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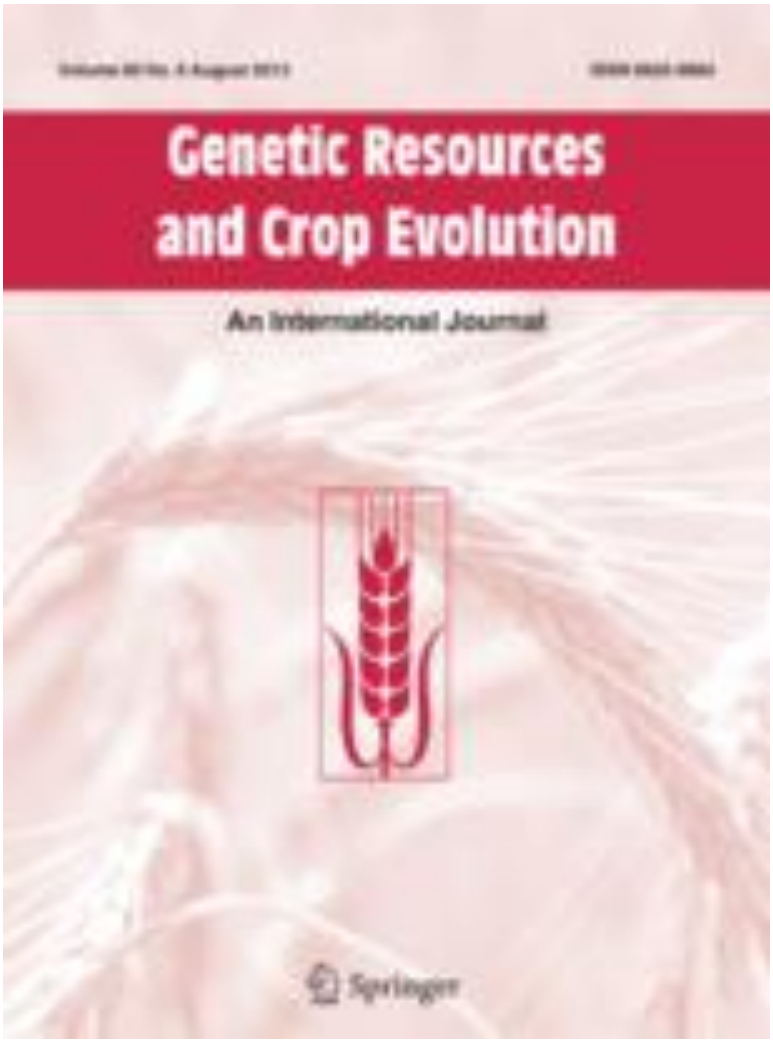

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RESEARCH ARTICLE

Genetic diversity, seed traits and salinity tolerance of *Millettia pinnata* (L.) Panigrahi, a biodiesel tree

Ni Luh Arpiwi · Guijun Yan · Elizabeth L. Barbour · Julie A. Plummer

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Abstract The leguminous tree, *Millettia pinnata* (pongamia) produces oilseed suitable for biodiesel production. Assessment of oil production and genetic, morphological and physiological traits are required. Collections from the Forest Products Commission in Kununurra, Western Australia were compared with accessions from India, Indonesia, Queensland and the Northern Territory in Australia. Molecular diversity, examined using the internal transcribed spacer region, indicated distinctiveness of genotypes from Java, Indonesia. Seed traits varied across trees with the smallest seeds from Indonesia and the largest from Kununurra. Oil content varied across trees with a minimum of 28 % in an Indonesian accession and the highest of 45 % from Kununurra. Major fatty acids across trees were oleic (51 %), linoleic (19 %), palmitic (11 %) stearic (6 %), linolenic (4.5 %) and behenic (4.5 %) acids. Seed weight and oil content per seed of developing seeds increased with a sigmoid pattern and oleic acid was the major fatty acid throughout seed development. Waterlogging and salinity tolerance were assessed. Four month-old seedlings from Kununurra, Western Australia and India were exposed to: non-saline drained control, saline drained, non-saline waterlogged and saline waterlogged treatments. Seedlings were waterlogging tolerant. Salt, applied in weekly increments of 50 mM, led to reduced survival, height growth rate, leaf number and stomatal conductance and increased concentrations of leaf Na^+ and Cl^-. Salinity tolerance was 200 mM NaCl under saline drained and 150 mM NaCl under waterlogged conditions. *Milletia pinnata* diversity could be exploited for selection of superior genotypes for oil production on marginal land.

Keywords Fatty acid composition · Genetic resources · ITS region · *Millettia pinnata* · Oil content · Pongamia · Salinity tolerance

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Introduction

Millettia pinnata (L.) Panigrahi syn. *Pongamia pinnata* (L.) Pierre is a medium sized leguminous tree.

Millettia pinnata seed contains oil, which is inedible but useful for biodiesel (Mukta et al. 2009). With the depletion of world oil reserves, this biodiesel tree could contribute to renewable energy (Scott et al. 2008) especially if it could be grown on marginal land unsuitable for food production.

Millettia pinnata grows in India, across Asia (China, Malaysia, Indonesia, Japan, the Philippines), Polynesia and tropical regions of Australia (Queensland and the Northern Territory) and it has been introduced into New Zealand and the USA as an ornamental tree (Scott et al. 2008). Many biodiesel crops have had previous uses, often as ornamentals, but as they emerge as biofuel crops, selection, breeding and production systems for feedstock are essential to gain maximum economical and environmental benefits (Azam et al. 2005). Initial steps require procurement of a germplasm collection, establishment and then phenotypic characterization for selection of important traits. The Forest Products Commission of Western Australia has a substantial collection of mature, seed bearing trees which were established from an Indian collection at the Frank Wise Research Station, in 1999 in Kununurra, in northern Western Australia. Studies of genetic diversity are limited to trees within certain states of India (Sahoo et al. 2010; Sharma et al. 2011) and a broader investigation of diversity is required.

Previous molecular genetic studies of *M. pinnata* used AFLP, TE-AFLP (Sharma et al. 2011) and ISSR (Sahoo et al. 2010). Sequencing of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA has been used for phylogenetic study of the Tribe Millettieae including *P. pinnata* (Hu et al. 2002). The ITS region evolves rapidly, has high sequence variation and contains many informative sites. It is relatively small, being under 700 bp (Baldwin et al. 1995), and therefore is suitable for species comparison between (Stappen et al. 1998) and within species (Ritland et al. 1993). Both TE-AFLP and AFLP indicate a high level of genetic diversity of *M. pinnata* collected from different locations in Delhi, India (Sharma et al. 2011) whilst ISSR indicates narrow genetic diversity within the trees from several regions of Orissa, India (Sahoo et al. 2010). Identifying and capturing the genetic diversity across *M. pinnata* is essential for future exploitation of this species and analysis of the ITS sequence is a suitable approach.

Seed oil quantity and quality are essential characters for biodiesel feedstock production. In particular, seed size, oil content and fatty acid composition are important criteria for selection of superior trees. Seed traits need to be characterized across a broad range of phenotypes and compared with molecular genetic diversity. In other crops, seed oil and fatty acid content vary during development (Hathurusingha et al. 2011; Saoussem et al. 2009; Saldivar et al. 2011). This needs to be investigated in *M. pinnata* to determine peak oil and the optimum fatty acid composition for biodiesel quality. This information is very important in determining an appropriate time for harvesting.

