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NRC Research Press

ARTICLE

Comparative analysis of genetic variation in kava (*Piper methysticum*) assessed by SSR and DArT reveals zygotic foundation and clonal diversification

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Abstract: Kava (*Piper methysticum*) is a major cash crop in the Pacific. The aim of this study was to assess genetic variation among 103 accessions of kava using SSRs and DArTs. Genetic structure was determined using clustering analyses (WPGMA) and principal coordinate analyses (PCA). Thirteen SSR primers and 75 DArT markers were found polymorphic, and the two types of markers generated similar clustering patterns. Genetic distances ranged from 0 to 0.65 with an average of 0.24 using SSRs and from 0 to 0.64 with an average of 0.24 using DArT. Eleven genotypes were identified with SSR while 28 genotypes were identified with DArT markers. By combining the two sets of markers, a total of only 30 distinct genotypes were observed. In the Vanuatu archipelago, noble cultivars originating from different islands clustered together within a very narrow genetic base despite their diversity of morphotypes. SSR and DArT fingerprints allowed the identification of kava cultivars unsuitable for consumption, so called "two-days", and clearly differentiated the wild types classified as *P. methysticum* var. *wichmannii* from the cultivars as var. *methysticum*. Molecular data reveals that all noble cultivars evolved by the predominance of clonal selection. Although they are represented by clearly distinct morphotypes, these cultivars are genetically vulnerable and their potential to adapt to forthcoming changes is limited. These newly developed markers provide high resolution and will be useful for kava diversity analyses and quality assessment.

Key words: kava, clonal selection, kavalactones, genetic variation, SSR, DArT.

Résumé : Le kava (*Piper methysticum*) est une culture de rente importante dans le Pacifique. L'objectif de cette étude était d'apprécier la diversité génétique de 103 accessions de kava à l'aide de marqueurs SSR et DArT. La structure génétique a été déterminée à l'aide de classification hiérarchique (WPGMA) et par analyse en composantes principales (ACP). Treize amorces SSR et 75 marqueurs DArT se sont révélés être polymorphes et les deux types de marqueurs ont donné des agrégations similaires. Les distances génétiques varient de 0 à 0.65 avec une moyenne de 0.24 pour les SSR et de 0 à 0.64 avec une moyenne de 0.24 pour les DArT. Onze génotypes sont identifiés par SSR alors que 28 génotypes sont identifiés par DArT. En combinant, les deux types de marqueurs un total de 30 génotypes distincts sont observés. Dans l'archipel du Vanouatou, les cultivars dits nobles et originaires d'îles différentes se regroupent entre eux et présentent une base génétique très étroite malgré leur variabilité morphologique. Les empreintes SSR et DArT permettent de différencier les formes sauvages identifiées comme *P. methysticum* var. *wichmannii*. Les données moléculaires révèlent que les cultivars nobles ont évolué par sélection clonale. Bien que représentés par des morphotypes distincts, ces cultivars sont génétiquement vulnérables et leur potentiel d'adaptation aux futurs changements est extrêmement faible. L'utilisation de ces marqueurs pourrait s'avérer fort utile à l'avenir pour les études de diversité et le contrôle de la qualité.

Mots-clés : kava, sélection clonale, kavalactones, variabilité génétique, SSR, DArT.

Introduction

Kava is the traditional beverage of the Pacific obtained by cold water extraction of the roots of *Piper methysticum* Forst. Morphological, chemical, cytological, and genetic evidence indicates that kava derives from the wild species *Piper wichmannii* C. DC. through domestication and selection (Lebot and Lévesque 1996*a*). These two taxa are now considered as a single species, with the cultivars identified as var. *methysticum* and the wild forms as var. *wichmannii* (Applequist and Lebot 2006). The species is dioecious and decaploid ($2n = 10x = 130$) (Lebot et al. 1991). It is the only cultivated plant of economic importance with an area of distribution restricted entirely to the Pacific Islands. Wild kavas are found in Papua New Guinea (PNG), the Solomon Islands, and Vanuatu, while the cultivars are distributed in Melanesia (PNG, Vanuatu, and Fiji), Micronesia (Pohnpei and Kosrae), and Polynesia (Tonga, Samoa, French Polynesia, and Hawaii).

Specimens of var. *wichmannii* are found in cultivation and are seedless in Vanuatu, although it is known to produce seeds in PNG and the Solomon Islands. In contrast, kava cultivars are exclusively propagated by stem cuttings and do not reproduce sexually.

Received 13 November 2014. Accepted 10 March 2015.

Corresponding Editor: A. Civetta.

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Neither seeds nor fruits have ever been described in the field and are absent from all herbarium vouchers preserved in all major Herbaria of the world (Lebot and Lévesque 1989). Previous studies using isozymes and AFLPs revealed little genetic variation among cultivars grown throughout its area of distribution and, based on the limited sexual reproductive biology of the plant, suggested that all cultivars were most likely the result of human selection and preservation of somatic mutations in a few genetically similar, vegetatively propagated clones (Lebot et al. 1991, 1999; Jokhan and McLenachan 2004). There is, however, a need for a better assessment of the existing diversity using more accurate genetic markers to clarify the origin of these cultivars.

Despite low levels of genetic variation, kava cultivars present extensive morphological and chemical variation. A standardized list of eight morphological descriptors has been used to discriminate a hundred different morphotypes throughout the Pacific Islands (Lebot and Lévesque 1989; Lebot et al. 1999). Based on HPLC chemotypes, cultivars were separated into three distinct use-categories corresponding to traditional classification: nobles, medicinal, and two-days. These use-categories are based on the physiological effects of beverage made from the roots, which depend largely on the levels of six major kavalactones (Bilia et al. 2002; Singh and Singh 2002). Both noble cultivars, which are safe for daily drinking and medicinal cultivars, have a chemotype rich in kavain (K) and produce relaxing effects; however, noble cultivars have a higher K content. Medicinal and noble cultivars do not differ significantly in chemotyping and their classification refers to their uses as a medicinal plant or for daily consumption as a beverage, respectively. However, two-days cultivars, low in K and rich in dihydrokavain (DHK) and dihydromethysticin (DHM), are not suitable for consumption and are known for their side effects and nausea (Lebot and Lévesque 1989; Lasme et al. 2008).

Kava has been widely used in western medicine to treat mild and moderate anxiety (Pittler and Ernst 2003; Boerner et al. 2003; Connor et al. 2006). However, suspected cases of hepatotoxicity in Germany led health authorities in Europe to ban kava products (Campo et al. 2002). The ban led to the rapid collapse of the industry and resulted in losses of export revenues of nearly €4 million per year in the Pacific, affecting primarily smallholders (Gruenwald et al. 2003). It was recently suggested that these events were caused by the poor quality of the raw material, for example, by use of the wrong cultivar or the wrong plant parts in the manufacturing of a few German extracts (Teschke 2011). In Vanuatu, the *Kava Act* was passed in Parliament in 2002, which stipulated that only noble cultivars are authorized for trade, but the absence of quality control measures remains problematic (Teschke and Lebot 2011). While isozyme and AFLP markers did not enable discrimination between noble and two-days cultivars, chemical analyses and field experiments clearly indicate that chemotypes are genetically controlled (Lebot and Lévesque 1996*b*; Siméoni and Lebot 2002; Wang et al. 2015). Since suspected hepatotoxicity of kava might be caused by wrong cultivars or the incorrect plant parts being used in the extraction, there is an urgent need for quality control measures that can differentiate cultivars unsuitable for consumption (two-days) from the other use-categories (including nobles and medicinal). The present study aims at investigating the genetic relationship among kava cultivars and the correspondence among their morphological, chemical, and molecular traits.

Simple sequence repeats (SSRs) have been massively used for characterizing germplasm collections and identifying clones, because of their ease of use, high polymorphism, locus specificity, and codominance, even though they have a limited coverage of the genome. Diversity Array Technology (DArT) are dominant markers but are useful because they offer deep coverage of the genome and high effectiveness without the need for prior sequence information (Jaccoud et al. 2001; Wenzl et al. 2004), and they have been successfully used in polyploid species such as wheat (Akbari et al. 2006), banana (Risterucci et al. 2009), and sugarcane (Heller-Uszynska et al. 2011). These two molecular markers may contribute to a better understanding of the genetic relationships among *P. methysticum* cultivars. In this paper, we use SSRs and DArTs to study the variation within the narrow genetic base of kava to (*i*) assess and compare genetic diversity between cultivars, and (*ii*) understand how the morphological and chemical variation are genetically structured among cultivars.

