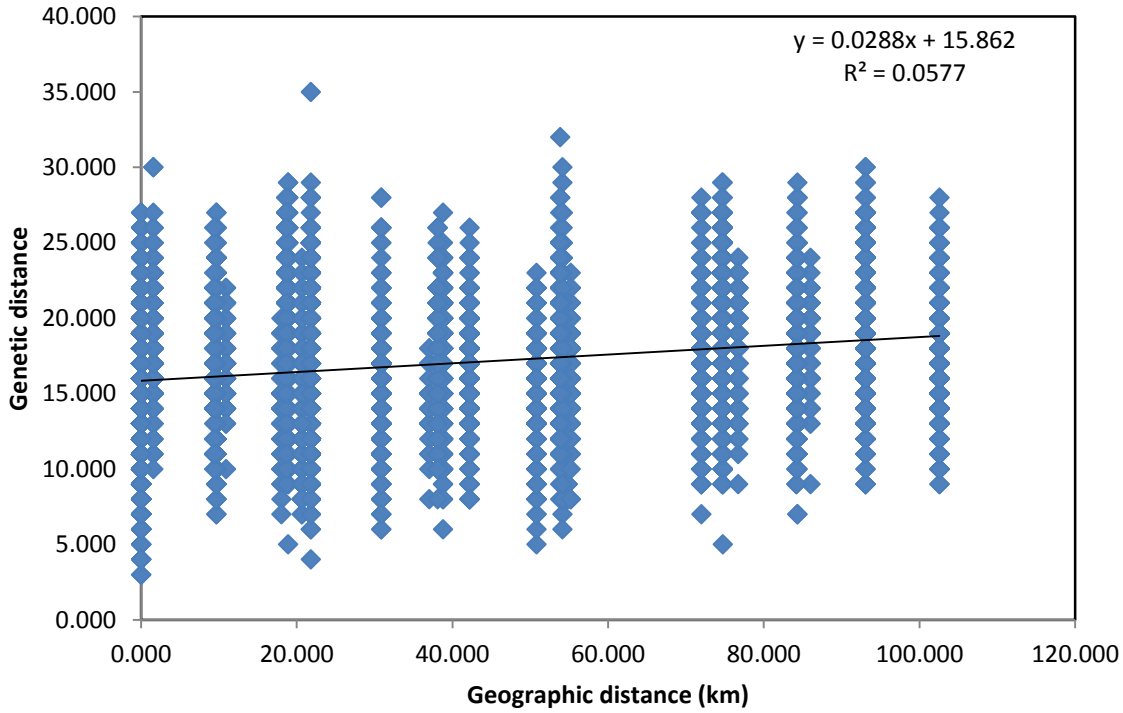

Fig. A4. Correlation between geographic and genetic distances calculated from pairwise comparisons among populations. Each vertical line of diamonds represents the samples from one site.

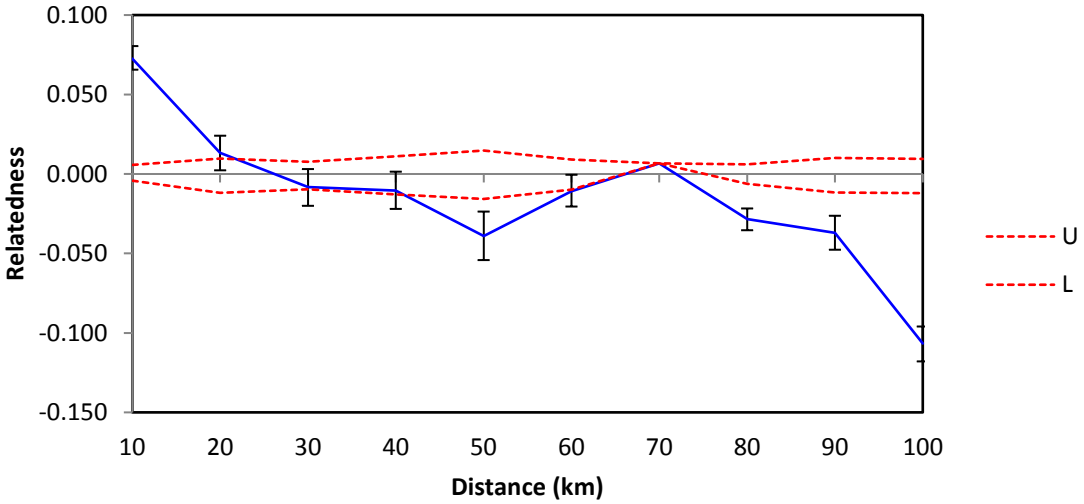

Fig. A5. Spatial autocorrelation analysis of eight sites with relatedness plotted against geographic distance up to 100 km. Upper (U) and lower (L) confidence limits.

References

Amos,W., Hoffman, JI, Frodsham, A., Zhang, L, Best, S. & Hill, AVS. (2007) Automated binning of microsatellite alleles: problems and solutions. *Molecular Ecology Notes* **7**, 10-14

Blacket, M.J., Robin, C., Good, R.T., Lee, S.F. & Miller, A.D. (2012) Universal primers for fluorescent labelling of PCR fragments – an efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources* **3**, 456–463.

Chapuis, M. & Estoup, A. (2006) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* **24**, 621–631.

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Leger, P., Lepais, O., Lepoittevin, C., Malausa, T., Revardel, E., Salin, F. & Petit, R.J. (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* **11**, 591–611.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. & Vogt, P.H. (1997) Multiplex PCR: Critical parameters and step-by-step protocol. *BioTechniques* **23**, 504-511.

Prince, L.M. (2015) Plant genotyping using fluorescently tagged inter-simple sequence repeats (ISSRs): Basic principles and methodology. *In*: 'Plant Genotyping Methods and Protocols' (J. Batley Ed.) pp. 63–76. Springer, New York.

Rossetto, M., McLauchan, A., Harriss, F.C.L., Henry, R.J., Braverstock, P.R., Lee, L.S., Maguire, T.L. & Edwards, K.J. (1999a) Abundance and polymorphism of microsatellite markers in tea tree (*Melaleuca alternifolia*, Myrtaceae). *Theoretical Applied Genetics* **98**, 1091–1098.

Rossetto, M., Slade, R.W., Baverstock, P.R., Henry, R.J. & Lee, L.S. (1999b) Microsatellite variation and assessment of genetic structure in tea tree (*Melaleuca alternifolia* - Myrtaceae). *Molecular Ecology* **8**, 633–643.

Rossetto, M., Harriss, F.C.L., Mclauchlan, A., Henry, R.J., Baverstock, P.R. & Lee, L.S. (2000) Interspecific amplification of tea tree (*Melaleuca alternifolia*-Myrtaceae) microsatellite loci-potential implications for conservation studies. *Australian Journal of Botany* **48**, 367–373.

Rychlik, W., Spencer, W.J. & Rhoads, R.E. (1990) Optimization of the annealing temperature of DNA amplification *in vitro*. *Nucleic Acid Research* **18**, 6409–6412.

Schuelke, M. (2000) An economic method for the fluorescent labelling of PCR fragments. *Nature Biotechnology* **18**, 233– 234.