Second generation biofuel production targets marginal lands to avoid the conflict between food production and first generation biofuel crops (Gressel 2008). Saline and waterlogged soils are typical of marginal lands as they reduce plant growth and productivity (Rengasamy et al. 2003). *Millettia pinnata* is found in coastal habitats (Kesari and Rangan 2010; Mukta and Sreevalli 2010) indicating it should cope with waterlogging and salt concentrations equivalent to sea water, which contains about 500 mM NaCl (Flowers et al. 1986). It is purportedly highly salt tolerant (Divakara et al. 2010; Kaushik et al. 2007; Kesari and Rangan 2010) however preliminary pot and field experiments examining salinity tolerance have yielded mixed results (Singh 1990; Tomar and Gupta 1985; Tomar et al. 2003). Studies of tolerance of waterlogging are lacking (Mukta and Sreevalli 2010). Knowledge is needed of the response to salt and waterlogging across a range of provenances.

A standard approach for salinity tolerance experiments under glasshouse conditions is to increase salinity until the maximum concentration is reached which is marked by salt injury (older leaves turning yellow, wilting, senescence, necrosis) or 50 % death of plants, then they are held for several more weeks at this concentration (Craig et al. 1990; Meddings et al. 2001; Van der Moezel et al. 1991). The gradual increase of salinity would mimic field conditions where soils seasonally dry out and salt concentration increases (Niknam and McComb 2000).

The aims of the present study were: firstly to investigate genetic diversity of *M. pinnata* from the Forest Products Commission collection in Kununurra in tropical, northern Australia and compare them with natural populations in Australia, India and Indonesia; secondly, to assess the existence of variability of

important biodiesel parameters such as seed traits, oil content and fatty acid composition to select high oil yielding trees; thirdly, to determine the appropriate time of harvest as indicated by the oil content and fatty acid composition in developing seeds; and finally, to investigate the tolerance of seedlings to salinity, waterlogging and the combined stress conditions and evaluate their suitability for planting in marginal land.

Materials and methods

Molecular genetic diversity using sequencing of ITS region

Plant material

The *M. pinnata* trees used were planted in 1999 at the Frank Wise Research Institute located in the Ord River Irrigation Area of Kununurra (Lat 15° 46′ S, Long 128° 44′ E) in tropical, northern Western Australia. Seed was purportedly sourced from Indian collections. In 1999 seedlings were grown under nursery conditions and transplanted into laser-leveled irrigated plots at 3.6 m × 6 m spacing. Two young leaves per tree were harvested from 30 mature trees from Kununurra for DNA extraction.

Seeds were also collected from natural populations in Australia (9 trees) using single tree collections per location. These included trees from Brighton, Gladstone, Graceville, Indooroopilly, Mackay, Toowong and two sites at Kelvin Grove in Queensland and at Katherine in the Northern Territory. To compare these with seeds from known international provenances, seeds were purchased from six Indian states including Andhra Pradesh, Madhya Pradesh, Punjab, Tamil Nadu, Uttar Pradesh and West Bengal and three provinces of Indonesia namely Sumatera, West Java and two sites in East Java. Seeds from Australia, India and Indonesia were germinated in a tray with potting mix, grown for 6 weeks and fully expanded leaves were harvested for DNA extraction.

DNA extraction and quantification

Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Clifton Hill, Victoria Australia). Approximately 100 mg of either frozen or fresh leaf samples were ground in liquid nitrogen with a mortar and pestle to fine powder. DNA was isolated according to manufacturer's instructions and examined by gel electrophoresis using 1 % agarose gel in Tris acetate-EDTA buffer with ethidium bromide at 120 V for 40 min. DNA was quantified using a nanodrop spectrophotometer (ND 10000, Biolab).

PCR amplification and purification

The ITS region consists of three components, namely the 5.8S subunit and two spacers (ITS 1 and ITS 2) (Baldwin et al. 1995) and the techniques used here were modified from Hu et al. (2002). The ITS regions were amplified via the polymerase chain reaction (PCR) using HotStarTaq *Plus* Master Mix Kit (Qiagen, Clifton Hill, Victoria, Australia) with ITS 4 (reverse) and ITS 5 (forward) primers (Integrated DNA Technologies, Coralville IA, USA). Sequence of the reverse primer ITS 5 was 5′-GGA AGT AAA AGT CGT AAC AAG G-3′ and the forward primer ITS 4 was 5′-TCC TCC GCT TAT TGA TAT GC-3′. Reaction mixtures (20 µl) containing 10 µl master mix (1 unit HotStarTaq Plus DNA polymerase, 1 × PCR buffer, 200 µM of each dNTP), 0.1 µM of each primer, 40 ng of DNA template and known volume of RNase-Free water were run in a thermocycler (Multigene Gradient, Edison, NJ, USA). A negative control containing all components of the reaction mixture except DNA template (replaced with water) was included to test reagents for DNA contamination. The temperature program for PCR had an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 51 °C for annealing, 1 min extension at 72 °C and 10 min final extension at 72 °C. Amplification products were visualized in 1.5 % agarose gel electrophoresis stained with ethidium bromide.

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen) according to manufacturer's instructions and were visualized in 1.5 % agarose gel following electrophoresis and staining with ethidium bromide at 120 V for 1 h. A DNA marker, 100 bp ladder (Axygen, Union City, California, USA) was run in each gel to examine the size of products. Concentrations of PCR products and purified PCR products were examined using a nanodrop spectrophotometer (ND 10000 Biolab).

DNA sequencing

The purified double-stranded PCR products were used as templates in sequencing reactions. The forward (ITS 5) and reverse (ITS 4) primers used for PCR amplification were also used for sequencing. Sequencing reactions (20 µl) contained 14–20 ng DNA template, 2 µl primer at 1 pmol/µl, 1 µl ABI PRISM big dye terminator mix, 7 µl 2.5× buffer and 8 µl of culture sterile water. The reaction was performed in a thermocycler (Multigene, Edison, NJ, USA) using a program with an initial activation step at 96 °C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 51 °C, 1 min at 72 °C with a final 10 min extension at 72 °C. Products were cleansed and then sequenced using an automated sequencer ABI Prism 3,500 Genetic Analyzer (Applied Biosystem) according to manufacturer's protocol. In addition to *M. pinnata,* DNA sequences of outgroup species were obtained from the gene bank (NCBI database). These included *Millettia pulchra* Kurz (accession #AF467479) and *Millettia thonningii* Baker (accession #AF467481).

Data analyses of sequences of ITS region

The DNA sequences were aligned using ClustalW (Thompson et al. 1994). Parsimony analysis was performed using PAUP 4.0 (Center for Biodiversity, Illinois Natural History Survey, Illinois, USA). Heuristic searches were employed with tree bisection-reconnection branch-swapping by step-wise addition and closest addition sequence. Five trees were held at each step and only the best tree was kept. Bootstrap analysis was performed to estimate the relative levels of support to individual clades with 1,000 replications using heuristic search.

Seed traits and oil content in mature seeds

Seed traits

Seed for seed traits, oil content and fatty acid composition in mature seeds were taken from the same sources as DNA extraction. Ten seeds per tree were weighed and their length, breadth and thickness measured using a digimatic caliper (Mitutoya, Japan).

Oil extraction and quantification

Seeds were dried in an oven at 45 °C until constant weight was obtained before grinding. Oil was extracted in n-hexane using the soxhlet method (Meher et al. 2006) in triplicates. Dry ground seeds (1 g) were packed in a thimble, placed in a soxhlet extractor and extracted under constant heat (65 °C) for 2 h. Hexane was removed using a rotary evaporator and crude oil content was measured and expressed as a percentage of dry seed weight.

