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GENETIC CONTROL OF KAVALACTONE CHEMOTYPES IN *PIPER METHYSTICUM* CULTIVARS

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Key Word Index—*Piper methysticum*; Piperaceae; chemotypes; kavalactones; HPLC; isozymes; genetic variation; clonal selection; effects of environment.

Abstract—The chemical composition of 121 cultivars of *Piper methysticum* originating from 51 Pacific islands, were investigated using HPLC. The results obtained for six major kavalactones (demethoxy-yangonin, dihydrokavain, yangonin, kavain, dihydromethysticin and methysticin) were submitted to cluster analysis and six distinct chemotypes were identified. Three field experiments demonstrated that chemotypes are not affected by environmental factors but are controlled genetically. Analysis of the isozyme variation for eight enzyme systems, revealed that these cultivars exhibited only three zymotypes. These are so similar that the differences in malate dihydrogenase and diaphorase could be explained as mutations. Few genes are thought to be responsible for the chemotype variation between cultivated clones. Because all cultivars are decaploids and sterile, it is suggested that mutagenesis might be used to induce genetic variability and to improve cultivar chemotypes. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Kava (*Piper methysticum* Forst. f.) is used in the Pacific to make a phychoactive beverage that is prepared by grinding the roots of the shrub. The active ingredients, called kavalactones, have diuretic, soporific, antiepileptic, spasmolytic, analgesic, local anaesthetic, bacteriocidal and antimycotic properties [1]. About 100 tons of dry roots are imported annually by European laboratories that are now facing difficulties related to variation in total kavalactone content and chemical composition of the raw material.

A study of the genetic variation existing between *P. methysticum* and its wild progenitor, *P. wichmannii* C. DC., has revealed remarkable variation in morphotypes and chemotypes [2]. Farmers cultivate numerous cultivars which exhibit differences in growth habit, morpho-agronomic traits, and physiological effects; *ca* 120 distinct morphotypes are recognized throughout Polynesia, Melanesia and Micronesia. An ecogeographical survey of the isozyme variation existing between *P. methysticum* and the wild species, *P. gibbilimbum* and *P. wichmannii*, has shown that kava was probably domesticated from a very narrow genetic base [3]. Cultivars are sterile and are propagated vegetatively by stem cuttings. These clones are all decaploids with $2n = 10x = 130$ chromosomes. Sterility is probably due to high ploidy levels and to the accumulation of mutations inhibiting sexuality in cultivars.

Kava is a very attractive cash crop for smallholders and research has been undertaken in order to select the best clones for the local drinking market and for the pharmaceutical industry. Because there are no sexual recombinations between cultivars, the future of breeding is uncertain. Clonal selection might be the only practical approach to the genetic improvement of this crop but its efficiency has yet to be demonstrated. It is therefore important to characterize and evaluate germplasm in order to assist the selection of the most promising cultivars.

Several authors [4–7] have observed that before chemical standards can be formulated, it is essential to study the variation in the active constituents and their relation to plant age, cultivar type and environmental parameters. A first attempt [5, 6] showed that the biogenetic activity is essentially the same in the various parts of the vegetative system but that it leads to different compositions in the roots. Only two cultivars were studied and their chromatograms were quite similar. Analyses carried out at different times of the year showed no seasonal changes in kavalactone content in either cultivar.

During our study, we addressed several questions. First, are the chemical composition and total kavalactone content of a plant dependent on the cultivar, the age of the plant, specific environmental factors or a combination of these variables? Second, when a sport of a cultivar presents a new desirable chemical composition, is it possibly to preserve this composition by cloning? We conducted various field experiments to

improve our understanding of factors controlling the biosynthesis of kavalactones. The aim of these experiments was to provide evidence for the efficiency of clonal selection and they contribute to achieving a better quality control of the commercialized drug materials.

The objectives of the present study were to examine quantitative variation of six major kavalactones and to analyse isozyme polymorphism for eight enzyme systems in 121 cultivars originating from 51 different islands of the Pacific. We attempted to elucidate whether chemotypes are affected by environmental factors and ontogeny, and whether they are linked to isozyme markers or dependent on cultivar and, thus, controlled genetically.