Materials and methods

Plant material and DNA extraction

Hereafter, we use the term cultivar to refer to a set of clones identified by farmers under a precise name in vernacular languages and managed together as one morphotype at the community level. A survey was conducted in six villages each located on a distinct island of Vanuatu. Thirty-eight cultivars identified by farmers were collected and for each, inquiries were made regarding their classification as noble or two-days kava. We also studied 46 cultivars from the national germplasm collection of the VARTC (Vanuatu Agricultural Research Technical Centre, Santo Island, Vanuatu). Nine cultivars originating from Hawaii, two from Tonga, one from Pohnpei, and two from PNG, originating from the Alia Point Nursery, Hilo, Hawaii (APN), were included for comparison. Five var. *wichmannii* accessions from Vanuatu were also included to assess the relationships between cultivars (var. *methysticum*) and wild kava (var. *wichmannii*). Overall, 98 cultivars and five wild kavas were studied (Table 1). Among these, 27 cultivars had already been analyzed by HPLC and their chemotypes determined (Lasme et al. 2008). Morphotypes of VARTC cultivars were described with eight morphological descriptors (A, C, I, L, E, P, S, N) previously tested to differentiate accessions efficiently (Lebot et al. 1999): T1

A – general appearance of the plant: 1 = erect, 2 = normal, 3 = prostrate;
C – stem coloring: 1 = pale green, 2 = dark green, 3 = green with purple shading, 4 = purple, 5 = black;
I – internode configuration: 1 = uniform, 2 = mottled, 3 = speckled, 4 = striated and mottled, 5 = striated and speckled;
L – leaf coloring: 1 = pale green, 2 = dark green, 3 = purple, 4 = yellow;
E – lamina edges: 1 = undulate, 2 = raised, 3 = drooping, 4 = regular, 5 = covered;
P – leaf pubescence: 0 = absent, 1 = present;
S – internode shape: 1 = short and tick, 2 = long and thin, 3 = long and thick, 4 = medium and thin;
N – node color: 1 = purple, 2 = green, 3 = black.

For each sample, a 1 mg leaf disc was dried in an oven for 48 h at 40 °C, ground, and incubated at 65 °C for 30 min in an extraction buffer (100 mmol/L tris pH = 8; 50 mmol/L EDTA; 500 mmol/L NaCl; 1.25% SDS; PVP 40000 1%, Na_2SO_3 1%). DNA was extracted using the Dneasy 96 plant kit (Qiagen, Venlo, the Netherlands), and DNA quality was checked on a 1.2% agarose gel before the genotyping procedure was started.

Simple sequence repeats (SSR)

Microsatellite-enriched libraries $(GA)_n$ and $(GT)_n$ were constructed using a single kava genomic DNA sample. The DNA fragmentation was performed by restriction with *Rsa*I, and the libraries were constructed according to Billotte et al. (1999), based on a hybridization capture methodology using biotin-labelled microsatellite oligoprobes and streptavidin-coated magnetic beads.

Selection of microsatellite motifs was performed by analyzing fragments sequenced using the ABI Big Dye Terminator v3.1 kit and which were separated and visualized on an ABI 3500xL genetic analyser (Applied Biosystems, Foster City, Calif., USA). In total, 120 colonies were sequenced from our microsatellite-enriched libraries and 53 SSR-containing DNA fragments obtained.

Table 1. The accessions of *Piper methysticum* var. *methysticum* and var. *wichmannii* used in this study.

No.*	Island	Site†	Name	Group‡	Chemotype§
VU115	Ambae	VARTC	Melomelo	N	—
VU116	Ambae	VARTC	Taritamaewo	T	—
VU319	Ambae	Lolosori	Tarimavute	T	—
VU320	Ambae	Lolosori	Tarimavute	T	—
VU321	Ambae	Lolosori	Sweet santo	N	—
VU322	Ambae	Lolosori	Borogoru	N	—
VU323	Ambae	Lolosori	Palarasul	N	—
VU324	Ambae	Lolosori	Melomelo	N	—
VU325	Ambae	Lolosori	Borogoru	N	—
VU326	Ambae	Lolosori	Melomelo	N	—
VU327	Ambae	Lolosori	Melomelo baraeto	N	—
VU328	Ambae	Lolosori	Kliss Han	T	—
VU329	Ambae	Lolosori	Melomelo bisuiboe	M	—
VU014	Epi	VARTC	Maklo	N	—
VU015	Epi	VARTC	Kelai	N	HQ
VU171	Gaua	VARTC	Tarivarusi	T	—
VU197	Maewo	VARTC	Borogoru memea	M	—
VU200	Maewo	VARTC	Malohubora	W	—
VU202	Maewo	VARTC	Malohuia	N	—
VU207	Maewo	VARTC	Vambu	T	LQ
VU012	Malekula	VARTC	Pade	N	HQ
VU209	Malekula	VARTC	Nelimliun	—	—
VU212	Malekula	VARTC	Silese	N	HQ
VU214	Malekula	VARTC	Ranapapa	—	LQ
VU215	Malekula	VARTC	Malog rock	T	LQ
VU217	Malekula	VARTC	Malog lilab	M	—
VU218	Malekula	VARTC	Malog velab	M	HQ
VU314	Malekula	VARTC	Silese	N	—
VU315	Malekula	Brenwe	Silese	N	—
VU316	Malekula	Brenwe	Tavandi	M	—
VU317	Malekula	Brenwe	Pia	N	—
VU318	Malekula	Brenwe	Mala'ma	T	—
VU350	Malo	Avunamalaï	Melomelo	N	—
VU351	Malo	Avunamalaï	Pia	N	—
VU352	Malo	Avunamalaï	Aïboïé	T	—
VU001	Pentecost	VARTC	Borogu	N	HQ
VU002	Pentecost	VARTC	Laklakh	T	LQ
VU006	Pentecost	VARTC	Sinibo	W	—
VU011	Pentecost	VARTC	Rongrongwul	T	LQ
VU103	Pentecost	VARTC	Fabukhai	T	LQ
VU104	Pentecost	VARTC	Fabularalara	T	LQ
VU110	Pentecost	VARTC	Sese	N	—
VU111	Pentecost	VARTC	Seselaralara	T	—
VU112	Pentecost	VARTC	Tarivarusi	T	—
VU120	Pentecost	VARTC	Gorogor entepal	M	—
VU122	Pentecost	VARTC	Laklakh	T	—
VU123	Pentecost	VARTC	Meleliap	W	—
VU158	Pentecost	VARTC	Abogae	T	LQ
VU161	Pentecost	VARTC	Borogoru tabal	N	—
VU340	Pentecost	Metaruk	Sini bogong	T	—
VU342	Pentecost	Metaruk	Laklakh	T	—
VU345	Pentecost	Metaruk	Abogae	T	—
VU348	Pentecost	Metaruk	Borogu tewewep	N	—
VU003	Santo	VARTC	Malogro	T	LQ
VU005	Santo	VARTC	Marino	T	LQ
VU126	Santo	VARTC	Fock	T	—
VU130	Santo	VARTC	Malovoke	T	LQ
VU136	Santo	VARTC	Makuku	—	—
VU137	Santo	VARTC	Palarasul	N	—
VU138	Santo	VARTC	Palasa	N	—
VU139	Santo	VARTC	Palisi	T	LQ
VU140	Santo	VARTC	Palisi	T	—
VU145	Santo	VARTC	Tudei	T	—
VU147	Santo	VARTC	Vakorokoro	—	—
VU303	Santo	Pesena	Palarasul	N	—
VU304	Santo	Pesena	Palisi	T	—
VU305	Santo	Pesena	Sotrin	N	—

Table 1 (*concluded*).

No.*	Island	Site†	Name	Group‡	Chemotype§
VU306	Santo	Pesena	Laklakh	T	—
VU307	Santo	Pesena	Wets kara	T	—
VU308	Santo	Pesena	Kliss han	T	—
VU312	Santo	Pesena	Borogu	N	—
VU313	Santo	Pesena	Borogu kara	M	—
VU354	Santo	Pesena	Kava echa	T	—
VU355	Santo	Pesena	Tudei	T	—
VU017	Tanna	VARTC	Pia	N	HQ
VU018	Tanna	VARTC	Malamala	M	—
VU019	Tanna	VARTC	Ahouia	N	HQ
VU155	Tanna	VARTC	Wichmanii	W	—
VU331	Tanna	Lamlu	Nekawa am	N	—
VU336	Tanna	Lamlu	Wapuil	N	—
VU337	Tanna	Lamlu	Seweu sawo	N	—
VU338	Tanna	Lamlu	Leay	N	—
VU339	Tanna	Lamlu	Tudey	T	—
VU009	Tongoa	VARTC	Puariki	N	HQ
VU008	Vanua Lava	VARTC	n.c.	—	HQ
VU175	Vanua Lava	VARTC	Maewo	W	—
VU010	n.d.	VARTC	Sentender	—	LQ
VU025	n.d.	VARTC	Bogania	N	HQ
VU415	n.d.	APN	n.c.	—	—
FM005	Pohnpei	APN	Rahmwagner	—	—
HW006	Hawaii	APN	Mo'i	—	—
HW007	Hawaii	APN	Honokan'iki	—	—
HW009	n.d.	APN	Papa'ele'ele	—	—
HW011	O'ahu	APN	Papa'kea	—	—
HW012	Hawaii	APN	Nene	—	—
HW013	n.d.	APN	Hiwa	—	—
HW017	Hawaii	APN	Opihikao	—	—
HW021	Kauai	APN	Hana ka pi'ai	—	—
HW023	Hawaii	APN	Papa éléélé pu'upu'u	—	—
PNG1	—	APN	Isa	—	—
PNG2	—	APN	Iwi	—	—
TO020	Vava'u	APN	Akau Huli	—	—
TO024	Vava'u	APN	Hina	—	—

*VU, Vanuatu; FM, Federated States of Micronesia; HW, Hawaii; PG, Papua New Guinea; TO, Tonga.
†VARTC, Vanuatu Agricultural Research and Technical Center, Santo, Vanuatu; APN, Alia Point Nursery, Hilo, Hawaii.
‡Use-categories: M, medicinal; N, noble; T, two-days (*P. methysticum* var. *methysticum*); W, var. *wichmannii*.
§Chemotype quality (Lasme et al. 2008): HQ, high quality corresponding to a high kavain (K) content; LQ, low quality corresponding to a low K content.

