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Characterization of tree-to-tree variation in morphological, nutritional and medicinal properties of *Canarium indicum* nuts

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Abstract As part of a feasibility study of the commercialization potential of *C. indicum* nuts as Agroforestry Tree Products in Papua New Guinea, preliminary characterization studies have examined the tree-to-tree variation in morphological traits (nut and kernel mass and kernel:nut ratio), as well as nutritional (carbohydrate, fat, protein, sodium, vitamin E) and medicinal traits (anti-oxidant activity, anti-inflammatory activity and phenolic content) of kernels from 18 to 72 trees in a small number of different villages of Papua New Guinea (East New Britain Province). There was continuous variation in these traits indicating opportunities for multiple trait cultivar development targeted at food and pharmaceutical markets. Certain traits, for example anti-inflammatory activity, in which tree-to-tree variation was highly significant, present greater opportunities than others, such as saturated:unsaturated fatty acid ratio. This intraspecific variation was greater within populations than between populations. The data presented has allowed the development of a strategy to domesticate *C. indicum* for cultivation in homegardens and cocoa–coconut agroforests, using a participatory approach aimed at the production of agroforestry tree products (AFTPs) to empower small-holders and enhance their livelihoods and income.

Keywords Kernel:nut ratio - Oil content and fatty acid profile - Tocopherol content (vitamin E) - Anti-oxidant and phenolic content - Anti-inflammatory activity

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Introduction

Worldwide, the domestication of indigenous fruits and nuts for the diversification of subsistence agriculture is playing a part in the achievement of the Millennium Development Goals, especially the enhancement of the livelihoods of the rural poor and the mitigation of environmental degradation in developing countries (Leakey *2001a*, *b*; Leakey et al. *2005a*, *2007*). To ensure that subsistence farmers benefit from this global initiative, a participatory approach to cultivar development has been implemented in West and Central Africa (Leakey et al. *2003*), and is being replicated in Southern

Africa (Akinnifesi et al. 2006) and the Solomon Islands (Pauku 2005). In West Africa, this is being rapidly taken up by village communities who are starting to make substantial income from their nurseries (Tchoundjeu et al. 2006) and to benefit in other ways as well (Schreckenberg et al. 2006). It is apparent that there could be similar opportunities and benefits from the domestication and commercialization of *C. indicum* (Nevenimo et al. 2007; in press).

Two key elements to the development of cultivars of priority tree species are, firstly, the identification of elite individuals in the wild or semi-domesticated population (Leakey and Page 2006) and, secondly, their propagation by vegetative techniques (Leakey et al. 1990). The former, requires the quantitative characterization of fruit, nut and kernel variation (Atangana et al. 2001; Waruhiu et al. 2004; Anegbeh et al. 2003, 2005; Leakey et al. 2005b, c), variation in nutritive value and other food properties (Leakey et al. 2005d), and an understanding of the interactions between different traits for multi-trait selection (Leakey et al. 2002; Atangana et al. 2002; Leakey 2005). For the indigenous nuts of the Pacific, similar quantitative data for the morphological variability of nuts and kernels has been collected for *Barringtonia procera* (Pauku 2005, 2006a, b) and *Canarium indicum* (Moxon et al. unpublished). The latter is examined here, together with new data on the nutritive and health-giving properties of *Canarium* kernels.

Methods and materials

The morphological variation of *Canarium* nut and kernel fresh weights were assessed in 1998 and 2002, from farmer-selected, unplanted trees in eight villages (Tinganagalip, Ravat, Gunanba, Rainau, Ulaula, Ratongor, Kabaira, Navunaram) in the Gazelle Peninsula of East New Britain (70–100 nuts from 41 trees) and early-yielding planted trees (5-year-old) in Vunatung Plantation (50 nuts from each of 31 trees), near Kokopo, respectively. This was repeated in 2004, using 50 nuts from 18 trees from three sites in East New Britain (Rabaul, Pomio and the Duke of York atoll). Nuts of the 2004 collection were also dried and sent to Australia for proximate analysis and the assessment of medicinal properties.

Proximate analysis

A 50 g sample of extracted nut kernel (excluding testa) from each of the 18 sampled trees was ground using a hand operated rotary cheese grater. From these ground samples moisture content was determined by Australian Official Analytical Chemists International (2000)—AOAC 925.40), fat (AOAC 948.22—a), protein (AOAC 950.48), ash (AOAC 950.49—A) and sugar (AOAC 32.2.07). Total carbohydrates and energy content were calculated in accordance with Food Standards Australia New Zealand (FSANZ**)** 1.2.8.1.

Fatty acid profile

Galip nut oil was extracted from kernel samples using a hand operated hydraulic laboratory press (Apex Corporation Ltd., London). The kernels (50 g), with the testa removed, were ground using a hand operated rotary cheese grater, wrapped in pure dupion silk and inserted into a stainless steel cylinder with a matching plastic plunger. The wrapped samples were then subjected to a pressure of 70 kPa for 5 min during which time oil was expressed from the sample through small holes in the cylinder. The cold pressed oil was clarified as it passed through the silk and was collected in an aluminium cup positioned below the extraction cylinder. At the end of each extraction the oil was decanted into a Nunc Cryosystem tube (Nalge Nunc International, Rochester, NY) flushed with nitrogen and stored at –20°C prior to analysis.

Fatty acid profiles of galip nut oil (100 mg) were obtained by preparing fatty acid methyl esters (FAMEs) using the International Standards Organisation (ISO) method 5509:2000 clause 5.0 (ISO 2000; Christopherson and Glass 1969). Samples were injected into a Varian 3400 Gas Chromatograph (Varian Corporation, USA) fitted with a flame ionisation detector and split injection port. A fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness: DB 23 from J&W Scientific, USA) was used with a nitrogen carrier gas flow rate of 12.6 cm s^{-1} and a split flow rate of 36 cm s^{-1}. The injector temperature was 240°C and detector was 260°C. The column temperature was programmed as followed: the initial

temperature was 160°C, this was immediately ramped at 1°C min^{-1} to 175°C and then ramped at 5°C min^{-1} to a final temperature of 250°C where it was held for 5 min. The FAME was performed using Star (version 4.5) integration software (Varian Associates Inc., USA) to measure peak areas and expressed as percentage of total FAME peaks detected.