RESULTS

Intraspecific variation

Several samples of the same extract, injected repeatedly through the HPLC column, have shown that the values obtained are reliable, since variation between samples was close to nil. This confirmed the accuracy of the method and allowed interpretation of the results. The compounds were divided into two groups, major and minor kavalactones. The former account for *ca* 96% of the total lipid extract. We therefore used only six major kavalactones for our study. These were numbered as follows to define chemotypes: 1 = demethoxy-yangonin (DMY); 2 = dihydrokavain (DHK); 3 = yangonin (Y); 4 = kavain (K); 5 = dihydromethysticin (DHM); and 6 = methysticin (M). Chemical compositions were coded, by listing in decreasing order of proportion, the six major kavalactones in the lipid extract. The first three kavalactones in the code usually represented over 70% of the total composition.

A first field experiment aimed at evaluating chemotype variation between 63 distinct cultivars belonging to the germplasm collection of the Department of Agriculture in Vanuata. The results of chemical analyses using HPLC are presented in Table 1. Although the 63 plants were planted together and uprooted from the same plot, the data indicate considerable variation between cultivars. Some cultivars had very low total kavalactone contents (min. KL% = 04.43, expressed in lipid extract percentage of the dry root system), while others produced very high yields of active ingredients (max. KL% = 18.70). These results obtained from cultivars grown in a homogeneous environment indicate that the variability in chemical composition is controlled by genotype rather than by external factors. Farmers, consumers and exporters of kava, are all aware of this variation. It is known from pharmacological studies [1] that each kavalactone has characteristic properties. The physiological effect of a chemotype is governed by its dominant kavalactone concentration.

The information gained from the first experiment needed to be confirmed. More than 120 cultivars collected from 51 different islands of Polynesia, Micronesia and Melanesia (including the 63 from Vanuatu) were analysed and their chemotypes identified. The resulting data matrix (121 cultivars × 6 major kavalactones) was submitted to cluster analysis and revealed six clusters and chemotype groups. Within each group, cultivars were not significantly different. Results are summarized by the dendrogram in Fig. 1 and the data presented in Table 2.

Chemotype 246531 (cluster 1), which is the biggest group, is a group of 32 cultivars used for daily drinking. Chemotype 426135 (cluster 2, 9 cultivars) is famous for producing a pleasant, fast and temporary physiological effect. Chemotype 264531 (cluster 3) is a group of 28 cultivars traditionally used for medicinal purposes. Chemotype 256431 (cluster 4) is a group of 26 cultivars known for their very pronounced physiological effect that can last for more than 12 hours. Chemotype 265431 (cluster 5) is a group of 16 cultivars, drunk occasionally for ceremonial purposes, and known for their very pronounced physiological effect. Chemotype 643251 (cluster 6) is a group of 10 cultivars that are always prepared from dried and not fresh roots, unlike the previous chemotypes.

Drinkers do not appreciate a high percentage of DHK (2) and DHM (5) but chemotypes with a high percentage of K (4) and a low percentage of DHM (5) produce a pleasant and desirable physiological effect. Chemotype variability is not due to the geographical origin of the cultivars, because most clones have been distributed, from Vanuatu, where they were domesticated [3], throughout the Pacific Islands. However, the chemotypic diversity of kava within Vanuatu is greater than anywhere else in the Pacific; this country is probably the area of domestication of most cultivars.

Table 1. Results of HPLC analysis conducted on 63 cultivars of *P. methysticum* from the germplasm collection of Vanuatu, Efate Island

	KL	DMY	DHK	Y	K	DHM	M
Mean	11.01	06.85	32.14	09.01	20.30	15.85	15.73
Min.	04.43	04.03	16.49	09.99	01.70	05.95	07.63
Max.	18.70	15.16	49.02	19.33	37.41	58.21	27.31
STD	03.28	02.08	06.66	03.18	08.54	10.03	04.62
CV%	29.79	30.36	20.72	35.29	42.06	63.28	29.37

DMY = demethoxy-yangonin; DHK = dihydrokavain; Y = yangonin; K = kavain; DHM = dihydromethysticin; M = methysticin; KL = total kavalactone content, as % dry matter.