Accession VU 115 was used in constructing microsatellite enriched libraries and was selected because it was a Vanuatu accession with very good DNA quality. The fragments were sorted using the SSR analysis tool (SAT) pipeline (Dereeper et al. 2007) with a cutoff for di- and tri-nucleotide motifs of eight and five repeats, respectively. Finally, a subset of 24 primer pairs was selected and synthesized (Sigma-Aldrich, Saint-Louis, Mo., USA). Each forward primer was designed with a 5′-end M13 extension, 5′-CACGACGTTGTAAAACGAC-3′ (Steffens et al. 1993). PCR was performed separately for each primer pair, in 10 μL with 25 ng DNA, 0.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.8 μmol/L reverse primer, 0.1 μmol/L M13-tailed forward primer, 0.1 μmol/L M13 primer fluorescently labelled with FAM, VIC, PET, or NED, and 0.5 U *Taq* DNA polymerase. The program was as follows: initial denaturation at 95 °C for 4 min; 10 cycles of touchdown PCR from 55 to 50 °C with 94 °C for 30 s, T°C for 60 s, and 72 °C for 120 s, 25 cycles of 94 °C for 30 s, 50 °C for 60 s, and 72 °C for 120 s; and a final elongation step at 72 °C for 8 min. Allele size was determined after separation on an ABI 3500xL capillary sequencer (Applied Biosystems~~, Foster City, Calif., USA~~).

Diversity Array Technology (DArT)

A genomic fragment library was generated using a combination of restriction enzyme digestion and adaptor ligation, followed by PCR amplification, according to the genome complexity reduction method described by Jaccoud et al. (2001). We used the restriction enzyme combination of *Pst*I/*Taq*I because it has been found to have high polymorphism in polyploid plants such as wheat (Akbari et al. 2006), banana (Risterucci et al. 2009), and sugarcane (Heller-Uszynska et al. 2011). The representation of a fragment of the genome was created using a subset of 26 accessions, including 24 kava cultivars selected to cover the wide geographic distribution and the variability of chemotypes present in our sample and two accessions of var. *wichmannii* from Vanuatu. The amplification products were cloned into a PCR 2.1-TOPO vector using a T/A cloning kit (Invitrogen, Carlsbad, Calif., USA), then transformed into *Escherichia coli* DH10B. Overall, 6144 individual clones were grown and spotted on GAPSII-coated microarray slides (Corning, Corning, N.Y., USA) using a Microgrid II arrayer (Biorobotics, Cambridge, UK). Then, targets were generated for each sample to genotype following the same complexity reduction method used for library construction (*Pst*I/*Taq*I). Finally, labeling and hybridization of the targets were processed as described in Jaccoud et al. (2001). Slides were scanned using a Tecan LS300 (Tecan, Männedorf, Switzerland) fluorescent microarray scanner.

Data analysis

For analysis of SSR markers, Genemapper was used for automated data collection, computation of allele size, and accurate

visualization of alleles. Due to the polyploid nature of kava, inferring allele frequencies can be problematic because co-dominant scoring and heterozygosity calculation are difficult. Each allele was thus scored as 1 (present) or 0 (absent). Considering that in this case a common absence of two units was not informative, asymmetrical weights were given to the two modalities (0, 1), reducing the risk of under-estimating dissimilarity. A distance matrix was calculated between all pairs of individuals using the Dice dissimilarity index, d_{ij}, (Dice 1945)

$$d_{ij} = \frac{n_{10} + n_{01}}{2n_{11} + (n_{10} + n_{01})}$$

where n_{11} is the number of fragments present in both i and j, and n_{01} and n_{10} are the number of fragments present in one accession but absent in the other.

For DArT analysis, individual slides were analyzed and the score of each marker was calculated for each sample using DArTsoft 7.4.5 (Diversity Arrays Technology P/L, Canberra, Australia). Markers were scored "0" for absence and "1" for presence in the genomic representation of the sample. For each marker, four quality parameters were computed: the between-cluster variance of the relative target hybridization intensity as a percentage of the total relative intensity variance (P value); the multivariate equivalent of the P parameter (Q value) (Storey and Tibshirani 2003); the call rate index (CR), which is the percentage of scored DNA samples; and the reproducibility using 23 independent repetitions for each marker. Q measures the fraction of the total variation across all individuals due to bimodality. It is calculated based on only one dimension as follows:

$$Q = \max\left(i \times (x - xloi)^2 + (N - i) \times (x - xhii)^2\right)/N \times \mathrm{var}(x) \times 100, 0 \le i < N$$

where x is the average value of the N points, $xloi$ is the average value of the i elements of lowest value, $xhii$ is the average value of the $N - i$ points of highest value, and var(x) represents the variance of the N points. Q is performed on multiple dimensions. P measures the amount of variation across individuals but is performed on one or more dimensions using the formula

$$P = \left(n1 \times (g - g1)^2 + n2 \times (g - g2)^2 / \sum i = 1 \Rightarrow n1 + n2(xi - g)^2\right) \times 100$$

where $n1$ and $n2$ are the number of points belonging to the crisp cluster1 and cluster2, respectively (decided by the highest membership value), g is the centre of gravity of the $n1 + n2$ d-dimensional x points, $g1$ and $g2$ are the centres of gravity of the $n1$ and $n2$ d-dimensional x points, respectively. Whereas Q uses only the values of data points to determine the best possible separation, P uses the partition found by the algorithm and evaluates its quality. Q and P are complementary and a substantial difference between these two values for the same clone reflects automatically the presence of artefacts. In the present study, both were high (>75) and genotyping data were reproducible.

For DArT markers, equal weight was given to both modalities (0, 1), since a common absence for two units was regarded as informative, considering the high level of ploidy of *P. methysticum*. A distance matrix was calculated between all pairs of accessions using the Sokal and Michener dissimilarity index, d_{ij}, (Sokal and Michener 1958) as the proportion of unmatching markers

$$d_{ij} = \frac{n_{10} + n_{01}}{n_{11} + n_{10} + n_{01} + n_{00}} = \frac{n_{10} + n_{01}}{N}$$

where n_{11} is the number of fragments present in both i and j, n_{01} and n_{10} the number of fragments present in one accession but absent in the other, and n_{00} the number of fragments "absent" in both i and j.

The distance matrices thus obtained were analyzed using weighted pair group method using a weighted average (WPGMA) to summarize the relationships as dendrograms and with principal coordinates analysis (PCA) to summarize them as points along eigenvectors. The analyses were performed using the DARwin software (Perrier and Jacquemoud-Collet 2006). To investigate further the relationships between genotypes within each group, a minimum spanning network was inferred that represents all possible minimum length connections among genotypes. We used the Network software (fluxus-engineering.com) to perform such analysis from the binary data of both markers type.

Finally, a specific focus was given to markers that may be useful for addressing the question of the origin of particular genotypes, i.e., whether they derive from recombination or from mutation within a clonal lineage. Assuming that the global phenetic tree (dendrogram) enables pinpointing ancestral states (those alleles that are spread across several long branches), it is possible to evaluate phylogenetic relationships. In doing so, the various transitions from an 'ancestral' state to a 'derived' state can be inferred from the following:

DArT 1 → 0: there are diverse ways of losing a DArT marker, including mutations at one or more restriction sites, as well as loss of the chromosome (segment) that bears the marker.

SSR 1 → 0: there are several ways of losing an SSR allele, including a repeat length expansion or deletion as well as loss of the chromosome (segment) that bears the marker.

SSR 0 → 1: there are a few ways of yielding a new SSR allele, including a repeat unit expansion or deletion from 'neighboring' alleles.