Anti-oxidant and phenolic content

Coarsely ground kernel samples (1.0–1.1 g) were weighed into 50 ml tubes. Fat was extracted by the addition of two successive aliquots of heptane, the first with an overnight extraction with vigorous agitation and the second with 5 h extraction with vigorous agitation. The heptane residue was allowed to evaporate and then 10 ml of 50% ethanol was added. This was incubated for 48 h, with vigorous agitation. Samples were then centrifuged at 3,000*g* for 20 min to remove particulates and clarify the solution. These extracts were then assayed directly, using the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) decolourisation assay for anti-oxidant capacity (Re et al. *1999*) and the Folin-Ciocalteu method for phenolics (Kim et al. *2003*). Anti-oxidant capacity was measured in mg ascorbate equivalents per gram sample, while phenolic content was measured in the units of mg catechin equivalents per gram sample.

Vitamin E

Tocopherol content (α, β, γ, δ isomers) was determined by High Pressure Liquid Chromatography methods (Australian Government Analytical Laboratory Method Code: VL-291) as developed by de Leenheer et al. (*2000*) and Indyk (*1988*). Five gram of each sample was accurately weighed into a 250 ml flask and 60 ml alcoholic KOH is added. The solution is then placed in a water bath at 80°C for 30 min to saponify. The solution was cooled and then transferred to a 500 ml separating funnel containing brine. Extraction was made using petroleum ether with five aqueous washes, each shake and wash followed by collection and combining of organic phases. The petroleum ether extract was then reduced under rotary evaporation followed by nitrogen and the sample made up to 10 ml in a volumetric flask with methanol. Tocopherol isomers within the extract were then separated by Normal phase HPLC on a 5 μ Silica Novapak column using an iso-propanol in tri-methyl pentane mobile phase. Detection was made using fluorescence with excitation at 292 nm and emission at 350 nm. Quantification was then made against tocopherol isomer standards whose concentration were determined by absorbance measurements. Results are expressed to two significant figures in units of μg/100 g.

Anti-inflammatory activity

Ten kernel samples were supplied frozen, and these were stored at −80°C until use. Due to the matrix of the material, a solubility trial was carried out to determine the most effective solvent. Two representative samples were diluted in methanol, DMSO, ethanol, and acetone, at a concentration of 10 mg ml^{-1}. The samples were not soluble in methanol, slightly soluble in DMSO, soluble in ethanol after vigorous mixing, and easily soluble in acetone. Therefore, it was decided that samples would be diluted in acetone.

Prostaglandin E_2 assay

3T3 Swiss Albino fibroblast cells were plated out into 96-well tissue culture plates, in phenol-red free Dulbecco's modified Eagles medium containing 10% foetal bovine serum, 3.5 mg ml^{-1} glucose, 2 mM l-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (1×10^5 cells/ml, 100 μl/well). The cells were cultured overnight at 37°C, 5% CO_2. The samples, as prepared above, were tested undiluted, and at 1:5 and 1:25 dilutions. 1.5 μl of sample was added to the wells, at a final concentration of 150, 30, and 6 μg ml^{-1}. Cells + samples were incubated (37°C, 3 h), before the addition of calcium ionophore A23187. Following a further 20 min incubation, the plate was centrifuged (1,000*g*, 3 min) and the supernatants removed. Included on the plate was a positive control (aspirin; 100 μM), and acetone control, both with and without calcium ionophore A23187 (Sigma C7522). It should be noted that when added to the wells, some of the sample solution precipitated. Therefore, the actual concentration available to the cells is unknown. Given the insolubility of the samples, and toxicity of most other alternative solvents to the cells, an alternative method was not available. The

supernatants were diluted by serial dilution (1:500) in enzyme immunoassay (EIA) buffer, and assayed for PGE_2 using the Prostaglandin E_2 EIA Monoclonal Kit (Cayman Chemical Catalog No. 514010), according to the kit protocol.

Results

The individual trees sampled in 8 villages in East New Britain varied significantly ($P = 0.001$) and continuously (7.3–14.7 g) in their mean nut mass (Fig. 1). Mean kernel mass also varied continuously from 1.4 g to 3.7 g. Although the number of trees sampled per village was small there was significant tree-to-tree variation ($P = 0.001$) within a village, while that between villages was not significant (Fig. 2). There was similar and significant tree-to-tree variation in mean kernel mass in trees from the young Vunatung plantation in 2002 (Fig. 3). However, trees in this plantation had significantly lower kernel mass than those from the naturally regenerated village trees. Kernel:nut ratio was weakly related to kernel mass (Fig. 4), such that trees with a high kernel mass, do not necessarily have a high kernel:nut ratio (i.e. thin and easily-cracked shells and a single kernel).

Fig. 1 Nut mass of *Canarium indicum* from individual trees in East New Britain, Papua New Guinea, in ascending order