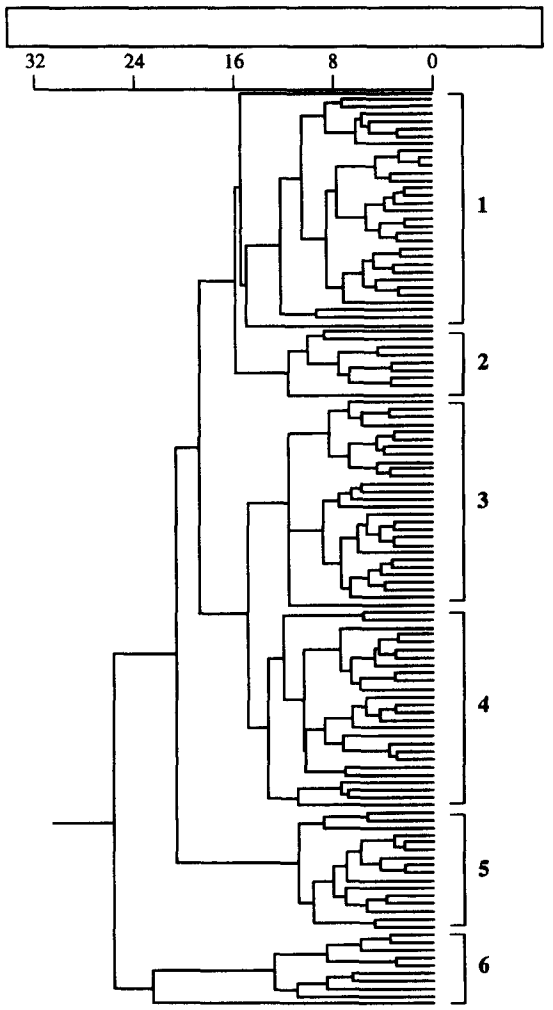

Fig. 1. Dendrogram obtained by cluster analysis (using Euclidean distance and UPGMA) of the matrix 121 cultivars × 6 major kavalactones of *P. methysticum*.

Intra-clonal variation

Cultivars Vila and Small leaf were analysed when harvested from the first experiment and their chemotypes were, respectively, 256431 and 423615. In the second experiment, eight plants, propagated by stem cuttings of these two cultivars were planted the same day, in a row, and harvested exactly two years later. The results from this experiment are presented in Table 3. Variation between plants of the same cultivar was limited. These results show that kavalactone composition is very homogeneous within the clone and that chemotype is consistent. The coefficients of variation obtained for the mean of eight plants indicate the vegetative propagation of a selected cultivar leads to a high probability of preserving the same chemotype by cloning the mother plant.

Variation with environment and ontogeny

For the third experiment, clones from different cultivars harvested from the first experiment were sent to a different island and planted in a row the same day. After one year, one plant from each cultivar was harvested every five months and the analytical results compared with the chemotypes of the mother plants from the first experiment. The data presented in Table 4 are the mean of four plants harvested at 13, 18, 23 and 28 months. Kavalactone content was highest after 18 months on average. This content remained stable during the subsequent growth of the plant, although the rootstock biomass continues to increase over time (a shrub can live for up to 15–20 years). These results also confirm that the major kavalactone composition is not related to environment. No variation was observed between plants of the same row harvested every five months; it is therefore concluded that chemical composition is not related to season or ontogeny.

Isozyme variation and genetic variability

Neutral molecular markers, such as isozymes, are not affected by environmental factors, and provide an assessment of the degree of genetic variation existing between cultivars. One objective of the present work was to determine whether isozymes could be used to fingerprint and differentiate between cultivars exhibiting a particular chemotype.

