Genetic fingerprinting of Fijian kava

Anjeela D Jokhan[1], Patricia A McLenachan[2]
[1]*Department of Biology, The University of the South Pacific, Suva, Fiji*
Jokhan_ad@usp.ac.fj
[2]*Allan Wilson Centre for Molecular Ecology and Evolution, Massey University, Palmerston North, New Zealand*
p.a.mclenachan@massey.ac.nz

ABSTRACT

DNA analysis of 15 kava cultivars with 20 primer pairs showed no observable difference in the fingerprint pattern. This indicates that while the Fijian kava cultivars have morphological differences, genetically they are very similar. Similar results have been obtained with Hawaiian kava cultivars. This result indicates that when studying diseases such as the dieback disease in kava, a focus on environmental factors may be needed as controlling factors of the diseases.

1 INTRODUCTION

Kava (commonly known as yaqona in Fiji) is a dicot plant belonging to the family Piperaceae. The family is mainly tropical and comprises five genera and over 2000 species. The genus *Piper* is the most numerous in this family and includes several aromatics such as black pepper (*Piper nigrum, L.*) (Onwueme and Papademetriou, 1997).

Cultivated kava is classified as *Piper methysticum* Frost F. and is closely related to *Piper wichmanni* C. DC. There is some evidence to suggest that kava was derived from *P. wichmanni* through centuries of natural and human selection (Lebot and Lindstrom, 1992). While *P. wichmanni* reproduces sexually by producing seeds in the wild,
P.methysticum is unable to set seeds and thus relies entirely on humans for its propagation and dispersal.

There is also some evidence to suggest that kava (*P. methysticum)* originated in Vanuatu. Vanuatu has the greatest number of cultivated varieties. Of the 118 kava cultivars found in the Pacific, 80 are found in Vanuatu alone (Lebot *et al*. 1999).

Kavalactones are the main psychoactive and pharmaceutical constituents of kava. Over 15 kavalactones have been identified in kava but only 6 of these are present in large quantities. Different parts of the plant (such as roots, stumps and leaves) contain different amounts and types of kavalactones (Onwueme and Papademetriou, 1997).

Fiji is closer to Vanuatu and is thought to have been colonised before Hawaii (Gray and Jordan, 2000) so it was reasonable to expect there may be more diversity in the cultivars from Fiji compared to those from Hawaii. Human mitochondrial genome studies have shown that the diversity of haplotypes found in Hawaii is a subset of that found in Central Polynesia (REF) and this may be reflected in domesticated plants taken with the colonisers. Fifteen Fijian kava cultivars were chosen on the basis of their morphological and chemical differences for genetic analysis.

In addition to the widely known recreational, social, cultural and religious uses of kava, it has also been used as a folk medicine for a long time. Kava has been used for conditions such as urino-genital inflammation (in Samoa, Hawaii and Vanuatu), gastro-intestinal upset (in Hawaii, Fiji and Vanuatu), headache (in Hawaii, Fiji, Samoa and Vanuatu) and many other conditions in the Pacific Island countries (Onwueme and Papademetriou, 1997).

More recently, kava has been harvested in Fiji for the Western "health food" market. A capsule is made from dried ethanol or acetone kava extracts (whereas tradtionally kava is prepared in water). However such preparations have been implicated in the deaths of a small number of people through liver damage and kava preparations are now banned in most Western countries (Moulds and Malani, 2003). This ban, coupled with the effects of dieback disease has had a detrimental economic effect on the kava industry.

In Fiji there are some 30 kava cultivars and these have been identified based on their morphological characteristics and the geographical area from which they come from. Some of these plants have significant morphological differences, not only in terms of pigmentation but also in leaf structure and branching patterns. The cultivars also exhibit chemical differences, for example one cultivar originally from Vanuatu is known as 2-day kava because the effects last for 2 days. Another cultivar is known for its weaker effects.

(Lebot *et al.* 1999) undertook a study of Hawaiian kava. They investigated chemical differences in relation to the cultivar, plant age and the different parts of the plant and also used AFLP to generate genetic fingerprints for the cultivars. The fingerprints showed that 22 cultivars were monomorphic for 21 pairs of primers which indicates that the cultivars are genetically extremely closely related. Lebot and his co-workers concluded that environmental factors are more likely to influence the morphological characteristics of the plants. They futher suggested that morphological variation in the Hawaiian cultivars is controlled by one or two genes only and that such variation might have arisen from somatic mutations, selected clonally from a single source of kava species.