Transesterification and fatty acid analysis

Transesterification and fatty acid methyl ester (FAME) analysis was adapted from Mukta et al. (2009) with heptadecanoic acid methyl ester (Sigma Aldrich, Sydney, Australia) as an internal standard. Fatty acid profile was determined using a Shimadzu GC-2010 gas chromatograph fitted with flame ionization detection (FID) and a BPX 70 column (50 m × 0.22 mm inner diameter, 0.25 µm film thickness; Alltech Australia). Initial oven temperature was 200 °C held for 15 min, then increased by 5 °C per min to 220 °C and held for 10 min with total run time of 30 min. Detector and injector port temperatures were maintained at 250 °C and 285 °C respectively. Helium was used as a carrier gas with a flow rate of 0.65 ml min^{-1} and a split ratio of 49.9. Injection volume for GC analysis was 2 µl. Manual peak integrations were performed using GC post run analysis. Identification of FAMEs were confirmed using gas chromatography—mass spectrometry (GC-MS) on a Shimadzu GC 2010 using the same column and same temperature program as GC FID. Fatty acid methyl ester contents were expressed as percentage of total fatty acid in each sample.

Data analysis

Seed traits, oil content and fatty acid composition, were subjected to general analyses of variance (ANOVA) using Genstat 14th edition (VSN International Ltd) to check overall significant differences. Oil content across trees were categorized into low ($<x-1$ s), medium ($x-1$ s to $x+1$ s) and high ($>x+1$ s) oil content groups, where x and s are mean and standard deviation respectively (Mukta et al. 2009). A correlation coefficient between seed traits and oil content was generated using Microsoft Excel, t and *P* values were calculated for each correlation. Fatty acid composition was reported as proportion of each fatty acid to the total fatty acid in each sample. The diversity of fatty acid composition was examined by

cluster analysis using Primer 6 software. Euclidean distance matrix was used for cluster analysis based on unweighted pair-group method using arithmetic averages (UPGMA).

Seed traits and oil content in developing seeds

Changes in seed weight, oil content per seed and fatty acid composition of developing seeds were measured by harvesting 10 pods from three trees monthly from 6 to 11 months after flowering (MAF) during year of 2009. In 2008, flowering in Kununurra occurred in October and seeds matured in September 2009. At six MAF, seeds were large enough to accurately measure morphological traits, oil content per seed and fatty acid composition.

Salinity and waterlogging tolerance

Plant material

Seeds of *M. pinnata* from the Forest Products Commission collection in Kununurra (KN) and four states of India including Madhya Pradesh (MP), Punjab (PJ), Tamil Nadu (TN) and West Bengal (WB) were germinated and 4 month-old seedlings were used in the experiment.

Experimental design

The salinity experiment was conducted in the Forest Products Commission Research Nursery at the University of Western Australia, Shenton Park Field Station (Perth, WA) in a screen house. There were four treatments, five provenances and six plants per provenance in a tank experiment. Each plant was treated as a replicate. Treatments were control, i.e. non-saline drained (C), saline drained (S), non-saline waterlogged (W) and saline waterlogged (SW). Six seedlings of the same height and leaf number from each provenance were randomly arranged in the tanks for acclimatization 2 weeks before imposing treatments. The initial salt concentration was 50 mM NaCl and this was increased in weekly increments of 50 mM to up to the concentration when salt injury symptoms, such as older leaves turning yellow, necrosis, wilting and senesce were observed. This approach was adapted from Craig et al. (1990) and Van der Moezel et al. (1991). Plants were held at this concentration for 3 weeks before salt solutions were leached from the media with fresh water. Control and saline drained tanks were connected to pumps which circulated water from reservoirs. Plants were watered for 15 min (2x/day) using an automatic watering system. Waterlogging was achieved by filling tanks up to 1 cm below the surface of potting media. A soluble fertilizer solution (quarter strength, Advance Grow, Dutch Master, Adelaide, Australia) was applied during the first and the 2nd week of acclimation. This was increased to half strength for the rest of experiment and changed weekly. The soluble liquid fertilizer contained nitrogen (N, 2.6 %), soluble potash (K, 6 %), calcium (Ca, 2.20 %), iron (Fe, 0.045 %), phosphoric acid (P_2O_5, 0.75 %), magnesium (Mg, 0.70 %), sulphur (S, 0.005 %), copper (Cu, 0.004 %), manganese (Mn, 0.015 %), molybdenum (Mo, 0.004 %) and zinc (Zn, 0.005 %).

Measurements

Survival and height of the main stem were measured weekly. Dead plants were recognized when all foliage permanently wilted (Craig et al. 1990). Growth rate was calculated from plant height per week (Meddings et al. 2001). Stomatal conductance was measured in week 4 at noon on a sunny day on the youngest fully expanded leaf using a Leaf Porometer (Decagon Devices, Pullman USA). The youngest fully expanded leaves were harvested at week 5 for ion analysis (Na^+, K^+ and Cl^-). Leaf samples were oven dried at 65 °C for 72 h and ground with a ball mill. Ions were extracted in 0.5 M HNO_3 by shaking for 48 h at 20–25 °C. Na^+ and K^+ were determined in dilutions of the extracts using a flame photometer (PFP7, Jenway, Essex, UK) and Cl^- using a Buchler-Cotlove Chloridometer (Buchler Instruments, Model 4-2000, NJ, USA). Three lateral roots were removed per plant at week 5 to examine aerenchyma at 4–10 cm from the root tips. Roots were transversely sectioned and mounted onto a glass slide and examined under a microscope.

Data analysis

Analysis of variance was performed using Genstat 14th edition (VSN International) to identify overall significant differences between treatments, provenances and interactions. Percentage of survival was

calculated based on 30 replications. Results of each treatment were compared pairwise with the rest following a simple *t* test (Johnson and Bhattacharyya 1996).

Results

Genetic diversity

The nucleotide sequence of the ITS region indicated genetic diversity across the species. The aligned ITS sequences of 49 genotypes from *M. pinnata* and two out-groups (*M. pulchra* and *M. thonningii*) consisted of 661 base pairs (bp). Among these, 509 bp were constant, 105 bp were parsimony uninformative and 47 bp were parsimony informative.

A heuristics search of nucleotide characters from the entire ITS region generated a parsimony tree with length of 207, which contained five clades with more than 50 % bootstrap supports and 33 single individuals (Fig. 1). The two out-group species, *M. pulchra* and *M. thonningii,* were separated from the in-group with 99 % bootstrap support. Clade I consisted of two individuals (# 12 and 13) from Kununurra. Clade II included five individuals from Kununurra namely # 2, 11, 14, 15, and 16. Clade III contained individual # 56 from Kununurra and a tree from Sumatera (Indonesia). Clade IV included four individuals from Indian accessions, namely Andhra Pradesh, Tamil Nadu, Madhya Pradesh and Punjab. Clade V was the most distinct among trees studied and only contained trees from Indonesia (West Java, East Java 1 and East Java 2).

Seed traits and oil content of mature seeds

There was very high variation in seed traits across trees ($P < 0.001$; Table 1). Generally, the shortest and the lightest seeds were from Indonesia which were 18.29 mm and 1.17 g respectively while the heaviest seeds were from India. Oil content varied (Table 2) with the lowest range of 28–36 % in Indonesia, through the mid-range collections from India (30.3–38.0 %) and Queensland and the Northern Territory in Australia (33.0–40.3 %) to trees with high oil content (# 3, 5, 14 and 80) and the highest oil content of 45 % (# 4) in Kununurra.

There was a high positive correlation (all $P < 0.001$) between seed weight and seed length ($r = 0.67$), seed breadth, ($r = 0.53$) and seed thickness ($r = 0.7$). There was no significant correlation between these seed traits and oil content (Table 3).

Peaks detected by GC-FID consistently indicated nine fatty acid methyl esters (FAMEs) present in the oil. The FAMEs were identified by GC-MS and listed according to their retention time from the earliest to the latest as follows: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidic (C20:0), 11-eicosenoic (C20:1), behenic (C22:0) and lignoceric (C24:0) acids respectively. The internal standard peak (heptadecanoic acid methyl ester) appeared at 9.8 min between palmitic and stearic acid peaks.