More than 200 leaf samples corresponding to the different cultivars were electrophoresed and their zymograms analysed for eight enzyme systems. Resolution and banding intensity were constant regardless of the origin of the leaf material used. Malate dehydro-

Table 2. Chemotypes of *P. methysticum* identified by HPLC of 121 cultivars originating from 51 islands. Analysis of quantitative variation of major kavalactones carried out by cluster analysis

Cluster	Chemotype	DMY (1)	DHK (2)	Y (3)	K (4)	DHM (5)	M (6)
1	246531	5–7	29–40	6–10	18–29	9–16	10–19
2	426135	6–12	22–30	8–11	30–38	5–9	10–17
3	264531	5–8	26–32	5–13	9–18	7–13	18–23
4	256431	4–5	31–44	7–12	7–13	18–23	10–17
5	265431	4–5	27–43	7–9	11–14	14–21	14–26
6	643251	6–9	11–18	14–17	18–26	9–14	24–33

DMY = demethoxy-yangonin; DHK = dihydrokavain; Y = yangonin; K = kavain; DHM = dihydromethysticin; M = methysticin; as % dry matter.

Table 3. Variation measured between clones and comparison with mother-plant (control) from the germplasm collection of *P. methysticum*

Cultivar	DMY (1)	DHK (2)	Y (3)	K (4)	DHM (5)	M (6)	Chemotype
Vila (control)	04.62	31.54	10.43	11.09	21.57	20.71	256431
Mean of eight plants	06.23	33.70	11.66	14.17	17.70	16.52	256431
CV%	06.39	02.85	04.77	05.86	06.58	04.61	
Small leaf (control)	08.06	22.16	15.09	35.20	06.36	13.13	423615
Mean of eight plants	07.96	25.52	13.98	33.19	06.71	12.98	423615
CV%	01.17	02.28	03.21	01.06	02.16	02.17	

genase (MDH) and diaphorase (DIA) were the only variable enzyme systems and only three different zymotypes were observed for all cultivars (electrophoregrams are presented in Fig. 2). The zymotypes were so similar that the differences in MDH and DIA could be explained as mutations. More than 90 samples, representing 59 cultivars originating from Polynesia and Micronesia, that correspond to four distinct chemotypes exhibited only one zymotype (third experiment). More variation existed in Melanesia where 61 cultivars collected from Papua New Guinea and Vanuatu revealed all three zymotypes (Table 5).

The discriminating ability of enzyme electrophoresis depends on the number of polymorphic loci that can be resolved. For the eight enzyme systems resolved for *P. methysticum*, there are at least 16 loci and probably more, owing to the high level of ploidy ($2n = 10x$). There are several possible explanations for the absence of variability at the isozyme level. The most plausible hypothesis to account for low levels of genetic variation is that this species consists of sterile clones resulting from human selection of somatic mutants, rather than from sexual reproduction, which would have produced a greater diversity of zymotypes. If this hypothesis is valid, then only a few genes are responsible for the chemical variation between cultivars and none of these are linked with loci controlling isozyme markers. No correlation exists between morphotypes and zymotypes. All the Polynesian cultivars, for example, possess the same zymotype, although there are clear morphological and chemical differences (Table 5).

DISCUSSION

Our study has provided a better understanding of the factors affecting chemical variation in *P. methysticum* cultivars. Several specimens of the same cultivar growing in different soils and under different environments were analysed. In addition, different plants of the same clone grown under the same conditions were harvested at different ages, in order to study variation in relation to ontogeny. All these tests indicated that variability in kavalactone composition is related to genotype, rather than to environmental factors. When different cultivars are planted in an homogeneous environment, they produce a range of chemotypes that can be preserved and propagated by cloning. However, vegetative propagation is also the most likely source of genetic variability and chemical variation is probably controlled by very few genes as revealed by the limited isozyme polymorphism observed between cultivars. Mutations appear to be the main factor in the germplasm diversifi-

Table 4. Chemotype variation of *P. methysticum* with ontogeny and environment. Comparison between results obtained for the local check (E) from the Vanuatu germplasm collection (Efate 2200 mm) and mean (S) of four plants harvested at 13, 18, 23 and 28 months from Santo Island (3200 mm)