DArT 0 → 1: there are a few (if not a single) ways of generating a new DArT allele, i.e., a given sequence between two well-defined restriction sites.

While the former three types can be considered prone to homoplasy, the latter case (DArT from 0 to 1) can be considered indicative of a unique process that helps establish common ancestry through synapomorphy.

Results

Survey of polymorphic markers

From the SSR library, a total of 24 primer pairs were designed and tested on a subset of 12 cultivars of diverse origins. From these primer combinations, 13 produced clear peaks and precise sizing of PCR products (to within 1 bp), allowing allele detection in the sample to be easily scored (Table 2). These 13 SSR primers were T2
then used to genotype all of the accessions in the study. A high degree of polymorphism was revealed and all 13 loci were polymorphic within the material surveyed. A total of 60 alleles were detected, the number of alleles per locus varied from 2 (CirPm006) to 9 (CirPm001), with an average of 4.6 per locus (Table 2). DArT clones were declared as polymorphic when the following criteria were met: reproducibility >97% and call rate >80%. After sorting, the percentage of polymorphic clones detected on the array was low (1.4%), with a total of 89 polymorphic clones identified within the 103 kava accessions. Of these, 14 redundant markers were removed by sequencing a total of 75 polymorphic. The overall quality of these 75 DArT markers detected was high, with an average call rate of 95.6% and an average scoring reproducibility of 99.9%.

Overall genetic structure

Overall, 59 SSR markers (i.e., allele levels) and 75 DArT markers were obtained for the 103 accessions of *P. methysticum*. Genetic

Table 2. Sample list of primers developed for *Piper methysticum*. Indicated are the 5′ to 3′ sequence for both forward (F) and reverse (R) primers, the optimal reannealing temperature (Tm), the microsatellite motif observed in *P. methysticum* 'Isa', the number of alleles (A), and the range of the size of the fragments observed among the 103 accessions of the study.

Marker code	Genbank acc. No.	Primer sequences (5′-3′)		Tm (°C)	Repeat motif	A	Allele size range (bp)
mPmCIR001	KF01518	F	TTCTGCCATTTTCATTTC	52	$(ct)_{19}$	9	164–188
		R	GAAGATGTGAGGGCTTTT				
mPmCIR006	KF01519	F	GGTGAAGTGATACTGCTGA	52	$(gt)_8$	2	220–222
		R	ATGAAGAAAGGGAGATGC				
mPmCIR010	KF01520	F	TTGGTTCACCTCAATCAC	52	$(gt)_9$	3	223–225
		R	AATGATAAGAAATTGGAACCT				
mPmCIR103	KF01521	F	TTCAAACGAGTAATCAATTC	51	$(gt)_9$	5	158–172
		R	AACTAGCACAGTCCCTCA				
mPmCIR115	KF01522	F	GCATGTCTTGGTTTGTTC	52	$(tc)_{10}$ $(ttttc)_3$	3	164–167
		R	TCATGCCAACTAAACTCC				
mPmCIR126	KF01523	F	TACGGCTCGTGTTACTTC	52	$(gt)_9$	3	245–251
		R	CATTATTGGGCTCTTTCA				
mPmCIR127	KF01524	F	GTTGCGGTGATGGTAGAG	55	$(ga)_8$ $(tttc)_4$	4	305–323
		R	CACACTTCAACCGATTAACA				
mPmCIR143	KF01525	F	GTTCTGCTTCAGATCACG	52	$(ga)_{12}$	5	205–226
		R	ACCAATTTCTTGACCAATC				
mPmCIR159	KF01526	F	CATAAAGCAAGGTTGTCC	52	$(ct)_8$	3	213–217
		R	GTATTGCCATTGGATGAG				
mPmCIR160	KF01527	F	CCCAAAGCTATTCACAAA	52	$(ga)_{10}$ $(gga)_5$	5	244–254
		R	ATTCCTCCATTTCACCTT				
mPmCIR162	KF01528	F	AAAGGTTTTGAGCATGAA	51	$(ag)_{18}$	6	204–222
		R	GCAGAAGTTCCAGTTGTT				
mPmCIR163	KF01529	F	GCCATGAGCGTATATGAA	53	$(ag)_6$ $(tg)_6$	4	260–290
		R	AAAGAGGATGTGGAGGAG				
mPmCIR172	KF01530	F	TCTATAATACCTGCGAGTCC	52	$(ag)_{17}$ $(ggga)_3$	6	204–226
		R	CAGTAATTGGCTCATGCT				
mPmCIR185	KF01531	F	AAAGGCGAGAAATGTAGG	53	$(ga)_8$	2	223–227
		R	TGTGCGCTATAAAAGGTC				
mPmCIR186	KF01532	F	CCAACTAATTTTGCTGCT	52	$(ct)_8$	4	185–188
		R	GCTCTTCCACCATTGTAA				

distances among accessions ranged from 0 to 0.65 with an average of 0.24 using SSRs and from 0 to 0.64 with an average of 0.24 using DArT. Within our sample, 11 genotypes were identified with SSR and 28 genotypes with DArT markers. By combining the two sets of markers, a total of only 30 distinct genotypes were observed.

Genetic relationships between genotypes were first determined by performing a WPGMA cluster analysis using either DArT (Fig. 1*a*) or SSR data (Fig. 1*b*). Dendrogram topologies given by the two marker systems showed a high degree of similarity. The five wild var. *wichmannii* from Vanuatu clustered into two groups (Pw I and Pw II) well separated from the cultivars of var. *methysticum*. All the var. *methysticum* accessions clustered into three well-defined groups in accordance with their geographical provenance, with the exception of the cultivars Isa (PNG1) and Iwi (PNG2). These two cultivars, both originating from Papua New Guinea, displayed two distinct and genetically distant genotypes, which appeared isolated and unstable in terms of proximity between the DArT dendrogram (Fig. 1*a*) and the SSR dendrogram (Fig. 1*b*). Accessions from Polynesia and Micronesia, including eight cultivars from Hawaii, two from Tonga, and one from Pohnpei (FSM), clustered together into a single group (Pom). Cultivars from Vanuatu clustered into two groups (Vu I and Vu II) composed of 36 and 48 cultivars, respectively, originating from different islands of Vanuatu.

To further describe the relationships within our sample, multidimensional PCA was carried out (Fig. 2). The first two eigenvalues of the PCA explained 73.5% of the total variance in the DArT data and 74.9% in the SSR data, and the two marker systems produced similar representations. The cultivars and wild kavas were clearly distributed into five groups, identical to those given by the WPGMA (Pw I, Pw II, Vu I, Vu II, and Pom). The PCA obtained with both markers place Isa (PNG1), a var. *methysticum* accession, in closer proximity to the var. *wichmannii* from Vanuatu. Since memberships in these five groups were clear, matrices of inter- and intragroup genetic distances were computed (Table 3). Using a normalized Mantel statistic, a high correlation ($R = 0.72$, $P < 0.001$) was observed between the pairwise distances obtained by the two marker systems. The major difference between the two matrices was the lower distance obtained with DArTs (0.20) between the cultivar Iwi (PNG2) and the cultivars from Polynesia (Pom group) compared to SSRs (0.42), explaining the different positions of this cultivar in the two dendrograms (Figs. 1*a* and 1*b*).

Although all the groups were separated from each other by many markers, few were exclusive or particular to one group (Table 3). The three groups Vu I, Vu II, and Pom were structured by polymorphism observed at 38 DArT markers and 20 SSR markers spread over eight SSR loci. However, they displayed only three, five, and two specific modalities in DArTs and one, one, and three private alleles in SSRs. The search for markers with high phylogenetic information led to the identification of five DArT markers (Table 4). All five markers were absent in the var. *wichmannii* accessions and the two cultivars from PNG. With one exception (201634mp in Vu I), the markers were uniformly (either absent or present) within each cultivar group. Assuming the simplest model of molecular evolution, which is that each marker emerged only once and then stayed in each clonal lineage where it was present, the three groups of cultivars may have different zygotic origins.

Intragroup genetic distance

Very low polymorphism was observed within each group of cultivars. Average intragroup genetic distance ranged from 0 to

Fig. 1. WPGMA dendrograms of the genotypes observed among 98 *Piper methysticum* var. *methysticum* cultivars and five *P. methysticum* var. *wichmannii* accessions based on (*a*) Sokal and Michener dissimilarity index calculated with 75 DArT markers and (*b*) Dice dissimilarity index calculated with 59 dominant SSR markers spread over 13 loci. The numbers of cultivars encountered per genotype is indicated. The numbers at the tips of the horizontal lines of the dendrogram refer to the number of cultivars presenting an identical fingerprint.