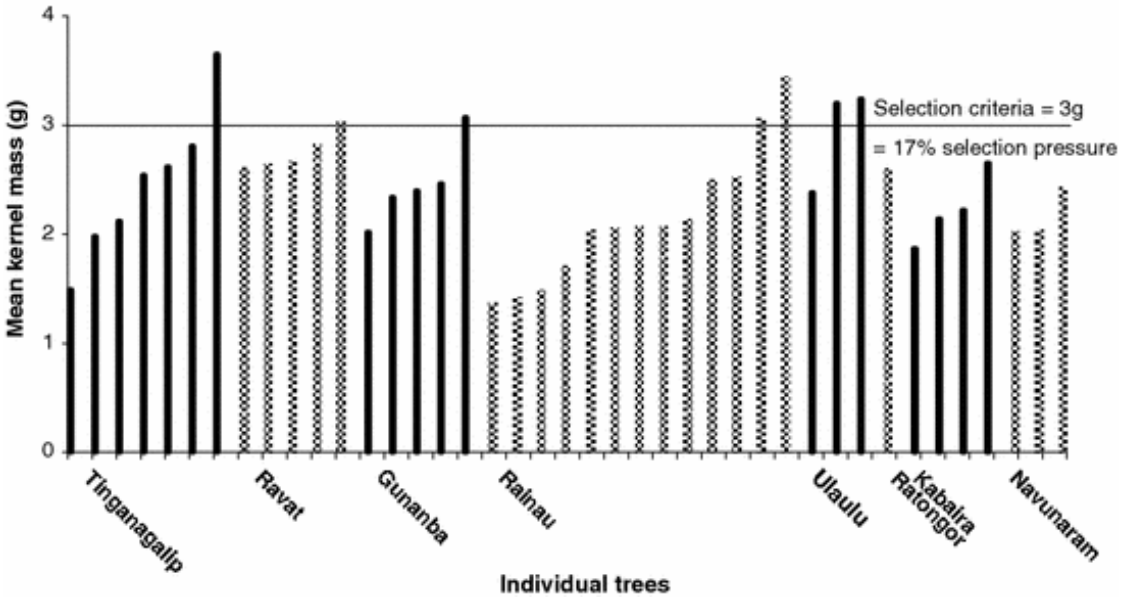

Fig. 2 Kernel mass of *Canarium indicum* from individual trees in villages in East New Britain, Papua New Guinea

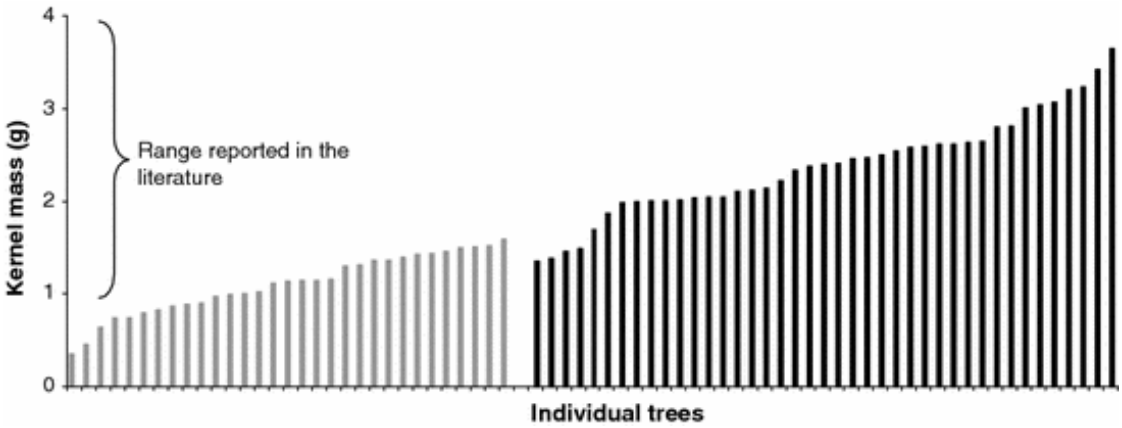

Fig. 3 Kernel mass of *Canarium indicum* from two collections in PNG (Grey = 2002 from young Vunatung plantation; Black = 1998 from older farm trees)

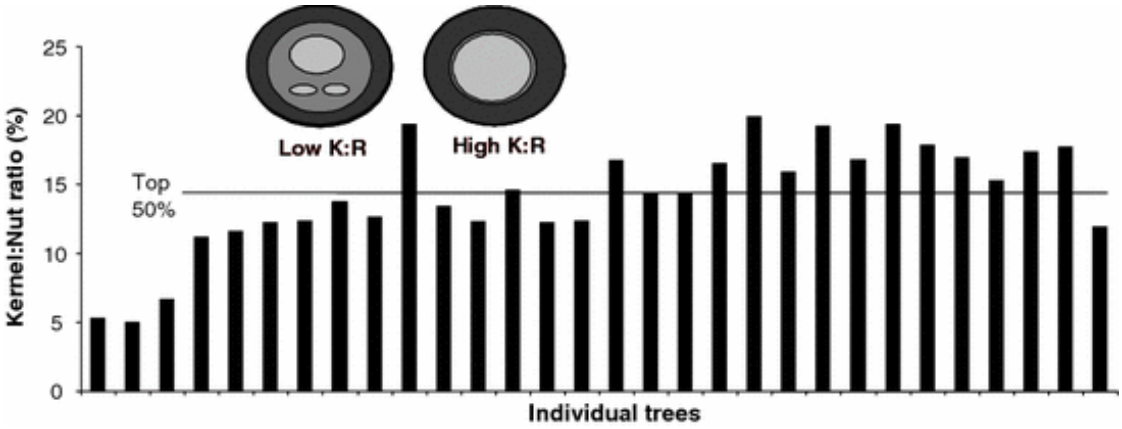

Fig. 4 Kernel:nut ratio (%) in *Canarium indicum* from Vunatung Plantation (2002), in kernel mass order

Continuous variation was also found for fat content (Fig. 5), fatty acid profile (Fig. 6), Saturated and unsaturated fats (Fig. 7), carbohydrate content (Fig. 8), protein content (Fig. 9), tocopherol content—especially beta-tocopherol (Fig. 10), sodium content (Fig. 11), anti-oxidant capacity (Fig. 12). These analyses were done on single samples so statistical analysis was not possible. However, analysis of anti-inflammatory activity was replicated and the tree-to-tree variation was highly significant (Fig. 13). The differences in anti-inflammatory activity were not significant between three dosage rates.