Fatty acid methyl ester composition varied across trees ($P < 0.001$). The major fatty acid was oleic (51 %), followed by linoleic (19 %), palmitic (11 %), stearic (6 %), linolenic and behenic (both 4.5 %), lignoceric (1.4 %) and 11-eicosenoic and arachidic acid (both 1.2 %). The range in the proportion of major fatty acids was 36.8–62.7 % for oleic, 40–24.1 % for linoleic, 8.9–15.2 % for palmitic and linolenic was 2.3–8.8 %.

Cluster analysis of the euclidean distance matrix of fatty acid composition indicated a dendrogram consisting of 3 groups (Fig. 2), with a distance value >12. The first group contained three individuals from Indonesia including East Java 1 and 2, and West Java. The second group was the largest and contained individuals from all other accessions. The third group contained a single tree (# 63) from Kununurra.

Seed traits and oil content in developing seeds

Increases in seed weight and oil content per seed in developing seed followed a sigmoidal curve (Fig. 3a). Seed weight at 6 MAF was 0.37 g, this nearly tripled by 7 MAF, there was no increase at 8 MAF, but then it resumed growth from 9 MAF and substantially increased to 1.45 g prior to maturity at 11 MAF. Oil content per seed was 0.07 g at 6 MAF and it increased four-fold by 7 MAF, then remained relatively constant for 3 months with a final increase to 0.51 g at maturity (11 MAF).

The composition of fatty acid methyl esters changed during seed development (Fig. 3b). Oleic acid increased from 42 % 6 MAF to 52 % 7 MAF, was relatively stable until 9 MAF and then increased to 61 % 11 MAF. Linoleic acid varied between 13 and

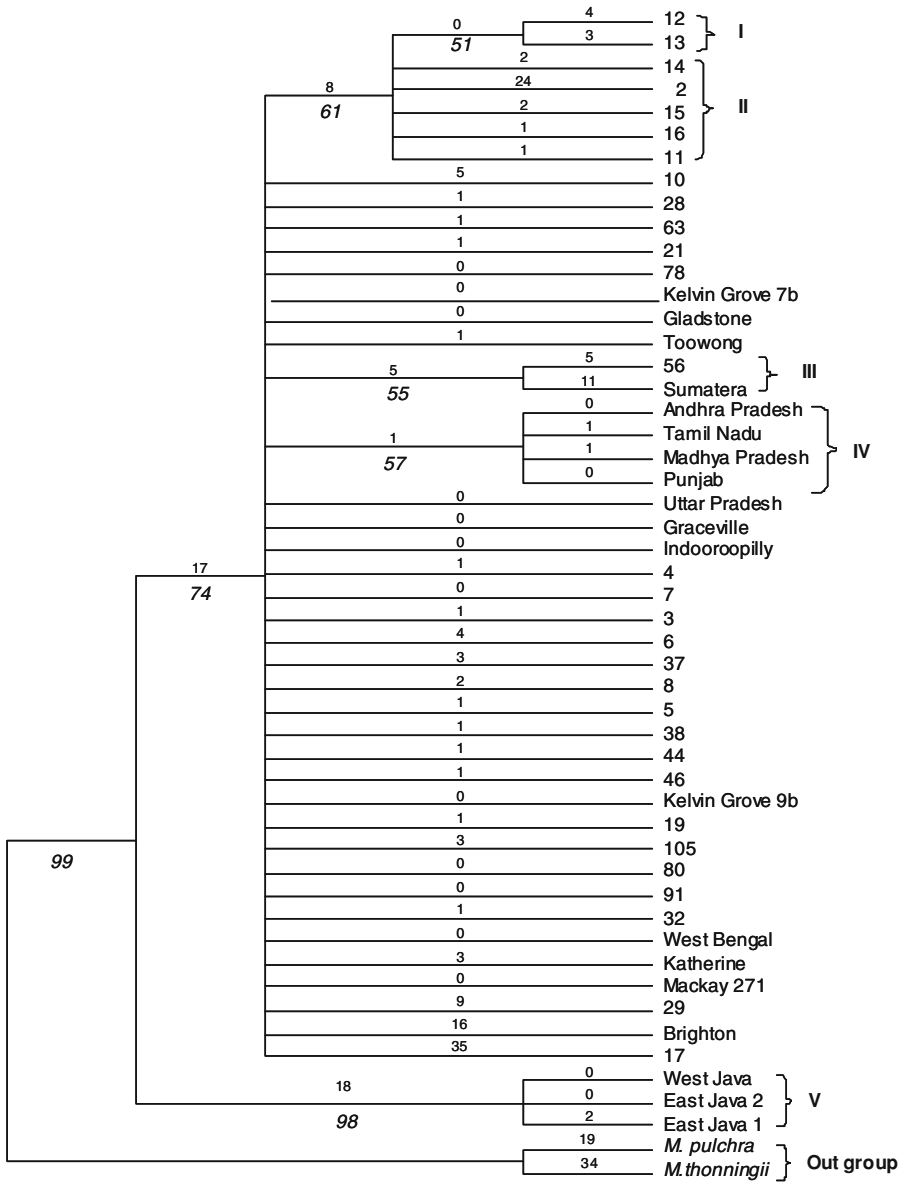

Fig. 1 Phylogenetic parsimony tree generated from PAUP 4.0 based on internal transcribe spacer (ITS) nucleotide sequence data. Tree length = 207, CI = 0.8937 and RI = 0.8151. Numbers above branches indicate branch length and numbers below branches in italics indicate bootstrap values of 1,000 replications

20 % up to 9 MAF then decreased to 13 % at maturity. Palmitic and stearic acid tended to decline from 6 MAF and were 13 % and 9 % respectively at maturity. Linolenic decreased from 7 % at 6 MAF to 3 % at maturity. Other FAMEs were minor components which also slightly increased during seed development except arachidic which remained unchanged at <1 %.

Salt and waterlogging tolerance

Waterlogging alone did not reduce plant survival but it was influenced by salt concentration ($P < 0.05$). There was no influence of provenance and no interaction between provenances and treatments for survival. Saline drained seedlings survived up to 250 mM NaCl held for 1 week, while survival for saline waterlogged seedlings was reduced at 200 mM NaCl and higher concentrations (Table 4). Holding seedlings in saline drained conditions at 250 mM NaCl for 2 weeks reduced survival to 80 % and holding the plants for 3 weeks further reduced survival to 37 %. After 1 week under saline waterlogged conditions at 200 mM NaCl survival declined to 86 %, and after 2 weeks at 250 mM NaCl survival reduced

Table 1 Seed traits (length, breadth, thickness and weight) of *M. pinnata* from four accessions

Accession	Length (mm)	Breadth (mm)	Thickness (mm)	Weight (g)
Kununurra	21.79 ± 0.28	15.61 ± 0.21	6.74 ± 0.13	1.31 ± 0.04
Australia (QLD and NT)	21.06 ± 0.85	14.70 ± 0.47	7.00 ± 0.21	1.44 ± 0.13
India	22.28 ± 2.11	14.54 ± 0.87	7.85 ± 0.70	1.53 ± 0.11
Indonesia	18.29 ± 1.03	13.35 ± 1.04	7.06 ± 0.65	1.17 ± 0.17

Values are mean of ten seeds plus minus standard errors

QLD Queensland, *NT* Northern Territory

Table 2 Oil content of *Millettia pinnata* seeds from four accessions

Accession	Number of trees (n)	Oil content (% w/w)	Number of trees in category		
			Low	Medium	High
Kununurra	30	38.57 ± 0.56	1	24	5
Australia (QLD & NT)	9	37.22 ± 0.52	1	8	0
India	6	34.06 ± 0.47	4	2	0
Indonesia	4	32.50 ± 0.89	2	2	0

Each oil content was determined as grams of oil per gram dry ground seed sample expressed as the mean percentage ± standard error and values for each tree were categorized into low (<33.9 %), medium (33.9–40.6 %) and high (>40.6 %)

QLD Queensland, *NT* Northern Territory

Table 3 Correlation matrix for seed traits and oil content

	Seed length	Seed breadth	Seed thickness	Seed weight	Oil content (%)
Seed length (mm)	1.00				
Seed breadth (mm)	0.37	1.00			
Seed thickness (mm)	0.30	0.18	1.00		
Seed weight (g)	0.67***	0.53***	0.70***	1.00	
Oil content (%)	0.22	0.38	−0.11	0.15	1.00

*** Indicates very high significance of interaction ($n = 49$, $P < 0.001$)

substantially to 34 % and after 3 weeks most plants were killed leaving only 3 % alive.