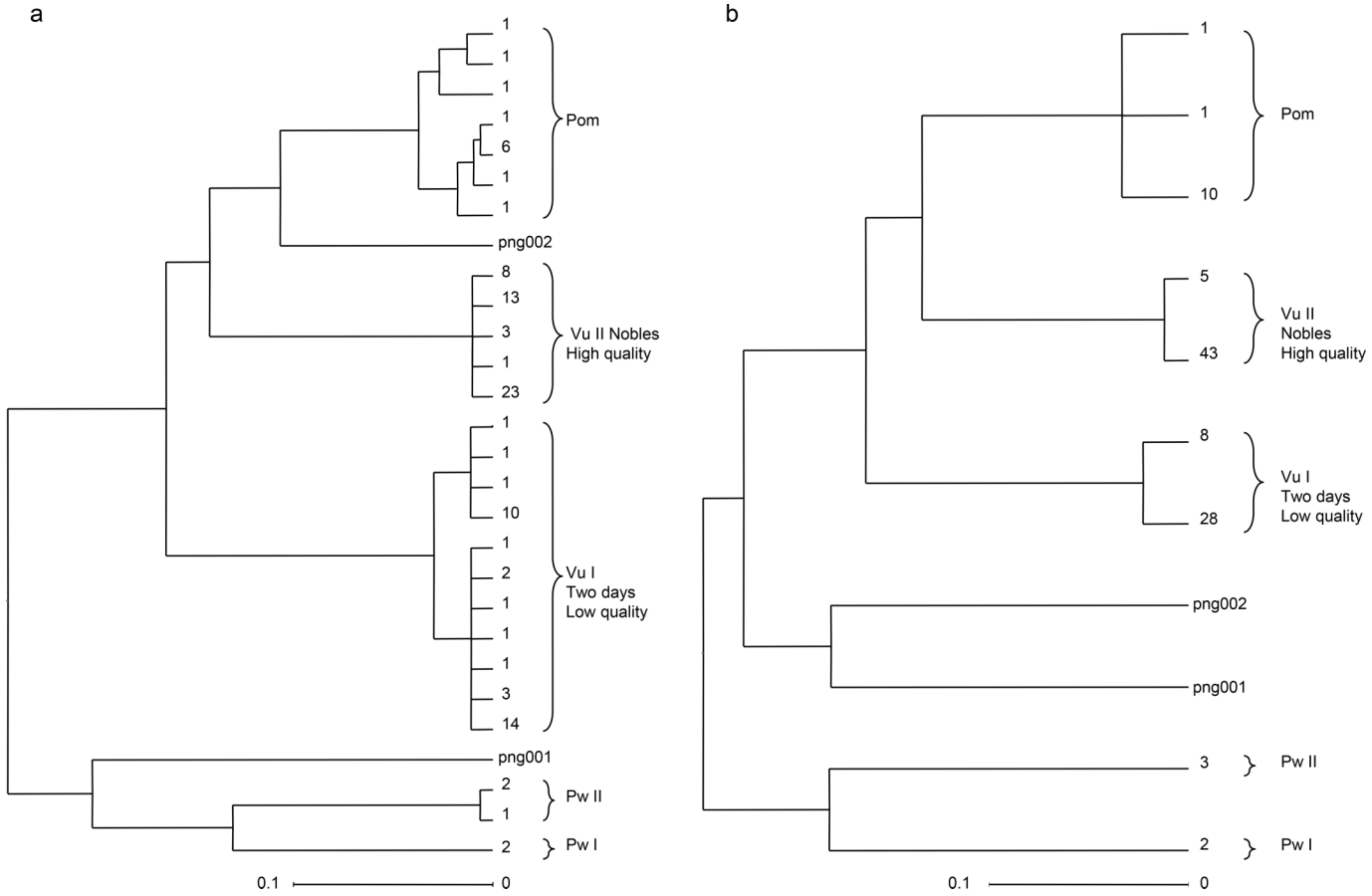

Fig. 2. Principal coordinate analysis of the variation for 98 *Piper methysticum* var. *methysticum* and 5 *P. methysticum* var. *wichmannii* accessions using (*a*) DArT and (*b*) SSR.

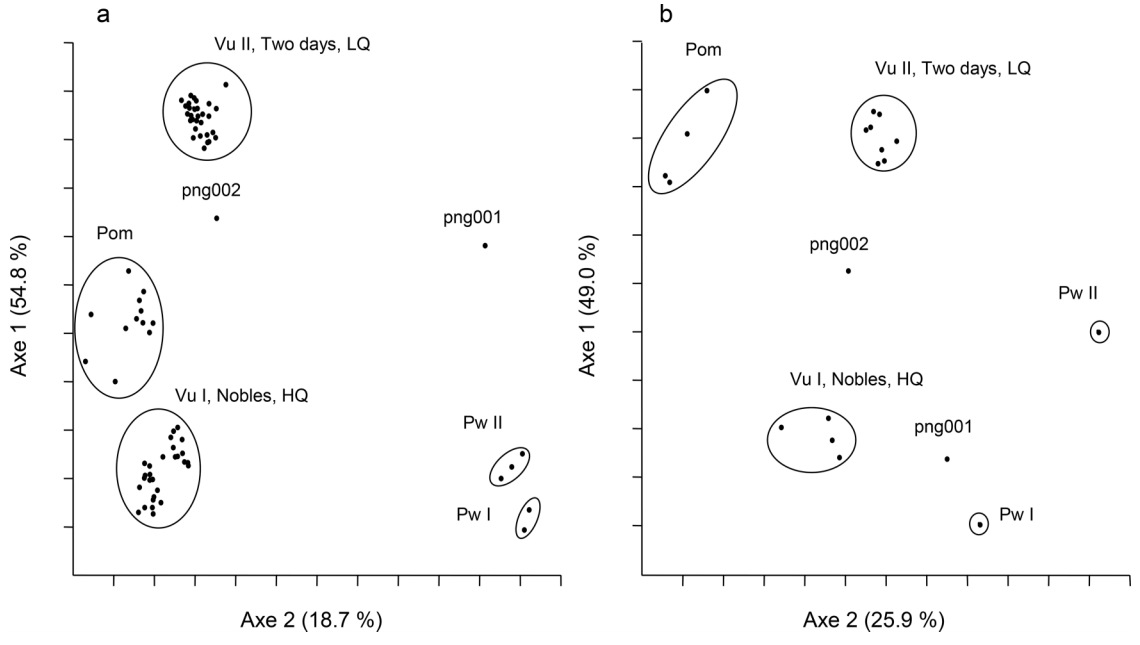

Table 3. Mean intragroup dissimilarity (diagonal in boldface type), mean dissimilarity (below the diagonal) and number of polymorphic markers (above the diagonal) between the different groups of *Piper methysticum* using (*a*) DArT and (*b*) SSR.

(*a*) Group	Pw I (3)*	Pw II (5)	'PNG1' (3)	'PNG2' (1)	Vu I (3)	Vu II (5)	Pom (2)
Pw I	**0**	21†	29	39	36	45	42
Pw II	0.29	**0.004**	34	40	35	42	39
PNG1	0.37	0.46	—	24	45	38	35
PNG2	0.55	0.57	0.37	—	29	20	15
Vu I	0.49	0.47	0.60	0.36	**0.025**	31	22
Vu II	0.61	0.55	0.54	0.24	0.35	**0.014**	23
PoM	0.60	0.56	0.51	0.20	0.27	0.27	**0.026**
(*b*) Group	Pw I (4)*	Pw II (5)	'PNG1' (8)	'PNG2' (4)	Vu I (1)	Vu II (1)	Pom (3)
Pw I	**0**	18†	27	28	19	24	26
Pw II	0.36	**0**	25	24	19	18	26
PNG1	0.55	0.49	—	19	24	27	29
PNG2	0.58	0.48	0.37	—	17	16	18
Vu I	0.42	0.44	0.50	0.37	**0.021**	13	15
Vu II	0.54	0.41	0.58	0.36	0.34	**0.008**	12
PoM	0.60	0.60	0.63	0.42	0.39	0.30	**0.023**

*Number of markers specific to each group.
†Number of polymorphic markers between groups are based on the most frequent genotype from each group.

Table 4. Distribution of modalities among DArT markers selected for their high phylogenetic information.

Marker	Pw I	Pw II	PNG1	PNG2	Pom	Vu I	Vu II
201634mp	–	–	–	–	–	Rare	+
201868mp	–	–	–	–	–	+	+
204244mp	–	–	–	–	–	–	+
204597mp	–	–	–	–	+	+	–
205244mp	–	–	–	–	+	–	+

Note: For each marker, the modality absence (–) correspond to the ancestral state, while the modality presence (+) correspond to the derived state.

0.023 with SSRs and from 0 to 0.026 with DArTs. By combining the two set of markers, we constructed three minimum spanning networks to illustrate the genetic polymorphism observed within each group of cultivars (Fig. 3). The majority of the polymorphism observed was detected using DArT markers while a unique SSR locus exhibited polymorphism in each group.