While work on kava has focused on the chemistry and pharmacological aspects, very little work has been done at the molecular level for this economically important plant of the Pacific. To date no such work has been done in Fiji. The work of Lebot and his co-workers (1999) confirm the need to take a close look at the Fijian kava cultivars in the local environment. The main objective of this study was to establish if there were significant genetic differences among cultivars and if so could they be linked to their response to the kava dieback disease. Therefore DNA

fingerprinting was carried out to determine genetic differences among Fijian kava cultivars.

2 MATERIALS AND METHODS

The samples were collected from the Ministry of Agriculture farm at Naduruloulou in Fiji. Strips of leaves (20 mm x 50 mm) were sealed in small plastic bags with silica gel and taken to the Allan Wilson Centre for Molecular Ecology and Evolution at Massey University.

2.1 DNA EXTRACTION

Total genomic DNA was prepared from the dried samples using a CTAB protocol (Doyle J.J. and Doyle J.L, 1990) and the DNA was resuspended in water. At a later date, fresh leaf material was collected and extracted at USP in Suva. Fresh leaf gave more DNA that had less colour than dried material. DNA extracted from both fresh and dried leaf was suitable for subsequent analysis.

2.2 ITS - AMPLIFICATIONS

The internal transcribed spacer (ITS) region sits between the genes encoding the 18S and the 26S ribosomal (r) RNAs and includes the rRNA gene for the 5.8S rRNA. The sequence of the 5.8S rRNA gene is very conserved, but the spacer regions on either side of it are variable and have been used extensively for phylogenetic studies in plants. The ITS region was amplified using the standard primers ITS5 and ITS4 (Baldwin *et al* 1992), in 2 cultivars, Yaqonu Karawa and Cokobawa Vula. The reaction was carried out in 20µl containing 250 µM dNTPs, 10 pmol of each primer, 1XQ solution, 1X Qiagen PCR buffer, 1U of Qiagen Taq polymerase and 3-30 ng of DNA (usually a 1/10 dilution of the extracted DNA). The thermocycling profile was as follows: one cycle of 95^{o}C for 3 mins; 35 cycles of 95^{o}C for 30 secs, 48^{o}C for 30 secs, 72^{o}C for 1 min; one cycle of 72^{o}C for 5 mins. The products were cleaned enzymatically (2µl SAP, 1µl Exo1, 30mins at 37^{o}C, 15 mins at 80^{o}C) and sequenced on an ABI 377 automatic sequencer at Massey University.

2.2.1 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM TECHNIQUE (AFLP)

Polyacrylamide gels were prepared, 6 µl samples of the loaded in the wells with a spade-tip loading tips (0.37 mm) with dye (bromophenol blue). The DNA standard used was the GibcoLifetech 100bp ladder diluted 100 fold. The gel was run at 55 W till the remaining dye from (bromophenol blue) was about 60 mm from the base of the gel. Silver staining was used to stain the polyacrlamide gel.

Initially 3 DNA samples were used for primer screening using AFLP. The three samples were chosen because they were different in the following way:

2.2.1.1 Yagona Yalu - this cultivar is also known as the '2-day' kava, thought to be the same as the '2-day' kava found in Vanuatu. This is a strong kava, the effect of which lasts for 2 days after consumption, unlike the others, the effects of which wear off much sooner. The plant has vigorous growth with many shoots arising from the base of the plant, which is distinctively different from the other plants.

2.2.1.2 Yagona mama - this variety has thick inter-nodes with variegation. The leaves and petioles are green and soft. It is thought to be a mild kava. These plants appear more herbaceous than the others.

2.2.1.3 Yagona Vula - this cultivar has thin, variegated stem but this plant appears more woody than yagona Mama.

The primers used for screening were 4 Mse1 primers: (M-CAA, M-CTG, M-CTC and M-CTA) and 5 EcoR1 primers: (E-AAA, E-ATA, E-AGC, E-ATA and E-AGC), 20 combinations in all.

3 RESULTS AND DISCUSSION

The ITS sequences of both cultivars were identical and this sequence was identical to the GenBank accession for *Piper methysticum* (AF275194).