Control height growth rate (HGR) was fairly constant at approximately 9 mm/week. Treatments reduced HGR ($P < 0.001$) but there was no influence of provenance ($P > 0.05$). Waterlogging reduced HGR by about a third. Generally HGR decreased with increasing NaCl concentration or prolonged salt treatment (Fig. 4a). Under saline drained conditions of 50–150 mM NaCl, HGR was halved to about 4.5 mm/week. When NaCl concentration was increased to 250 mM plant growth was reduced to about 2 mm/week. Combined salt and waterlogging conditions resulted in more severe growth reduction. At 50 mM NaCl HGR was again halved, it decreased to a third at 100 mM and 150 mM NaCl, and plants did not grow at 200 mM NaCl and above.

Treatments influenced leaf number (Fig. 4b; $P < 0.01$). Control plants commenced the experiment with 8 leaves and finished with 10 leaves. Non-saline waterlogged plants reduced leaf number slightly with more reduction by salt treatment. Increasing NaCl concentration under saline drained conditions gradually reduced leaf number and it halved after 1 week at 250 mM NaCl. Combined salt and waterlogging conditions reduced leaf number more than salt or waterlogging alone and prolonging the treatment caused more reduction in leaf number. For example, leaf number reduced by 6 and 7 leaves at 200 and 250 mM NaCl respectively. Leaf number further

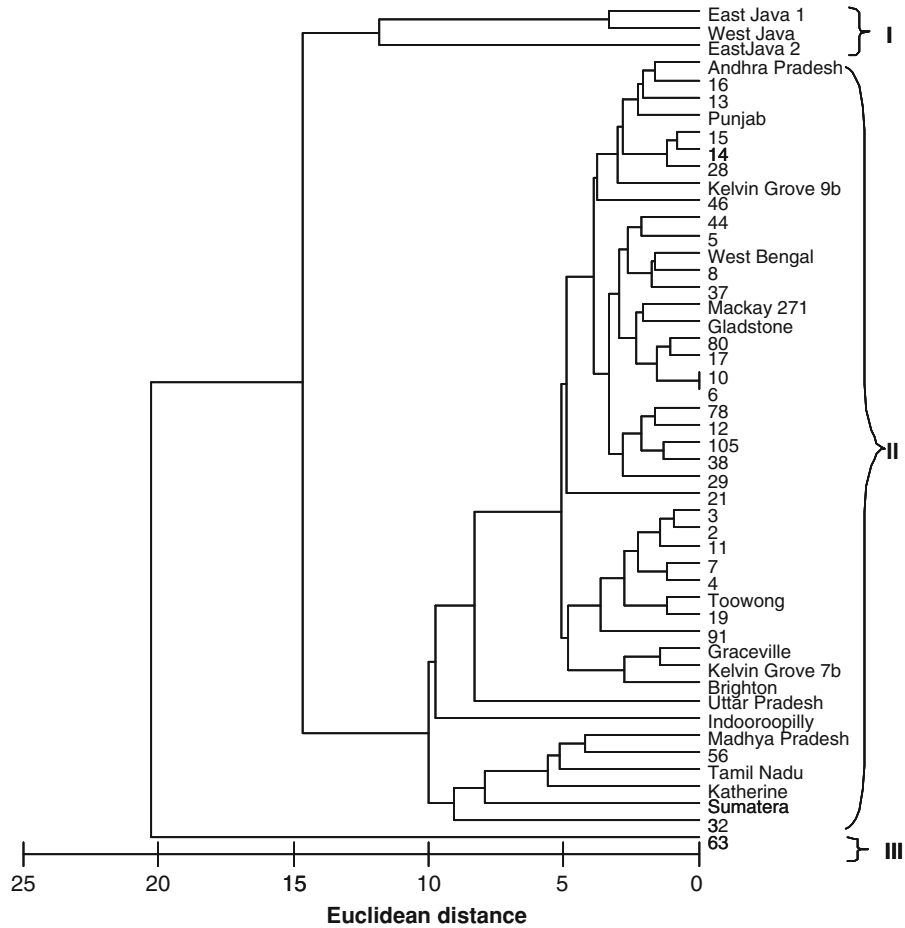

Fig. 2 Dendrogram of Euclidean distance matrix based on group average of fatty acid methyl ester composition (%) of 49 individual trees of *M. pinnata* from four accessions: Kununurra, Australia (QLD and NT), India and Indonesia

reduced so that after 3 weeks at 250 mM NaCl most plants had no leaves.

Stomatal conductance, measured at week 4 (salt at 200 mM NaCl), was influenced by treatment ($P < 0.001$) but not by provenance ($P > 0.05$). Control stomatal conductance was 176 mmol $m^{-2}s^{-1}$ and it decreased by 64 % in non-saline waterlogged conditions and was further decreased by 86 % in saline drained and by 94 % in saline waterlogged conditions. There was only an interaction between treatment and provenance under non-saline waterlogged conditions on stomatal conductance (Fig. 4c; $P < 0.001$). Plants from Kununurra and Tamil Nadu had the smallest reduction of 55 % in stomatal conductance, this was further reduced in plants from Punjab and Madhya Pradesh (66 % reduction) and plants from West Bengal (80 % reduction).

Leaf ion concentrations were measured at week 5, 1 week after imposition of 250 mM NaCl and treatment influenced concentrations of Na^+, Cl^- and K^+ (Fig. 4d; $P < 0.01$). Leaf Na^+ concentration increased 4.5-fold in saline drained and tenfold under saline waterlogged conditions. Leaf Cl^- concentration increased about threefold in both saline drained and saline waterlogged conditions. Neither leaf Na^+ nor Cl^- concentrations were influenced by non-saline waterlogging. Leaf K^+ concentration was not influenced by either saline drained or saline waterlogged conditions and it reduced by 22 % under non-saline waterlogging. No aerenchyma was observed in the roots under non-saline waterlogged and saline waterlogged conditions.