Cultivar	DMY (1)	DHK (2)	Y (3)	K (4)	DHM (5)	M (6)	Chemotype
Malogro (E)	04.07	35.10	06.75	11.15	23.71	19.23	256431
Mean (S)	07.21	30.40	07.90	13.99	20.13	10.37	254631
Marino (E)	05.93	30.81	09.71	11.67	18.09	23.79	265431
Mean (S)	07.51	28.29	11.02	13.95	18.15	21.04	265431
Merei (E)	05.24	38.85	09.80	11.83	18.07	16.21	256431
Mean (S)	06.41	34.64	10.14	14.62	18.85	15.32	256431
Fock (E)	05.62	30.03	12.17	15.10	15.74	21.33	265431
Mean (S)	07.59	31.31	11.78	15.42	16.18	17.69	265431
Kar (E)	08.25	34.10	08.11	22.30	10.89	16.36	246513
Mean (S)	07.87	29.88	11.11	24.53	12.06	14.53	246531
Thyei (E)	04.90	37.20	10.75	11.25	20.02	15.87	256431
Mean (S)	06.88	38.07	12.38	13.77	17.32	03.55	254631
Visul (E)	05.44	38.28	10.00	21.11	11.64	13.53	246531
Mean (S)	08.77	27.37	10.30	30.13	10.38	13.12	426351
Yevoet (E)	09.26	26.82	09.34	22.05	10.59	22.94	264531
Mean (S)	09.94	20.46	10.32	30.20	07.53	21.72	462315

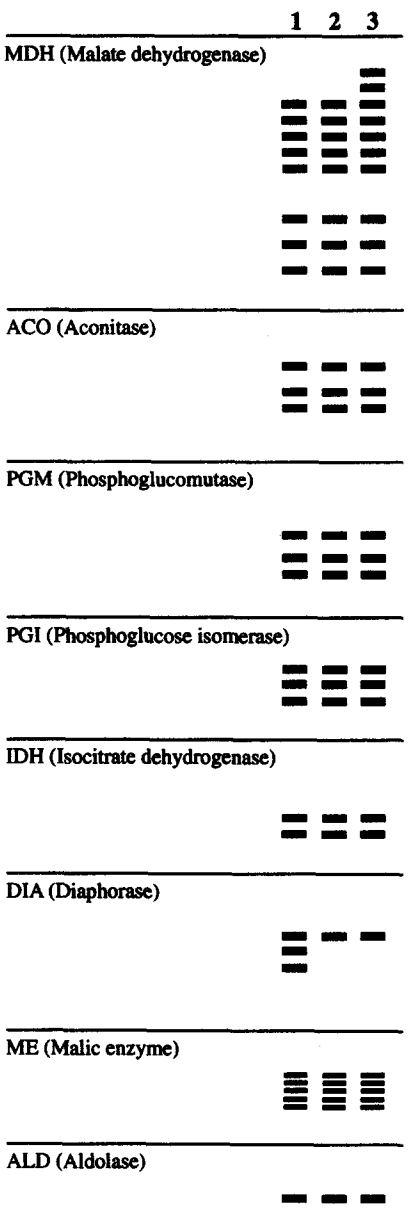

Fig. 2. Schematic representation of three zymotypes observed in cultivars of *P. methysticum* for the eight enzyme systems investigated.

cation process and this raises the question of the responsible enzymatic steps involved in the biosynthesis of the kavalactones sustaining the chemotypes.

Kavalactones are formed from phenylpropanes and from acetyl coenzyme building blocks, viz. from one phenylpropane and two acetate units [1]. Kavalactones can therefore be considered as variants of flavonoids that are made up of one phenylpropane unit and three acetate units. Kavalactones also have the characteristics of compounds whose biosynthesis indicates polyacetate chains and an acetate metabolism. The formation of kavalactones has been explained by two biosynthetic processes, one starting from cinnamic acid and resulting in styrylpyrones, like dehydrokawain, the other beginning with the alcohol corresponding to a given styrlpyrone, which develops into styryldihydropyrones, like kavain [1, 8]. Primary chemical differences between the kavalactones involves the presence or absence of double bonds at the 5,6- or 7,8- positions and the presence or absence of substituent groups in the phenyl ring. Consequently, there are two possibilities of variation, the degree of hydrogenation or the number of non-aromatic double-bonds, and benzenoid substitution.