In each network, the polymorphism observed was fully congruent with the hypothesis of a clonal lineage, except for three DArT markers present twice (205429mp and 204736mp in Vu I, 204681mp in Vu II). These rare discrepancies may be explained by the occurrence of homoplasy or caused by partial genotyping errors due to the high-throughput detection of the DArT method. Then, the genotypes involving a unique individual discriminated by a single DArT marker should be handled with particular caution. Nevertheless, similar subdivisions were found using both SSRs and DArTs, supporting the possibility of slight but real genetic distinctions within each group. In group Pom, two genotypes (FSM005 and HW012) were distinguished from others by both SSRs and DArTs. In group Vu I, a subgroup of 13 cultivars was separated from others by polymorphism at three DArT markers and among these cultivars, eight were also discriminated by one locus in the SSR analysis. In group Vu II, a subgroup of 16 cultivars was separated from others by one SSR polymorphism, and among these cultivars, 13 were also discriminated by one marker in the DArT analysis.

Correspondence among molecular, quality, and morphological traits

The group Vu I corresponds to two-days cultivars of low quality, while the group Vu II corresponds to noble and medicinal cultivars of high quality. The only exceptions to this pattern were cultivar VU025 on one side (medicinal cultivar of high quality in Vu I) and cultivars VU111, VU122, and VU339 on the other (two-days cultivars in Vu II). The morphological characterization based on eight descriptors (A, C, I, L, E, P, S, N) allowed the identification of 28 distinct morphotypes among the 45 Vanuatu cultivars (data not shown). The molecular differentiation between Vu I and Vu II is congruent with the classification based on chemotypes ($R = 0.91$, $P < 0.001$) or use-categories ($R = 0.89$, $P < 0.001$) (Table 5). However, morphological traits displayed variation between cultivars without any correlation with molecular classification. However, cultivars characterized by speckled internodes (I = 3) and regular lamina edges (E = 4) corresponded to the Vu I group, while cultivars with mottled internodes (I = 2) and drooping lamina edges (E = 3) corresponded to the Vu II group. Within the Vu I group, the same I and E traits displayed some degree of association with molecular variation as opposed to the other traits.

Discussion

The genetic structure among accessions of kava observed with the two types of markers are highly similar. They clearly differentiate two-days cultivars, unsuitable for consumption from nobles. Correlation between DArT and SSR results, as observed in this study, has also been found in other studies (Hurtado et al. 2008; Laidò et al. 2013). Nevertheless, the level of polymorphism revealed at the DArT loci (only 75 polymorphic clones for a total of 6144) was quite low in comparison to that observed with SSRs (100% of polymorphic loci). One explanation might be related to the choice of the restriction enzymes used for our library. However, as the number of polymorphic clones was extremely low, we tested the quality of the array by genotyping two outgroups (*Piper auritum* and *P. aduncum*). The results were positive, with 852 polymorphic clones identified (data not shown).

Another explanation for the low polymorphism at DArT markers could be their dominant nature of these markers, combined with the high ploidy level of our species. Such low polymorphism was also encountered previously with AFLPs (Lebot et al. 1999; Jokhan and McLenachan 2004). Heller-Uszynska et al. (2011) underscored the challenge of capturing the polymorphic fraction of polyploid species with DArTs, where multiple copies of homologous chromosomes reduce polymorphism visibility. In our case, to detect polymorphism at a given marker locus, the allele that generates the marker must be absent in all 10 copies of homologous chromosomes for a plant to release a global "absence" signal. There is, therefore, a tension between the necessity for the marker to be frequent enough to be captured during the library preparation phase but rare enough to produce polymorphism. A second problem associate in studying species in which the genetic structure is discontinuous, as is the case with vegetatively propagated species. The components that are under-represented in the panel that serves for library generation have little chance of contributing specific markers, in contrast with clonal groups that may be over-represented. This makes the conclusions more fragile for the smaller clusters, such as the Pom group in this study.

Lasso (2008) underlined the importance of setting the right genetic distance threshold for identification of clones. Unfortunately, the absence of established heteroclonal individuals rendered this problematic for kava. Considering the high ploidy level of the species, mutations in the kava genome are enhanced and the hypothesis of a single origin of the different groups followed by clonal differentiation is reasonable. However, both marker systems revealed very low intragroup diversity (0–0.032) and high intergroup diversity (0.26–0.60) among kava cultivars.

Each group of cultivars (Vu I, Vu II, and Pom) encompasses a few distinct genotypes discriminated by a low number of markers. Such low intragroup genetic diversity suggests that each group correspond to a single clonal lineage. On the other hand, the

Fig. 3. Polymorphism observed between genotypes within each group of *Piper methysticum* cultivars represented by a minimum spanning network. Each main genotype is represented by the number of individuals sharing the same pattern. Each difference between genotype is labeled by the name of the locus and the presence (1) or absence (0) of the marker.

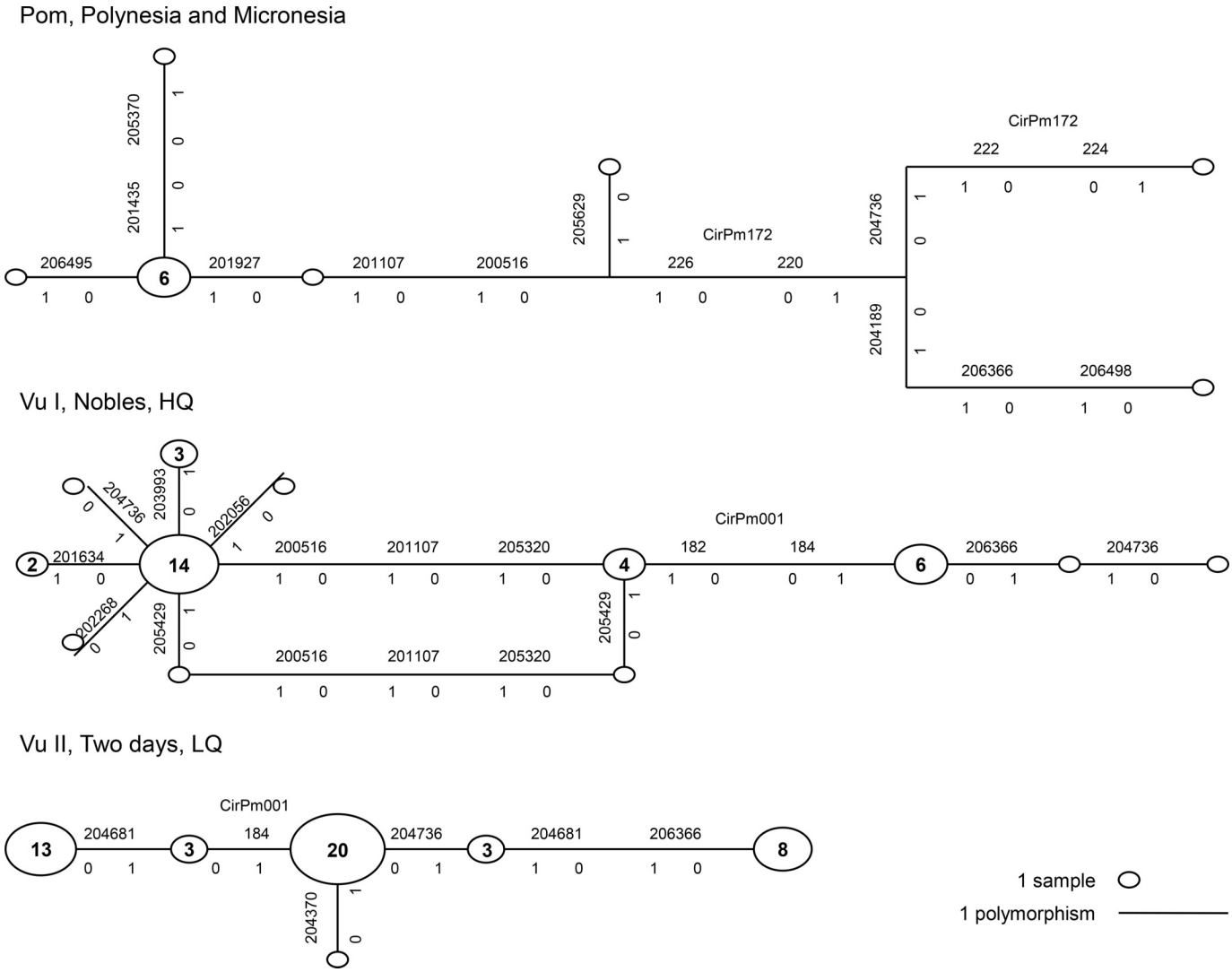

Table 5. Distribution of *Piper methysticum* cultivars from Vanuatu into groups Vu I and Vu II following their (*a*) chemotype (Lasme et al. 2008) and (*b*) use-category.