Discussion

The sequences of ITS region of 49 individuals indicated genetic variation between accessions of

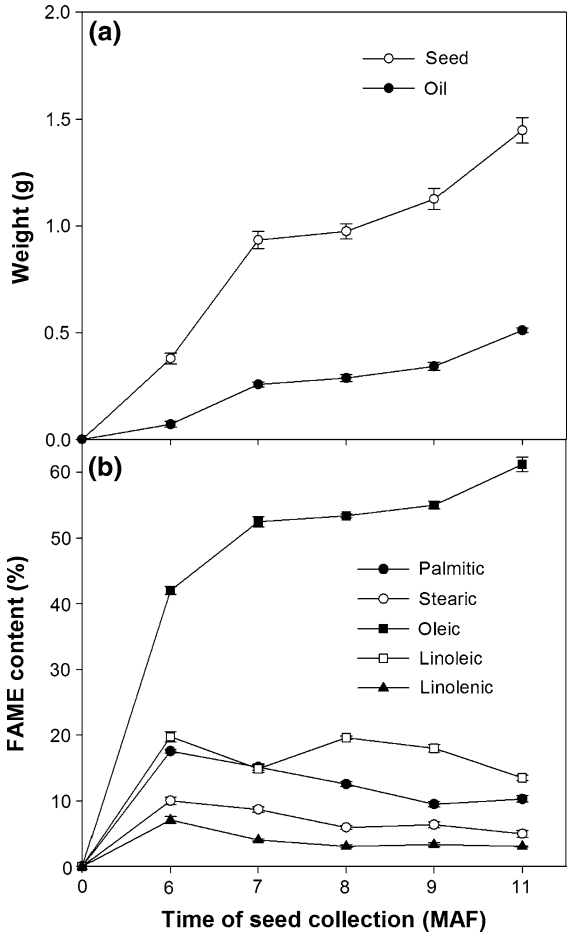

Fig. 3 **a** Seed weight and oil content per seed in developing seeds, **b** fatty acid methyl ester composition from 6 to 11 months after flowering (MAF)

M. pinnata. Three Indonesian accessions from across Java were placed in one clade, which was very distinct from the remaining individual trees especially those from India where the species is thought to originate (Sujatha et al. 2008). The genetic distance between Indonesian, Indian and Australian genotypes indicated that *M. pinnata* did not disperse naturally from India to Australia via the Indonesian archipelago. Scott et al. (2008) suggests that *M. pinnata* were probably introduced from India to Australia early in human history. The isolation of Javanese clades possibly suggests that the genotypes could have originated in Java but this requires further investigation.

Trees from Andhra Pradesh, Tamil Nadu, Madhya Pradesh and Punjab in India were in the same clade indicating they were closely related. Geographically, Andhra Pradesh and Tamil Nadu are adjacent coastal states. Coastal-grown *M. pinnata* may drop pods into estuarine rivers or directly into the sea. Pods can float for up to 3 months on saline water and then germinate under laboratory conditions and seedlings are often observed growing on beaches (Nakanishi 1988). Flotation is probably an effective dispersal method and the coastal proximity of Andhra Pradesh and Tamil Nadu could facilitate gene flow between populations. Seeds were also probably dispersed by humans along the coast as these plants have multiple uses in India including a traditional source of medicine (Chopade et al. 2008), fuel, fish poison, animal fodder, green manure and making farm implements (Mukta and Sreevalli 2010). The distance between Punjab in far north-western India and inland Madhya Pradesh may restrict natural gene flow, but not necessarily human dispersal. Further sampling within and between these states would be required to clarify these relationships.

None of the trees from Forest Products Commission collection in Kununurra were in the Indian clade of Andhra Pradesh, Tamil Nadu, Madhya Pradesh and Punjab, indicating their distinctiveness from these states in India. Improvement of the genetic diversity of the collection would require future seed sourcing from

Table 4 Survival of *M. pinnata* under different salt and waterlogging treatments

Treatments	Survival (%) at different NaCl concentration (mM)/time (week)						
	50/1	100/2	150/3	200/4	250/5	250/6	250/7
Control	100 a	100 a	100 a	100 a	100 a	100 a	100 a
Saline drained	100 a	100 a	100 a	94 ab	91 ab	80 b	37 c
Non-saline waterlogged	100 a	100 a	100 a	100 a	100 a	100 a	100 a
Saline waterlogged	100 a	100 a	100 a	86 b	77 b	34 c	3 d

Treatments were imposed for 1 week except the highest NaCl (250 mM) concentration which was kept for 3 weeks

Different letters following the means indicate significant differences according to *t* test (n = 30, $P < 0.05$)

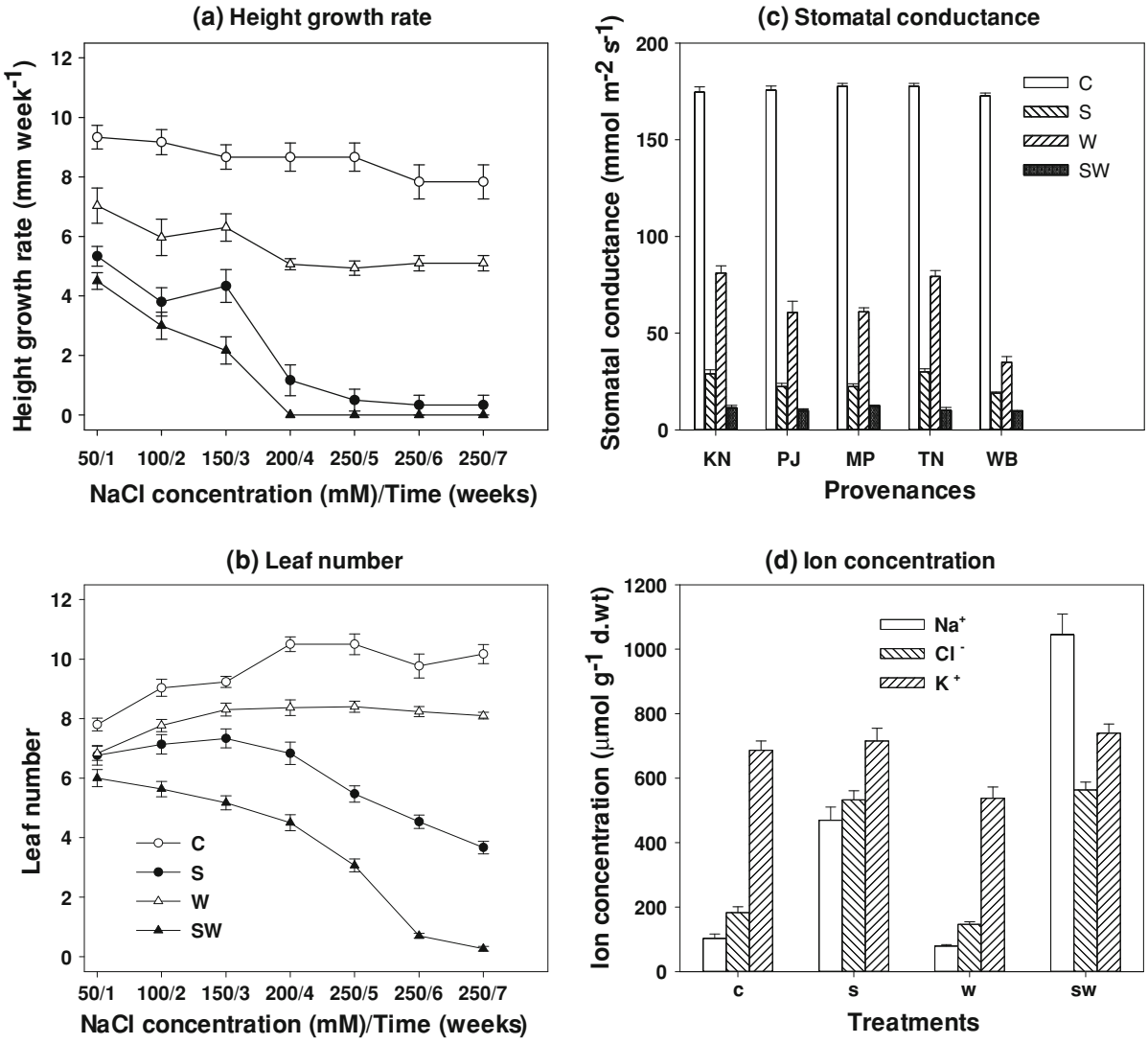

Fig. 4 **a** Height growth rate and **b** leaf number under non-saline drained control (*C*), saline drained (*S*), non-saline waterlogged (*W*) and saline waterlogged (*SW*) treatments with weekly increment of 50 mM NaCl. Each treatment was imposed for one week except at 250 mM NaCl which was held for 3 weeks. **c** Stomatal conductance of leaf from different provenances (KN = Kununurra, MP = Madhya Pradesh, PJ = Punjab, TN = Tamil Nadu, WB = West Bengal) at week 4 for control (*C*), saline drained (*S*), non-saline waterlogged (*W*), and saline waterlogged (*SW*) treatments at 200 mM NaCl. **d** Concentration of Na^+, Cl^- and K^+ in the youngest fully expanded leaf in week 6, 1 week after application of 250 mM NaCl. Values are means ± standard errors

these regions in addition to a wide range of other geographic and climatic conditions from across the *M. pinnata* range.