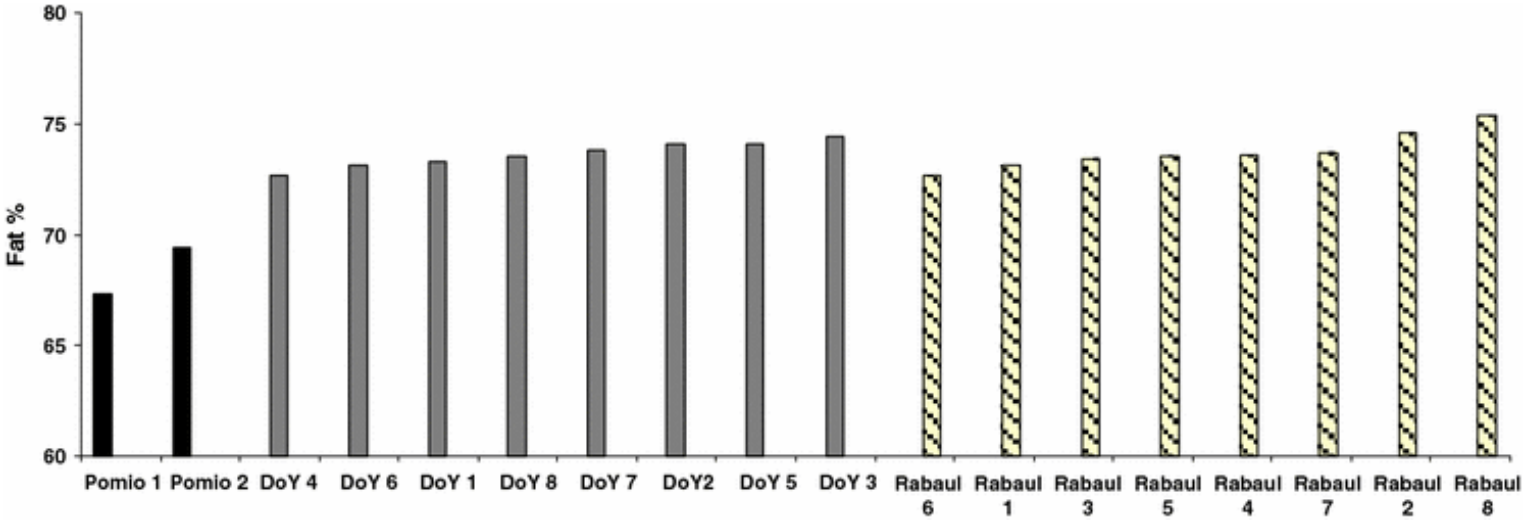

Fig. 5 Fat content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

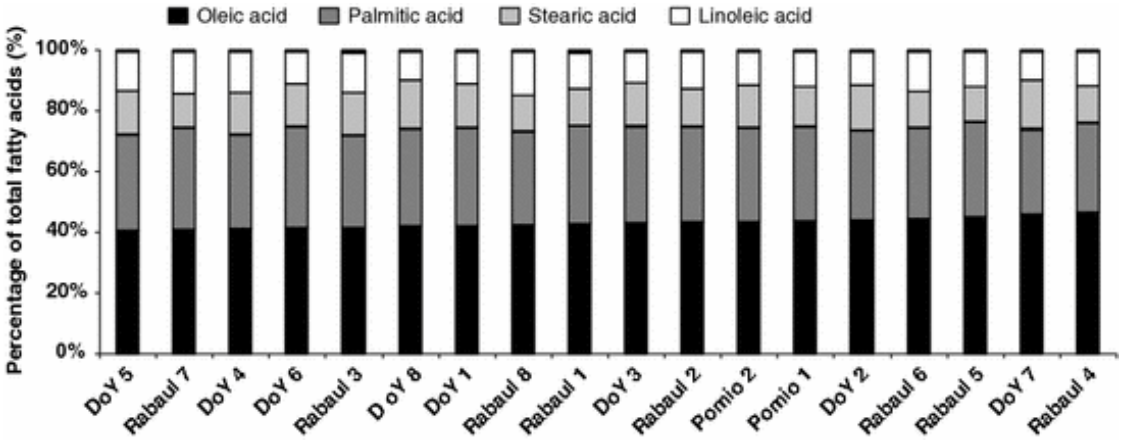

Fig. 6 Fatty acid content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

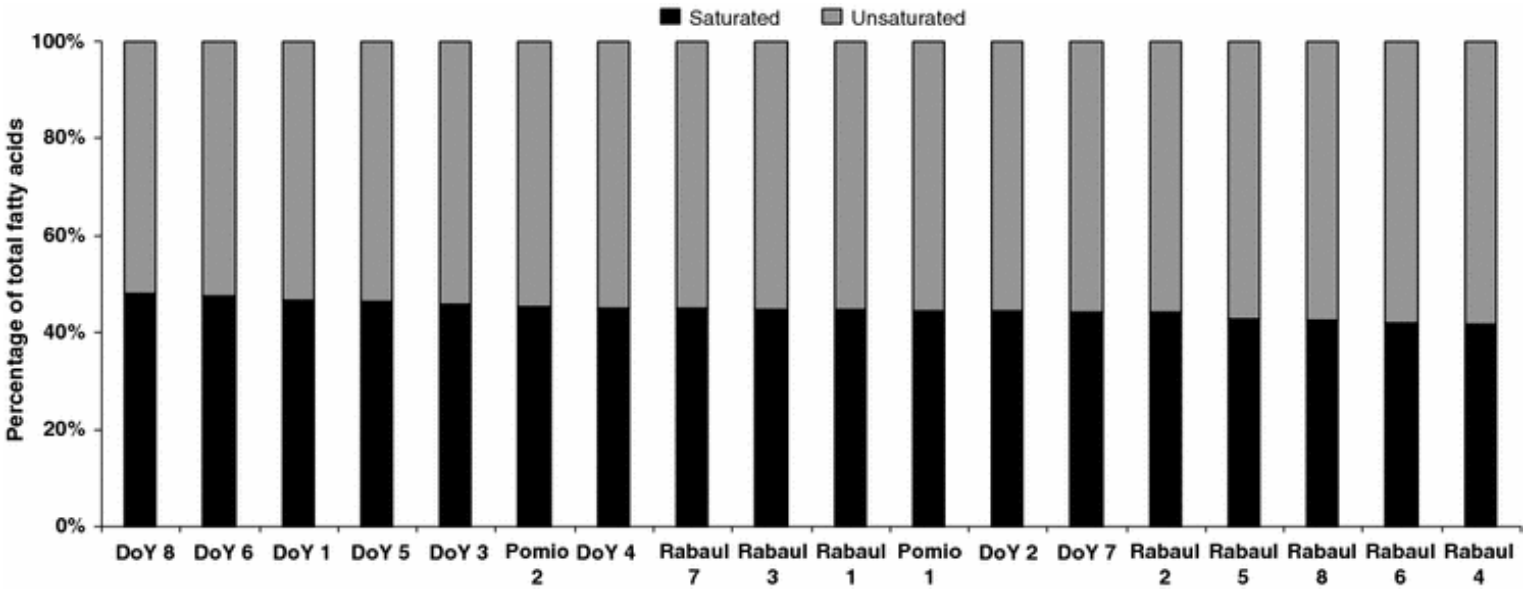

Fig. 7 Saturated to unsaturated fatty acid content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