(*a*)	Chemotype quality		
Group	Low	High	Total
Vu I	13	1	14
Vu II	0	9	9
Total	13	10	23
(*b*)	Use-category		
Group	Two-days	Noble, Medicinal	Total
Vu I	31	0, 1	32
Vu II	3	35, 7	45
Total	34	43	77

Note: (*a*): $\chi^2 = 19.22$, df = 1, $P_\chi < 0.001$, $P_E < 0.001$. V = 0.91. (*b*) $\chi^2 = 61.72$, df = 1, $P_\chi < 0.001$, $P_E < 0.001$. V = 0.89. Values of the χ^2 statistic and its P value (P_χ), the P value from Fisher's exact test (P_E) are given, as well as the coefficient of association Cramer's V.

range of intergroup dissimilarities is comparable to that in polyploid cross-pollinated species analysed with SSRs: 0.18–0.50 in sugarcane (Cordeiro et al. 2003; Selvi et al. 2003), and 0.19–0.59 in coffee (Moncada and McCouch 2004; Silvestrini et al. 2007). We can hypothesize that the two unique genotypes (PNG1 and PNG2) and the three groups of cultivars (Vu I, Vu II, and Pom) have each arisen from sexual reproduction from five founding lineages that further evolved by mutation over successive vegetative propagation. Scenarios with similar highly structured diversities have already been observed in other vegetatively propagated crops, such as sugarcane (D'Hont et al. 2002) and yam (Scarcelli et al. 2013).

The existence of multiple lineages is likely to reflect ancient events. True botanical seeds in *P. methysticum* have never been observed in Oceania since the first valid description by Forster 1786 (Smith 1981; Lebot and Lévesque 1989). This widespread sterility led to a revised taxonomy combining the two taxa (*P. wichmannii* and *P. methysticum*) into a single and unique species (Applequist and Lebot 2006). In Fiji, a country partly Melanesian and Polynesian, which has played an important role in the distribution of vegetatively propagated species across the Pacific, AFLP fingerprints have shown that all local cultivars are clones of a single genotype (Jokhan and McLenachan 2004). In Hawaii, AFLP

fingerprints produced using 21 pairs of primers revealed that kava has an extremely narrow genetic base in which morphological and chemical variation must be controlled by very few genes. It was hypothesized that most cultivars are most likely somatic mutants from a common clonal source introduced by the Polynesians (Lebot et al. 1999). The possible existence of multiple lineages was mentioned in a broad study based on isozymes produced using eight enzyme systems, which yielded three closely related zymotypes (nb8, nb9, nb10) in Vanuatu, while 93 accessions originating from Hawaii, Pohnpei, Kosrae, Fiji, Samoa, Tonga, and the Cook Islands exhibited a single zymotype (nb 10); these were, therefore, suspected to result from vegetative propagation of a single clone distributed by Polynesian voyagers during their migrations and subsequently diversified locally into sterile, but clearly variable morphotypes (Lebot et al. 1991). An association existed in Vanuatu between quality and zymotype (most two-day cultivars had zymotype nb9 and most noble cultivars had zymotype nb10), but it was disrupted when the other origins were considered.

Broader studies are necessary to clarify the lineages, their distribution, and their association with quality traits, especially for the Pom cluster. The markers that we have used provide a solid ground for future studies that will include broader sets of germplasm. They can moreover be expanded using the SSR libraries that we have generated.

Among the morphological traits, internode configuration and lamina edges appeared as the most related to the molecular marker-based structure among Vanuatu cultivars. They displayed contrast between the Vu I and Vu II lineages and differentiation between some of the few within-lineage components in Vu I. The other variable traits on general appearance, leaf color and internode shape appeared unrelated to the global structure and are likely to have more diverse and less phylogenetically relevant genetic control.

The presence of several distinct morphotypes sharing a common molecular profile illustrates that mutations exist that could be selected following clonal generations. Indeed, farmers from the Pacific Islanders are known to be prompt at tagging novel variants and integrating them into their varietal portfolio. It has been observed in other vegetatively propagated crops such as taro (Caillon et al. 2006), banana (Karamura et al. 2010), and yam (Scarcelli et al. 2011) that the selection of particular mutants is a continuous and conscious process. This selection is still dynamic and efficient in kava, where new cultivars and morphotypes have been regularly found in farmers' fields over a 20-year period (Lebot and Siméoni 2004).

A major result of our study is the detection of clear genetic variation between high-quality chemotypes and low-quality chemotypes from Vanuatu. Field experiments and analysis of kavalactones have demonstrated the stability of chemotypes across locations for a given cultivar and confirmed that chemotype is genetically controlled (Siméoni and Lebot 2002; Lasme et al. 2008). Nevertheless, this is the first study enabling genetic discrimination of cultivars differing in their chemical properties and quality. Our results are consistent with farmers' knowledge, by discriminating the noble and medicinal cultivars from the unsuitable two-days cultivars. After double checking in the VARTC germplasm collection, the unique discrepancy observed between genetic data, on the one hand, and chemotype, on the other, is thought to be related to mislabeling when the germplasm collection was replanted in 2009. The Pom group, of broad geographic origin, appears to be a heterogeneous assemblage of cultivars with very different chemical qualities and belonging to different chemotypes (Lebot and Lévesque 1989). Our results, which restitute the Pom group and display a little amount of variation within it, do not show the clear differentiation observed in Vanuatu, with poor quality cultivars (i.e., Akau Huli from Tonga) aggregating with good cultivars (i.e., Moi from Hawaii).

For psychoactive plants such as kava, morphological characters are less prone to human selection than are chemical characters. The chemotype is responsible for the quality of the physiological effects and the total kavalactone content, which also varies according to the cultivar used, determines its intensity (Siméoni and Lebot 2002; Wang et al. 2015). While noble and medicinal cultivars belong to a single clonal lineage, important variations in total kavalactones exist between and within these categories of cultivars (Lasme et al. 2008). So, although some environmental factors may play a role, it is likely that clonal selection is also exerted on the total kavalactone content as farmers judge the physiological effects of the beverage obtained by a single plant.

Acknowledgements

This research was financially supported by the ANR SYSTERRA program under the project No. ANR-10-STRA-007. Authors are thankful to the Vanuatu Agricultural Research Technical Center (VARTC) and to Ed Johnston (Alia Point Nursery, Hawaii) for the plant material made available for this study.

References

Akbari, M., Wenzl, P., Caig, V., Carling, J., Xia, L., Yang, S., et al. 2006. Diversity arrays technology (DArT) for high-throughput profiling of the hexaploid wheat genome. Theor. Appl. Genet. **113**: 1409–1420. doi:10.1007/s00122-006-0365-4. PMID:17033786.

Applequist, W., and Lebot, V. 2006. Validation of *Piper methysticum* var. *wichmannii* (Piperaceae). Novon, **16**: 3–4. doi:10.3417/1055-3177(2006)16[3:VOPMVW]2.0.CO;2.

Bilia, A., Gallori, S., and Vincieri, F. 2002. Kava-kava and anxiety: growing knowledge about the efficacy and safety. Life Sci. **70**: 2581–2597. doi:10.1016/S0024-3205(02)01555-2. PMID:12269386.

Billotte, N., Lagoda, P., Risterucci, A.M., and Baurens, F.C. 1999. Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits, **54**: 277–288.

Boerner, R., Sommer, H., Berger, W., Kuhn, U., Schmidt, U., and Mannel, M. 2003. Kava-Kava extract LI 150 is as effective as opipramol and buspirone in generalised anxiety disorder — an 8-week randomized, double-blind multi-centre clinical trial in 129 out-patients. Phytomedicine, **10**(Suppl. 4): 38–49. PMID:12807341.

Caillon, S., Quero-Garcia, J., Lescure, J.-P., and Lebot, V. 2006. Nature of taro (*Colocasia esculenta* (L.) Schott) genetic diversity prevalent in a Pacific Ocean island, Vanua Lava, Vanuatu. Genet. Resour. Crop Evol. **53**: 1273–1289. doi:10.1007/s10722-005-3877-x.

Campo, J.V., McNabb, J., Perel, J.M., Mazariegos, G.V., Hasegawa, S.L., and Reyes, J. 2002. Kava-induced fulminant hepatic failure. J. Am. Acad. Child Psych. **41**: 631–632. doi:10.1097/00004583-200206000-00001. PMID:12049436.

Connor, K., Payne, V., and Davidson, J. 2006. Kava in generalized anxiety disorder: three placebo-controlled trials. Int. Clin. Psychopharm. **21**: 249–253. doi:10.1097/00004850-200609000-00001.

Cordeiro, G.M., Pan, Y.-B., and Henry, R.J. 2003. Sugarcane microsatellites for the assessment of genetic diversity in sugarcane germplasm. Plant Sci. **165**: 181–189. doi:10.1016/S0168-9452(03)00157-2.

Dereeper, A., Argout, X., Billot, C., Rami, J.F., and Ruiz, M. 2007. SAT, a flexible and optimized web application for SSR marker development. BMC Bioinform. **8**: 465. doi:10.1186/1471-2105-8-465.