Seed traits and oil content were also useful in establishing genetic variation, especially as they are important components of yield (Kaushik et al. 2007; Kesari et al. 2008). Seed weight was naturally highly correlated with length, breadth and thickness and indicated as expected that all three parameters were important in determining seed size. Sunil et al. (2009) and Divakara et al. (2010) claim oil content and seed weight are under substantial genetic control, based on heritability in the broad sense but further research is required to estimate the narrow sense heritability of these traits in *M. pinnata* and to determine any influence from particular environmental factors.

Oil content was highly variable across trees. Among 30 trees from the Forest Products Commission collection at Kununurra, it ranged from 31 to 45 %. The lowest oil content of seed from Kununurra trees was much higher than in previous studies under Indian conditions. Across 21 accessions from Andhra Pradesh oil content ranges from 9.5 to 46 % (Mukta et al. 2009), and across 123 accessions from Andhra Pradesh and Orissa oil content ranges from 15 to 47 % (Sunil et al. 2009).

Based on oil content, the Forest Products Commission tree collection has seed which represent the high oil content range across *M. pinnata*. Trees # 3, 4, 5, 14 and 80 had high oil content (40.7–45 %). In addition to oil content, these trees also possessed high seed weight (1.3–1.7 g) and therefore they could potentially be used for mass propagation or for utilization in a breeding program. Oil yield is one of the most important traits determining the commercial success of *M. pinnata* as an energy crop.

Fatty acid composition of *M. pinnata* is suitable for biodiesel according to USA, German and European standards (Azam et al. 2005; Karmee and Chadha 2005), however quality can be improved. Oleic acid, a mono-unsaturated 18-carbon chain, is the most desirable fatty acid for good quality biodiesel, due to its low viscosity, low melting point of −20 °C, which makes it suitable for use under cold climatic conditions (Knothe 2008), and an acceptable cetane number of 57.6 (Naik et al. 2008), which indicates good ignition quality (Meher et al. 2006). Oleic acid was the major fatty acid across trees and generally constituted about half of the oil with a range from approximately one to two thirds.

The other major fatty acids, palmitic, stearic and linoleic, also possess good biodiesel properties (Knothe 2008). Among the nine fatty acids in *M. pinnata* oil, the only fatty acid not suitable for biodiesel is linolenic acid, which should not exceed 12 %. Linolenic acid has three double bonds and in engines this unsaturated fatty acid can be harmful (Azam et al. 2005) and its low cetane number of 22.7 indicates poor ignition quality (Knothe 2008). All trees had seed oil with <12 % linolenic acid.

Cluster analysis of fatty acid composition indicated close relationships among Indonesian genotypes. Trees from Java clustered together and were quite distinct from all other genotypes, whereas Sumatera grouped with trees from Kununurra, Australia and India. This supported the relationships indicated by genetic diversity of the ITS region, where trees from Java were also distinct from the remaining accessions. The Kununurra tree collection does not appear to include any Javanese material, but the small seed size and low proportion of oleic acid would make them less desirable for selection.

Agreement between the cluster analyses derived from molecular genetic data and differences in fatty acid composition supports research in other crops indicating genetic control of fatty acid composition. The stearic acid content of sunflower (*Helianthus annuus* L.) (Pérez-Vich et al. 1999) and the fatty acid content of peanut (*Arachis hypogaea* L.) (Norden et al. 1987) are primarily influenced by genotype. The high oleic acid content of oilseed rape (*Brassica napus* L.) has a very high heritability of 0.99 and is environmentally stable across different growing regions (Schierholt and Becker 2001), which supports selection of superior trees for breeding from this analysis.

Seed weight and oil content per seed in developing seeds followed a similar sigmoidal curve where gains were substantial up to 7 MAF, relatively stable for 2 months with a final increase towards maturity at 11 MAF. The highest seed weight and oil content per seed was at maturity similar to other crops, such as safflower (*Carthamus tinctorius* L., Gecgel et al. 2007), sunflower, winged bean (*Psophocarpus tetragonolobus* (L.) DC., Khor and Chan 1988) and almond (*Prunus dulcis* (Mill.) D.A.Webb, Soler et al. 1988) where oil content also generally increases towards maturity.

Fatty acid composition in developing seeds varied ($P < 0.001$). Oleic acid was the dominant fatty acid constituting about half the oil from 6 DAF to maturity, however oleic acid content increased substantially in the last months before harvest. This was at the expense of palmitic (C16:0) and stearic (C18:0) and to a lesser extent linoleic and linolenic (C18:3) acids.

Similarly in almond, increases in oleic acid from 85 days after fruit set to maturity at 176 days are accompanied by decreases in saturated fatty acids, especially linoleic acid (Soler et al. 1988). Fatty acid synthesis in *M. pinnata*, probably follows a similar pathway to other higher plants (Ohlrogge 1997; Thelen and Ohlrogge 2002), where palmitic acid (C16:0) is the precursor of stearic acid (C18:0), which is further desaturated to oleic (C18:1) then linoleic (C18:2) and linolenic acid (C18:3). Stearic acid is also

a precursor for very long chain fatty acids, such as arachidic (C20:0), behenic (C22:0), and lignoceric (C24:0) acids. Fortunately in *Millettia* the fatty acid biosynthetic pathway favors accumulation of oleic acid over the other compounds on this pathway, at least under the conditions examined.

Environmental conditions may alter the progress of seed development and fatty acid synthesis. This might be favorable and in *B. napus* warm and wet conditions can increase oleic acid content (Pritchard et al. 2000). In other oil seed species the influence of genotype, environment and their interaction also influence fatty acid composition, including oleic, linoleic and linolenic acids in soybean (*Glycine max* (L.) Mert., Primono et al. 2002; Zhe et al. 2010).

Under some circumstances the environment, in particular temperature, influences oil biosynthesis during seed filling. In sunflower, oleic acid increases in response to minimum night temperatures from 12.5 to 21.5 °C whereas it has little impact outside these temperatures (Izquierdo and Aguirrezabal 2008). In *B. napus*, wet conditions increase oleic acid content, but whereas Pritchard et al. (2000) report high temperatures increase oleic acid, Aslam et al. (2009) claim high temperatures decrease it. Thus the impact on fatty acid biosynthesis depends on the species and possibly the temperature range that plants are exposed to during seed development. Importantly, they highlight the significance of genotype comparisons within the proposed environment. The influence of the interaction between genotype and environment on oil content and fatty acid composition in *M. pinnata* was not investigated, and it will require examination of selected material across environments to distinguish genotype and environmental effects.