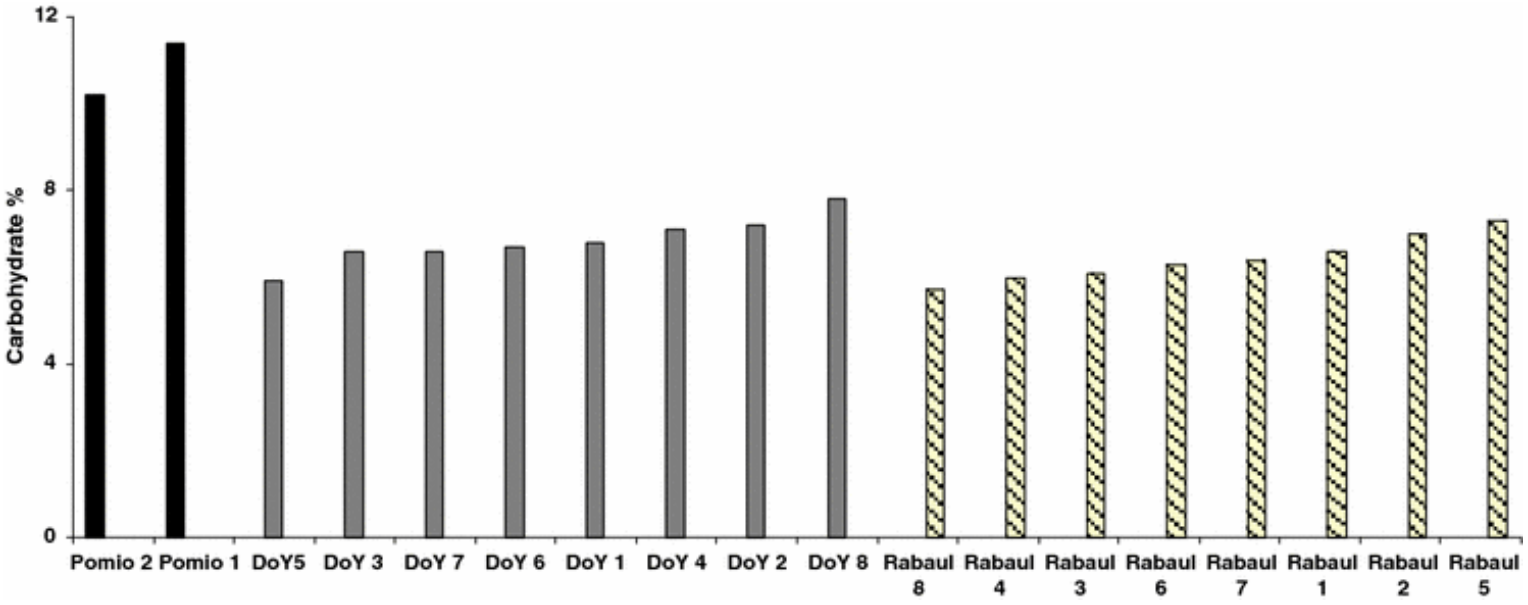

Fig. 8 Carbohydrate content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

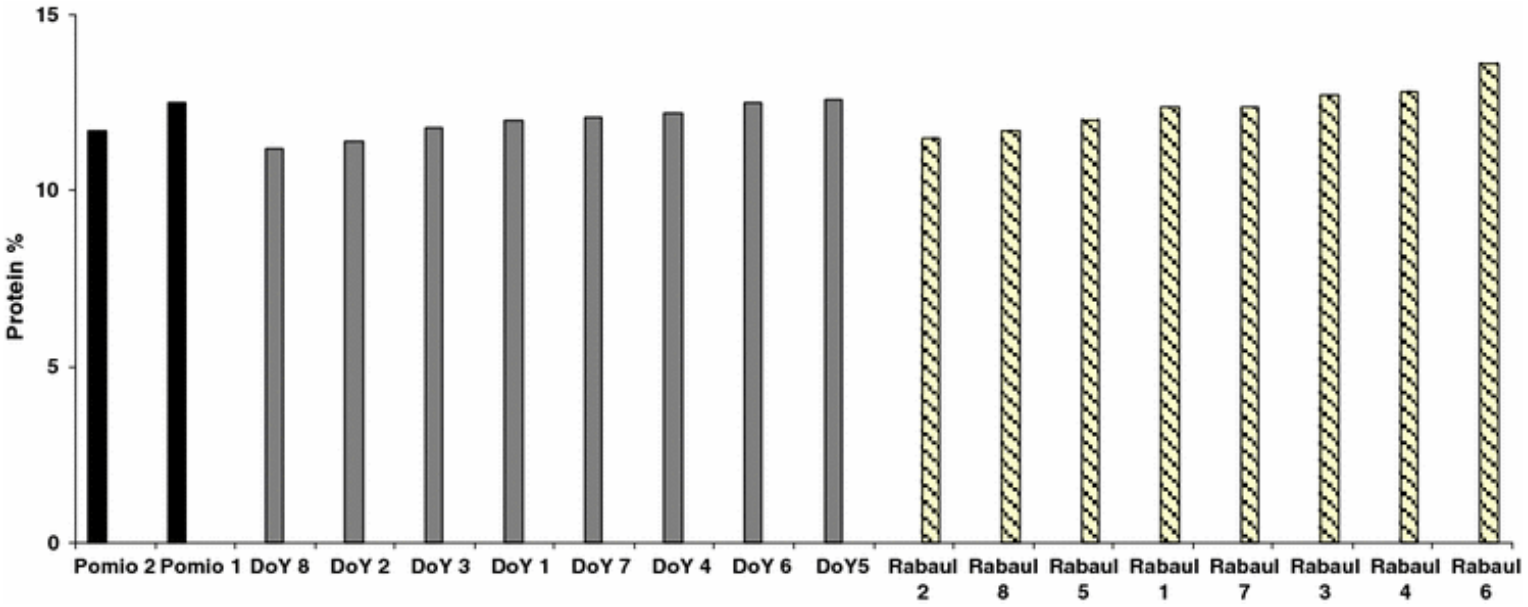

Fig. 9 Protein content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

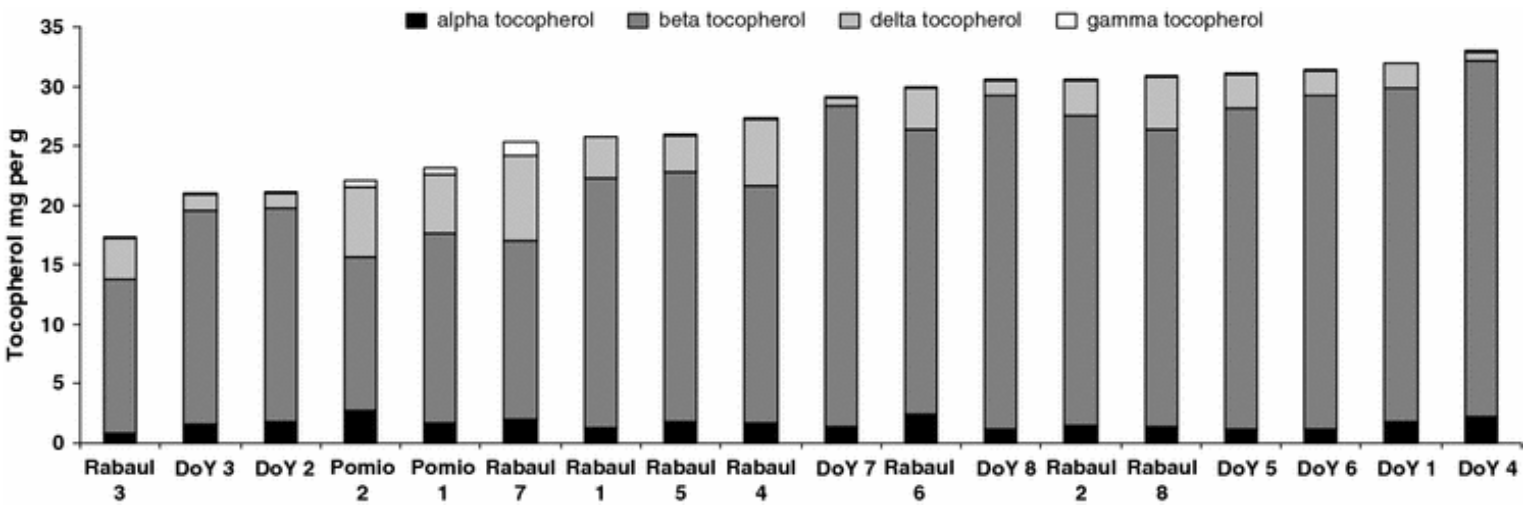

Fig. 10 Tocopherol content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