D'Hont, A., Paulet, F., and Glaszmann, J.C. 2002. Oligoclonal interspecific origin of "North Indian" and "Chinese" sugarcanes. Chromosome Res. **10**: 253–262. doi:10.1023/A:1015204424287. PMID:12067214.

Dice, L. 1945. Measures of the amount of ecologic association between species. Ecology, **26**: 297–302. doi:10.2307/1932409.

Gruenwald, J., Mueller, C., and Skrabal, J. 2003. In-depth investigation into EU member states market restriction on kava products. International Kava Executive Council report, 305p.

Heller-Uszynska, K., Uszynski, G., Huttner, E., Evers, M., Carlig, J., Caig, V., et al. 2011. Diversity arrays technology effectively reveals DNA polymorphism in a large and complex genome of sugarcane. Mol. Breed. **28**: 37–55. doi:10.1007/s11032-010-9460-y.

Hurtado, P., Olsen, K., Buitrago, C., Ospina, C., Marin, J., Duque, M., et al. 2008. Comparison of simple sequence repeat (SSR) and diversity array technology (DArT) markers for assessing genetic diversity in cassava (*Manihot esculenta* Crantz). Plant Gen. Res. **6**: 208–214. doi:10.1017/S1479262108994181.

Jaccoud, D., Peng, K., Feinstein, D., and Kilian, A. 2001. Diversity arrays: a solid state technology for sequence information independent genotyping. Nucleic Acids Res. **29**: E25. doi:10.1093/nar/29.4.e25. PMID:11160945.

Jokhan, A., and McLenachan, P. 2004. Genetic fingerprinting of Fijian kava. S. Pac. J. Nat. Appl. Sci. **22**: 43–45.

Karamura, D., Karamura, E., Tushemereirwe, W., Rubaihayo, P., and Markham, R. 2010. Somatic mutations and their implications to the conser-

vation strategies of the East African highland bananas (*Musa* spp.). Acta Hortic. **879**: 615–621.

Laidò, G., Mangini, G., Taranto, F., Gadaleta, A., Blanco, A., Cattivelli, L., et al. 2013. Genetic diversity and population structure of tetraploid wheats (*Triticum turgidum* L.) estimated by SSR, DArT and pedigree data. Plos ONE. doi:10.1371/journal.pone.0067280.

Lasme, P., Davrieux, F., Montet, D., and Lebot, V. 2008. Quantification of kavalactones and determination of kava (*Piper methysticum*) chemotypes using near-infrared reflectance spectroscopy for quality control in Vanuatu. J. Agric. Food Chem. **56**: 4976–4981. doi:10.1021/jf800439g. PMID:18540613.

Lasso, E. 2008. The importance of setting the right genetic distance threshold for identification of clones using amplified fragment length polymorphism: a case study with five species in the tropical plant genus *Piper*. Mol. Ecol. Resour. **8**: 74–82. doi:10.1111/j.1471-8286.2007.01910.x. PMID:21585721.

Lebot, V., and Lévesque, J. 1989. The origin and distribution of kava (*Piper methysticum* Forst. f. Piperaceae): a phytochemical approach. Allertonia, **5**: 223–380.

Lebot, V., and Lévesque, J. 1996*a*. Evidence for conspecificity of *Piper methysticum* Forst. f. and *Piper wichmannii* C. DC. Biochem. Syst. Ecol. **24**: 775–782. doi:10.1016/S0305-1978(96)00048-8.

Lebot, V., and Lévesque, J. 1996*b*. Genetic control of kavalactone chemotypes in *Piper methysticum* cultivars. Phytochemistry, **43**: 397–403. doi:10.1016/0031-9422(96)00209-9.

Lebot, V., and Siméoni, P. 2004. Is the quality of kava (*Piper methysticum* Forst. f.) responsible for different geographical patterns. Ethnobot. Res. Appl. **2**: 19–28.

Lebot, V., Aradhya, M., and Manshardt, R. 1991. Geographic survey of genetic variation in kava (*Piper methysticum* Forst. f. and *Piper wichmanii* C. DC.). Pac. Sci. **45**: 169–185.

Lebot, V., McKenna, D., Johnston, E., Zheng, Q.Y., and McKern, D. 1999. Morphological, phytochemical, and genetic variation in hawaiian cultivars of Awa (Kava, *Piper methysticum*, Piperaceae). Econ. Bot. **53**: 407–418. doi:10.1007/BF02866720.

Moncada, P., and McCouch, S. 2004. Simple sequence repeat diversity in diploid and tetraploid *Coffea* species. Genome, **47**(3): 501–509. doi:10.1139/g03-129. PMID:15190367.

Perrier, X., and Jacquemoud-Collet, J. 2006. DARwin software, version 5.0 for Windows. Available from http://darwin.cirad.fr/darwin. Accessed 20 April 2014.

Pittler, M., and Ernst, E. 2003. Kava extract versus placebo for treating anxiety. Cochrane Database of Systematic Reviews, 2, CD003383. doi:10.1002/14651858.CD003383. PMID:12076477.

Risterucci, A.M., Hippolyte, I., Perrier, X., Xia, L., Caig, V., Evers, M., et al. 2009. Development and assessment of diversity arrays technology for high-throughput DNA analyses in *Musa*. Theor. Appl. Genet. **119**: 1093–1103. doi:10.1007/s00122-009-1111-5. PMID:19693484.

Scarcelli, N., Tostain, S., Vigouroux, Y., Luong, V., Baco, M.N., Agbangla, C., et al. 2011. Genetic structure of farmer-managed varieties in clonally-propagated crops. Genetica, **139**: 1055–1064. doi:10.1007/s10709-011-9607-8. PMID:21898046.

Scarcelli, N., Couderc, M., Baco, M.N., Egah, J., and Vigouroux, Y. 2013. Clonal diversity and estimation of relative clone age: application to agrobiodiversity of yam (*Dioscorea rotundata*). BMC Plant Biol. **13**: 178. doi:10.1186/1471-2229-13-178. PMID:24219837.

Selvi, A., Nair, N.V., Balasundaram, N., and Mohapatra, T. 2003. Evaluation of maize microsatellite markers for genetic diversity analysis and fingerprinting in sugarcane. Genome, **46**(3): 394–403. doi:10.1139/g03-018. PMID:12834055.

Silvestrini, M., Junqueira, M., Favarin, A., Guerreiro-Filho, O., Maluf, M.P., Silvarolla, M.B., and Colombo, C.A. 2007. Genetic diversity and structure of Ethiopian, Yemen and Brazilian *Coffea arabica* L. accessions using microsatellites markers. Genet. Resour. Crop Evol. **54**: 1367–1379. doi:10.1007/s10722-006-9122-4.

Siméoni, P., and Lebot, V. 2002. Identification of factors determining kavalactone content and chemotype in kava (*Piper methysticum* Forst. f.). Biochem. Syst. Ecol. **30**: 413–424. doi:10.1016/S0305-1978(01)00093-X.

Singh, Y.N., and Singh, N.N. 2002. Therapeutic potential of kava in the treatment of anxiety disorders. CNS Drugs, **16**: 731–743. doi:10.2165/00023210-200216110-00002. PMID:12383029.

Smith, A.C. 1981. *Flora Vitiensis Nova*: a new flora of Fiji Pacific Tropical Botanical Garden, Lawai, Kauai, Hawaii. Vol. 2. 810p.

Sokal, R., and Michener, C. 1958. Statistical method for evaluating systematic relationship. Kans. Univ. Sci. Bull. **28**: 1409–1438.

Steffens, D., Sutter, S., and Roemer, S. 1993. An alternate universal forward primer for improved automated DNA sequencing of M13. Biotechniques, **15**: 580–582. PMID:8251153.

Teschke, R. 2011. Kava and the risk of liver toxicity: past, current, and future. AHPA Rep. **26**(3): 9–17.

Teschke, R., and Lebot, V. 2011. Proposal for a kava quality standardization code. Food Chem. Toxicol. **49**: 2503–2516. doi:10.1016/j.fct.2011.06.075. PMID:21756963.

Wang, J., Qu, W., Bittenbender, H., and Li, Q. 2015. Kavalactone content and chemotype of kava beverages prepared from roots and rhizomes of Isa and Mahakea varieties and extraction efficiency of kavalactones using different solvents. J. Food Sci. Technol. **52**(2): 1164–1169. doi:10.1007/s13197-013-1047-2. PMID:25694734.

Wenzl, P., Carling, J., Kudrna, D., Jaccoud, D., Huttner, E., Kleinhofs, A., and Kilian, A. 2004. Diversity Arrays Technology (DArT) for whole-genome profiling of barley. Proc. Natl. Acad. Sci. U.S.A. **101**: 9915–9920. doi:10.1073/pnas.0401076101. PMID:15192146.