The best time for harvest of *M. pinnata* was at maturity (11 MAF) because at this time seed had reached maximal weight, the highest oil content and seed oil contained the highest proportion of oleic acid. Oleic acid is the most suitable fatty acid for good biodiesel quality as mentioned earlier. It can also be used to enrich biodiesel for better biodiesel fuel properties (Knothe 2008). In addition mechanical harvesting would need to occur before the next flowering season 12 months after the previous flowering. In another biodiesel tree, *Calophyllum inophyllum* L., the highest oil content is also at maturity 77 days after anthesis and this is suggested for harvest time (Hathurusingha et al. 2011). However, in corn (*Zea mays* L.) kernels the highest oil accumulation occurs 20 days before maturation and it decreases at maturity (Saoussem et al. 2009). In five soybean genotypes, rapid oil accumulation occurs three to 7 weeks after flowering, then remains constant until maturity at 11 weeks after flowering (Saldivar et al. 2011). This emphasizes the importance of investigation on changes in oil accumulation during seed development and their impact on the appropriate time for harvest.

Millettia pinnata was non-saline waterlogging tolerant. Survival under non-saline waterlogged conditions was similar to controls but it was accompanied by a reduction in growth. Waterlogging causes hypoxia which reduces production of ATP leading to slower growth (Barrett-Lennard 2003). A common plant adaptation to waterlogging is formation of aerenchyma which provides interconnected air-filled channels in the root cortex for oxygen diffusion. This reduces the effects of waterlogging in woody plants, such as *Casuarina obesa* Miq. and *Eucalyptus camaldulensis* Dehnh. (Van der Moezel et al. 1988; Van der Moezel et al. 1989). The absence of aerenchyma in the root of *M. pinnata* indicated that an alternate mechanism of waterlogging tolerance may exist, such formation of long, superficial lateral roots (Mukta and Sreevalli 2010). However these would be less efficient than the adventitious roots of *Melaleuca cuticularis* Labill. and *C. obesa*, which contain aerenchyma and confer substantial waterlogging tolerance (Carter et al. 2006).

Millettia pinnata was less able to survive and grow in controlled salt conditions than expected, given earlier claims of high salt tolerance (Divakara et al. 2010; Kaushik et al. 2007; Kesari and Rangan 2010). It survived for short periods at 250 mM NaCl under drained conditions and at 200 mM NaCl under salt waterlogging. Combined salt and waterlogging resulted in higher plant death compared to either stress alone as is commonly observed (Craig et al. 1990; Meddings et al. 2001; Van der Moezel et al. 1991). In other moderately salt tolerance species, such as *Casuarina cunninghamiana* Miq., survival decreases substantially both under salt drained and salt waterlogged conditions with more deaths under combined treatment (Van der Moezel et al. 1989). There was also little to no difference in salt tolerance between provenances, which was unexpected given the geographical and genetic diversity of genotypes. It

may be that there was a lack of either salt tolerance or diversity for this trait, however further collection from saline areas is warranted given ecological (Kesari and Rangan 2010; Mukta and Sreevalli 2010) and field-based (Tomar and Gupta 1985; Tomar et al. 2003) evidence for salt tolerance within the species. In other species, such as *Acacia redolens* Maslin, *A. patagiata* R.S.Cowan et Maslin, *Eucalyptus occidentalis* Endl., *E. camaldulensis* and *C. obesa* salinity tolerance varies between provenances and it is often an adaptation to the salinity of the original provenance habitat (Craig et al. 1990; Van der Moezel and Bell 1990; Van der Moezel et al. 1991).

Salinity reduces plant growth by osmotic factors and ionic toxicity within the plant (Munns and Tester 2008). Reduced osmotic potential caused by salt outside the root inhibits water uptake leading to slower growth, an effect similar to drought. An immediate consequence of reduced osmotic potential is stomatal closure (Munns and Tester 2008) as seen in reduced stomatal conductance in *M. pinnata* under saline conditions. Increased concentrations of Na^+ and Cl^- in young leaves grown under saline drained and saline waterlogged conditions indicated poor ability to exclude these ions. Under saline-waterlogged conditions concentrations of Na^+ and Cl^- increased further due to increases in their rate of transport to the shoot, and this severely reduced plant growth and survival (Barrett-Lennard 2003; Teakle et al. 2007).

Growth reduction in *M. pinnata* occurred even under low salt concentrations of 50 mM NaCl. This early growth reduction indicated the powerful osmotic effect of salt outside the roots (Munns and Tester 2008). Reduction in growth and leaf number was more pronounced as the salt concentration increased. This longer term effect usually occurs when ionic toxicity exceeds the threshold levels that influence plant growth, due to accumulation of salt in plant tissue. Presumably more salts accumulated in older than in younger leaves because older leaves were no longer expanding and diluting salts, and this resulted in the death of older leaves. Even under 200 mM NaCl drained and 150 mM NaCl waterlogged conditions, the production of new leaves was slower than the death of old leaves, which reduced photosynthesis and the ability to supply carbohydrates for plant growth (Munns and Tester 2008).

Millettia pinnata was moderately salt tolerant, and may be able to be grown under drained conditions of 200 mM NaCl and waterlogged conditions of 150 mM NaCl. This salinity tolerance was substantially less than earlier claims (Divakara et al. 2010; Kaushik et al. 2007). In other species, such as *Eucalyptus botryoides* Sm. and *E. kondininensis* Maiden et Blakely, salt tolerance in the field is greater than under glasshouse conditions (Blake 1981) perhaps due to temporal and spatial variations in salinity. While there have been reported observations that *M. pinnata* is found along the seashore (Kesari and Rangan 2010; Mukta and Sreevalli 2010), survival may be enhanced by pockets of fresh water or less saline water, for example after dilution with rainwater or subterranean aquifers (Mensforth and Walker 1996). Alternatively, *M. pinnata* has less salinity tolerance as a seedling than at later, more mature stages when its deep roots can tap into fresher water supplies. Mature trees have a thick tap root that can grow down to 10 m depth, making it possible to extract deep ground water, even beneath other crops (Kesari and Rangan 2010).

The present study supports the salinity tolerance reported by Singh (1990) where *M. pinnata* survive well at 16.3 dS m^{-1} ($\approx$ 163 mM NaCl) but growth is reduced. The young seedlings (4 months old) investigated here are appropriate for plantation establishment. Seedlings are transplanted to the field at this age, and this represents a critical stage of development in saline and waterlogged production areas (Tomar and Gupta 1985). The salinity tolerance under drained conditions was 200 mM NaCl, under saline waterlogged was 150 mM and the non-saline waterlogging tolerance of the species is a good guide in selecting suitable planting areas for *M. pinnata* in marginal land. Areas with moderate salinity in the tropical, arid and semi-arid regions of the world are probably suitable. Lower salinity (150 mM NaCl) in waterlogged areas may also be suitable. Areas which are periodically or seasonally waterlogged with fresh water, such as along riverbanks and low-lying landscapes, would be preferable.

Conclusion

Genetic diversity of *M. pinnata* based on sequencing of the ITS region, revealed Indonesian genotypes were distinct from the rest of the genotypes. All seed oil contained nine fatty acids and by weight more than

half was oleic acid. Seed traits, oil content and fatty acid composition varied across trees. Five trees from the Forest Products Commission collection in Kununurra (# 3, 4, 5, 14 and 80) had large seed size and high oil content, and could be used as a source for mass propagation and breeding purposes. Seed weight, oil content per seed and proportion of oleic acid in developing seeds increased following a sigmoidal pattern. Eleven months after flowering seeds had maximum weight, the highest oil content and the greatest oleic acid proportion, indicating this was the ideal time for harvest. *Millettia pinnata* had moderate salinity tolerance of 200 mM NaCl under saline drained, 150 mM NaCl under waterlogged conditions and non-saline waterlogging tolerance. It is probably suitable for plantations in marginal lands along fresh water river banks exposed to periodic waterlogging, low-lying areas which are seasonally waterlogged, and in arid and semi-arid regions of the world where soils are moderately saline.

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