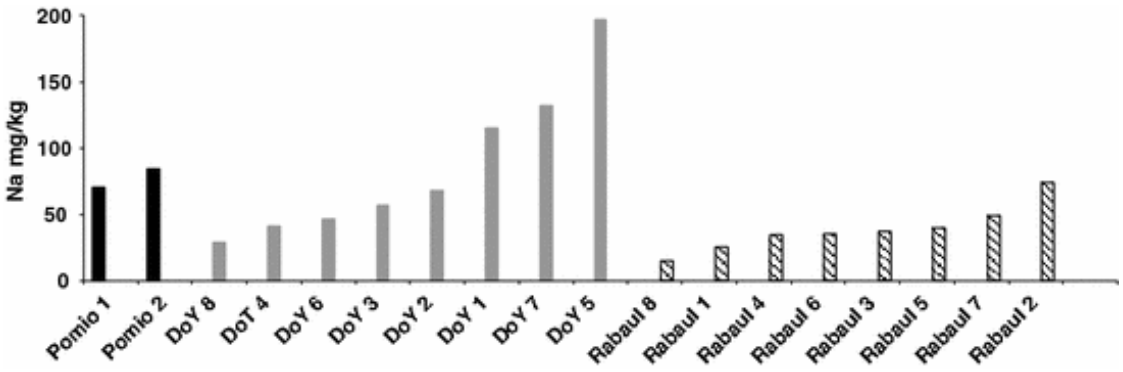

Fig. 11 Sodium content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

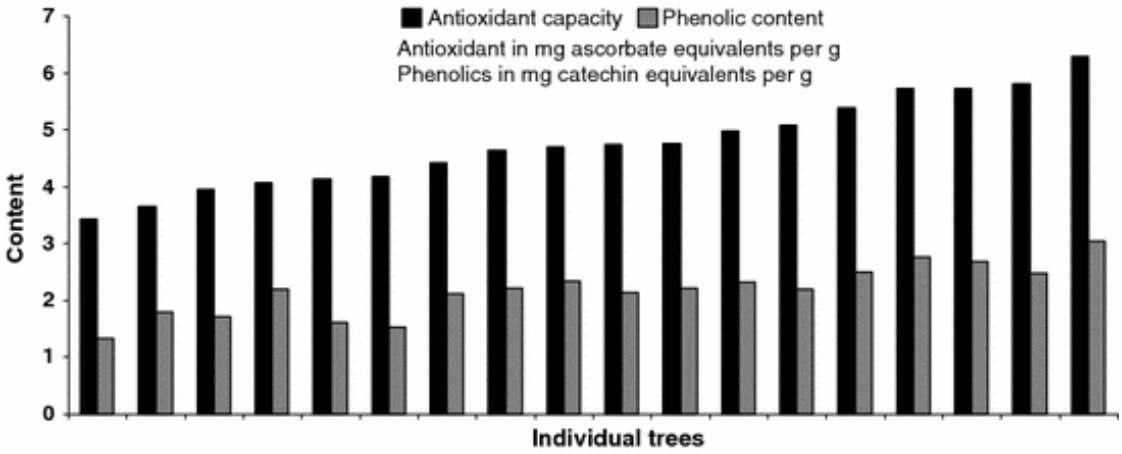

Fig. 12 Anti-oxidant and phenolic content of *Canarium indicum* kernels from individual trees in East New Britain, Papua New Guinea