Amplified fragment length polymorphism (AFLP) is a fingerprinting technique that supercedes other techniques such as the restriction fragment length polymorphism (RFLP) and random amplified polymorphism (RAPD because of its reproducibility, the requirement of small amounts of genomic DNA and the number of polymorphic bands that can be resolved per reaction (Vos *et al*. 1995, Lin *et al.* 1996; Mueller and Wolfenbarger, 1999). Total genomic DNA is cut with restriction enzymes, linkers are ligated to the fragment ends, then the restriction fragments are amplified, using selective primers These are primers complementary to the linkers but with 2 or more additional bases at the 3' end, which must be complementary to the restriction fragment for amplification to occur. Many different primer combinations can be used, each giving a fingerprint of a subset of the entire genome. Some primer pairs do not generate a fingerprint, hence the need for an intial screening process (Vos *et al*. 1995).

Results for the initial screening of primers using 3 cultivars only are as follows (Table 1), with a cross indicating that a scorable banding pattern was observed while the II shows the non-scorable banding pattern.

Almost no differences were observed in the fingerprint patterns for 3 cultivars and 20 primer combinations. Two primer combinations were chosen to apply to all 15 cultivars - M-CAA/E-AAA and M-CAA/E-AGC. *Macropiper excelsum,* (New Zealand kawakawa) DNA was included as a control. 46 and 55 scorable bands were observed with these primers respectively but the differences across the 15 cultivars was only one or 2 bands in each case, about a 2% difference. Differences of less than 5% are thought to be insignificant; ie not above the error margin of the method. The fingerprint for *Macropiper excelsum* was significantly different with very few shared bands.

Table 1. Primer pairs used for AFLP screening in kava.

	M-CAA	M-CTC	M-CTA	M-CTG
E-AAA	✓	✓	X	✓

E-ATA	✓	✓	X	✓
E-AGC	✓	✓	X	✓
E-ATT	✓	✓	X	✓
E-AGG	✓	✓	X	✓

These results indicate that the Fijian kava cultivars are genetically very similar. This observation is consistent with the results from Hawaiian kava.

Work currently being undertaken on another interesting Pacific plant, the bottle gourd (*Lagenaria siceraria*) is showing similar results. Bottle gourds have very diverse fruits, in colour, size and shape; this is unlikely to be due to phenotypic plasticity However cultivars from Africa, Asia and the Pacific show extremely similar genetic fingerprints (Andrew Clarke, pers. comm). Conversely, kumara cultivars show quite different fingerprint patterns (Andrew Clarke, pers comm). Kumara (*Ipomoea batatas* Linn.) is another important Pacific crop with a varied history, that is propagated vegetatively.

4 CONCLUSION

Although many of the kava cultivars looked different morphologically, they did not show any significant genetic differences.

Other methods could be used to investigate differences between the cultivars, eg. cDNA -AFLP, or a similar differential expression assay which generates a fingerprint of expressed products (Lin *et al*. 1996) Methods such as these may be a way to explore differences in kavalectone expression and may help with the isolation of the genes.

A better understanding of the causative agents of dieback disease in kava is also needed but these results indicate that a focus on environmental factors may be needed for controlling the disease.

REFERENCES

1. Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S. and Donoghue
2. Doyle, J.J. and Doyle, J.L 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**, 13 -15.
3. Gray, R.D. and Jordan, F.M. 2000. Language trees support the express-train sequence of Austronesian expansion. *Nature* **405** 1052 - 1055
4. Lebot, V., Merlin, M. and Lindstrom, L. 1992. Kava, The Pacific Drug. Yale University Press, New Haven, 255.
5. Lebot, V., Johnston, E., Zheng, Q.Y., McKern, D. and McKenna, D.J. 1999. Morphological, phytochemical and genetic variation in Hawaiian cultivars of 'Awa (Kava, *Piper methysticum*, Piperaceae). *Economic Botany* **53**, 407 - 418.
6. Lin, J.J., Kuo, J. and Ma, J. 1996. Effects of different primer pair combinations on the resolution of AFLP in plants with small genomes. *Focus* **18**, 66-69.
7. M.J. 1995. The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Gard* **82**, 247 - 277.
8. Moulds and Malani 2003 Kava; herbal panacea or liver poison? *MJA* **178**(9) 451-453
9. Mueller, U.G. and Wolfenbarger, L.L 1999. AFLP genotyping and fingerprinting. ***Tree*** **14**, 389 - 394.
10. Onwueme, I.C. and Papademetriou, M.K. 1997. *The kava crop and its potential*. RAP Publication,
11. Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frijters, A. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407 - 4414.