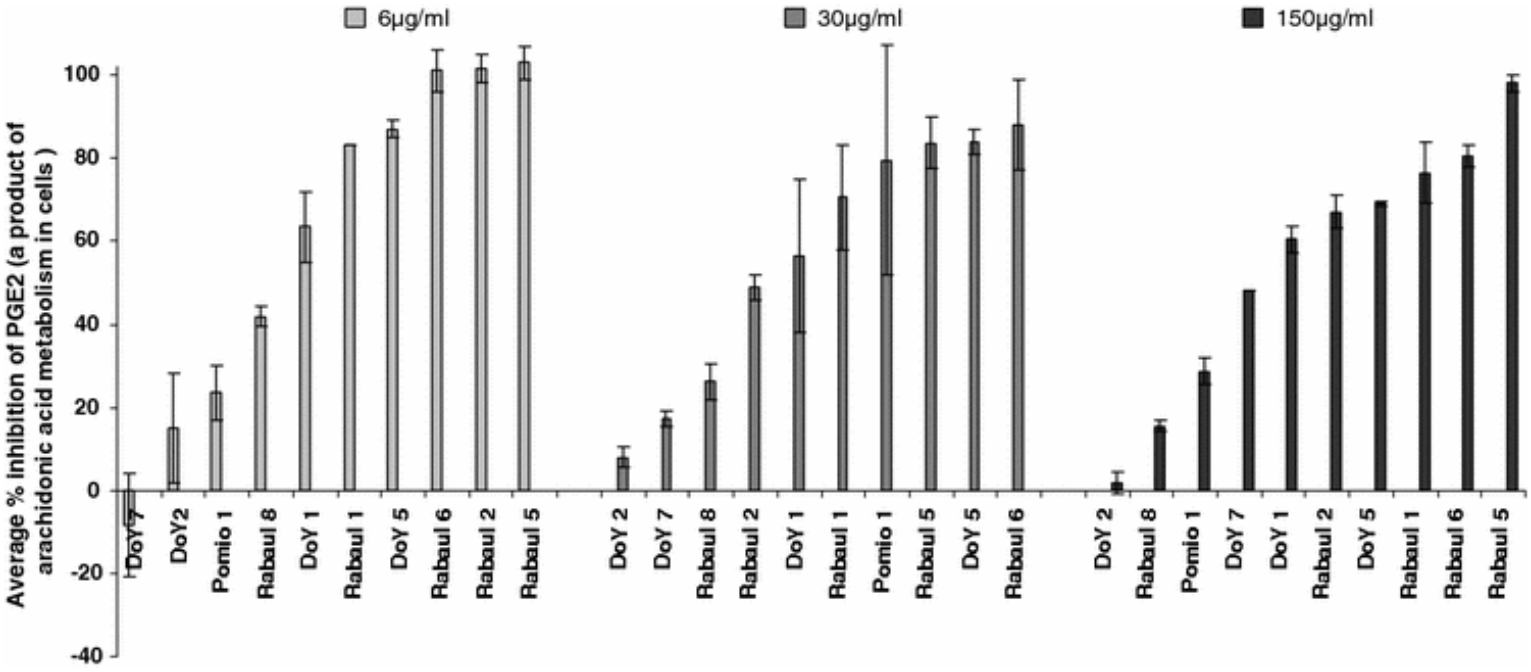

Fig. 13 Anti-inflammatory properties of *Canarium indicum* kernels (Inhibition of prostaglandin E2 [PGE2])

Strong negative relationships were identified between fat and carbohydrate contents and carbohydrate content and energy (Table 1). Strong positive relationships were however, identified between fat content and energy, total carbohydrate and sucrose contents and anti-oxidant activity and phenolic content (Table 1), weak negative relationships were found between fat and protein content and protein and carbohydrate contents (Table 1).

Table 1 Relationships between nut and kernel traits in individual trees of *Canarium indicum* from East New Britain, Papua New Guinea

	r^2
Fat content versus energy	0.993
Fat content versus protein content	0.037
Fat content versus carbohydrate content	0.823
Protein content versus carbohydrate content	0.046
Carbohydrate content versus energy	0.793
Fat content versus total tocopherol (Vit E)	0.077
Carbohydrate content versus sucrose content	0.766
Phenolics content versus anti-oxidant content	0.843

Discussion

The continuous variation in kernel mass has important implications for domestication, suggesting that considerable opportunities for cultivar development exist at the village-level. As found in other indigenous fruits and nuts, this means that village-level domestication can potentially enhance the livelihoods of village communities, while maintaining genetic diversity at the national level (Leakey et al. *2003*). In this study, selection of trees with mean kernel mass in excess of 3 g would result in considerable improvement in the quality and uniformity of marketable kernels. Screening a larger number of trees would almost certainly allow even greater benefits to be achieved, with the possibility of raising mean kernel mass above 4 g as kernels of this mass have been reported (Evans *1996*).

There was an unexpected and unexplained difference in morphological traits between unplanted village populations and a managed plantation population. The reasons for the generally smaller size of kernels from planted trees in Vunatung plantation are unclear, but possibly include:

- Genetic variation. This is a possibility as there is some evidence that the nuts germinated to establish the first planting were small.
- Poorly adapted planting stock, although this is unlikely as the planting stock was local.
- Excessive competition for resources between the first fruits from young trees.
- Some unusual environmental factor associated with that particular fruiting season.
- Error at the time of assessment.

Some of these possibilities could be tested by a reassessment of the trees.

A literature review (Nevenimo et al. *2007*) indicates that the grading of *Canarium* kernels and associated pricing structure for market is done on the basis of the kernel:nut ratio. However, on the basis of morphological data presented here this trait does not necessarily capture the trees which produce the biggest kernels (Fig. 4). Thus to identify elite trees it is important to include mean kernel mass among the selection criteria, in addition to kernel:nut ratio. However, it also has to be appreciated that there are economic criteria that indicate that kernel:nut ratio is important (Evans *1994*), particularly with regard to the ease and consequently labour costs, of extracting kernels (Table 2). In addition the larger and less damaged kernels resulting from selection of kernel:nut above 0.25 also attract a higher market price. Thus it is recommended that at least two kernel traits (kernel mass and kernel:nut ratio) should be used when selecting elite trees. Restricting selection to

these two morphological traits, would however, miss the opportunity to select trees to meet specific market opportunities. Selection should, therefore, also consider nutritive and medicinal properties. Proximate analysis found that the tree-to-tree variation in protein, carbohydrate and oil content were not large. A similar observation can be made in regard to fatty acid composition, but analysis of some medicinal traits identified greater variation.

Table 2 The relationships between kernel:nut ratio, kernel weight, cost of kernel extraction and market price in 1994 (After: Evans *1994*)

Kernel:nut ratio	Kernel wt	Cost of extraction per kg of kernels (1994)	Price paid for nuts in Kandrian (1994)
>0.25	3.5	US$ 0.26	US$ 0.36
0.21–0.25	2.5	US$ 0.36	US$ 0.30
0.16–0.2	1.8	US$ 0.50	US$ 0.24
0.1–0.15	1.2	US$ 0.75	–

Canarium kernels are very rich in oils (67.3–75.4%). These are commonly used as a medicinal product, for cooking, and in cosmetics and skin care products. Thus domestication activities should also recognise that there is continuous variation in oil content in tree populations (Fig. 5), and again higher levels of selection pressure could be used to increase or decrease the oil content of selected cultivars, through the identification of appropriate ideotypes (Leakey and Page *2006*). Higher oil content may be desirable for pharmaceutical, cosmetic or certain food uses, but lower oil content may be desirable for edible kernels. In the latter, however, it may be more important for health reasons to select for lower saturated:unsaturated fat content in which oleic and linoleic fatty acids are a high proportion (Figs. 6 and 7). From this study, it would seem that the potential for selection for healthier oil qualities may not be big, but this may reflect the limited geographic range tested, as well as the small sample size.

The low oil content of kernel samples from Pomio (Fig. 5) and the high carbohydrate content of the same samples (Fig. 8) can probably be attributed to their greater age, as they were collected during the May 2004 fruiting season, while the other samples were collected in September 2004. Seeds in general come in two types, those that store their reserves as starch and those that store their reserves as oils/fats. In both cases, reserves are hydrolysed to release energy for growth in the form of soluble sugars. Thus is would appear that the Pomio samples had converted some oils to carbohydrates during storage. This suggestion is supported by the strong relationships between oil and carbohydrate contents (Table 1), and energy and both carbohydrate and fat, in which the Pomio samples are clearly distinct, as well as in the relationship between sucrose and carbohydrate content.

In terms of the nutritive value of *Canarium* kernels there is relatively small variation between trees in protein content (Fig. 9), but greater variation in Vitamin E (tocopherols, especially beta-tocopherol—Fig. 10). There was also substantial variation in sodium content (Fig. 11), this being most variable in the population from the Duke of York atoll, perhaps reflecting saltier soils.

Finally, with regard to the medicinal properties, trees differed in their anti-oxidant capacity (Fig. 12), this being related to phenolic content (Table 1). The results of the anti-oxidant and phenolic assays may represent an underestimate due to inefficiencies in the extraction of the samples. It is recommended that the if the measurement of phenolic and anti-oxidant content of galip nuts is seen as an important area of research, that methods for reliable extraction be explored. *Canarium* kernel oil has been included in a product marketed as an anti-inflammatory (http://www.get-arthritis-relief-now.com), while patents on a product for the prevention and treatment of arthritis have been registered in a number of countries. However, there is no scientific evidence of the efficacy of *Canarium* nut oil in peer-reviewed literature. Inflammatory response is accompanied by the release of prostanoids, the predominant product being PGE_2 (Rang et al. *1995*). PGE_2 is a primary product of arachidonic acid metabolism in cells, and when cells are activated or exogenous free arachidonate is suppled, PGE_2 is synthesized *de novo* and released into the extracellular space. Consequently, this study tested *Canarium* nut oil

samples for their anti-inflammatory properties by measuring the inhibition of prostaglandin (PGE_2) produced from 3T3 Swiss Albino fibroblast cells, compared to an Asprin (100 μM) and acetone control, exposed to kernel oil and stimulated with calcium ionophore. An inhibition of PGE_2 production in these cells, relative to control cells not exposed to Galip nut oil, indicates the nut oil acts as an anti-inflammatory in vitro. Significant tree-to-tree variation in PGE_2 inhibition was found at three sample concentrations (6, 30 and 150 μg ml^{-1}), with two trees (Rabaul 5 and 6) consistently causing inhibition in excess of 80% (Fig. 13). These results indicate that kernel oil of these two trees inhibited PGE_2 production at a rate comparable to aspirin, however, due to the preliminary nature of these tests, there is a need for more detailed studies.

The lack of a dose response on anti-inflammatory activity is unexpected. It is possible that this represents a solubility problem as some precipitation occurred during the analytical process, and it is possible that only low concentrations of *Canarium* kernel oil remained dissolved in the small volumes required for the assay. Thus it is recommended that further in vitro testing occur to determine dose responses. Monitoring the chemical content of the extracts in parallel with the PGE_2 inhibitory activity would also allow some discrimination between the activities of the different nut oil samples.

It is clear from this study that the nuts and kernels from even a small population of individual trees of *Cananrium indicum* vary very considerably in morphological traits, while the site-to-site variation was not so great. This pattern of within and between population variation concurs with that of all other indigenous fruit/nut trees investigated to date (Leakey et al. *2005a*). This variability provides excellent opportunities for the domestication of the species through the development of multiple-trait cultivars from elite trees, using simple horticultural techniques. The data presented has allowed the development of a strategy to domesticate *C. indicum* for cultivation in homegardens and cocoa–coconut agroforests (Leakey, in press), using a participatory approach aimed at the production of agroforestry tree products (AFTPs) to empower small-holders and enhance their livelihoods and income. This strategy for rural development in the lowlands of Papua New Guinea aims to fulfil the needs of producers and consumers (Nevenimo et al. in press) and offers opportunities for wider scale marketing of *Canarium* kernels (Bunt and Leakey, in press).

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