Both major and minor kavalactones are present in variable concentrations in different parts of *P. methysticum*. Concentrations of kavalactones are typically highest in lateral roots and decrease progressively toward the aerial parts of the plant. There are striking differences between the chemical compositions of the roots and the leaves of the same plant. Kavain is notably absent from the leaves and this is explained by the immediate reduction of one double bond (7, 8) by ascorbic acid. Yangonin and dehydrokavain are found in the leaves but only in traces [6]. No unsaturated derivatives (e.g. kavain and methysticin) are found in significant quantities in the leaves, and dihydro derivatives are the only major components of the leaves. One possible explanation might be that the molecule of the basic skeleton, the styrylpyrone ring, is first developed in the roots and that the reductions of the double bonds at 5,6 and 7,8 occur subsequently in the leaves. Why only certain degrees of hydrogenation occur in kavalactones sustaining chemotypes in cultivars still has to be explained. Although the production of secondary metabolites in many plants has been linked to protection

Table 5. Geographic distribution of chemotypes and zymotypes of *P. methysticum* in Oceania

Country	Islands surveyed	Chemotypes	Zymotypes
Papua New Guinea	5	4, 5	1, 2, 3
Vanuatu	23	1, 2, 3, 4, 5	2, 3
Fiji	3	6	3
Tonga	2	3, 4	3
Samoa	3	1, 3, 4	3
Wallis and Futuna	2	1	3
Cooks	1	6	3
Tahiti and Marquesas	6	1, 6	3
Hawaii	4	1, 6	3
Pohnpei and Kosrae	2	1, 6	3

against predators, the evolution of kavalactone content and diverse chemotypes of cultivars does not appear to have resulted from natural selection.

Isozyme polymorphism has a simple genetic basis and is independent from human selection. Isozymes generally exhibit Mendelian inheritance and codominant expression. Because isozymes are proteins, they reflect alterations in the DNA sequence through changes in amino acid composition. Changes in amino acid composition alter the charge, thereby producing a change in electrophoretic mobility. Variation in electrophoretic mobility of a protein directly reflects changes in the DNA sequence of the structural gene for that protein and not polymorphism in one or more of the genes involved in the modification steps. Most evidence indicates that isozyme loci are widely distributed throughout the genome. Isozymes are therefore revealing changes in amino acid composition occurring throughout the genome and, in the case of *P. methysticum*, the absence of such changes.

Chemical variability in kava is largely the result of human selection and cloning of somatic mutations in genetically similar, vegetatively propagated cultivars. Polyploidy contributes to infertility and to absence of genetic recombinations, and enhances the survival of somatic mutants and periclinal chimeras. However, no significant correlation between chemotypes and zymotypes has been detected. Variation at the isozyme level does not obviously correspond to variation at the chemotypic level that was selected by farmers. It is possible that all kava cultivars originate from a single ancestral plant that has been repeatedly cloned, developed and dispersed by stem cuttings over great distances throughout the Pacific.

Kava is a traditional crop that is widely grown but not improved. Because the plant does not reproduce sexually, traditional genetic improvement, which requires sexual propagation, would be difficult or impossible. Further use of germplasm after screening is therefore limited, unless non-traditional methods for inducing genetic variability, such as mutagenesis, can be developed. If cultivars that produce more active ingredients can be selected, then more lucrative markets could be opened.

EXPERIMENTAL

Chemical analysis. More than 200 accessions, originating from 51 islands of the Pacific and corresponding to *ca* 121 different cultivars, were analysed for their chemical composition. While collecting roots for analysis, great care was taken to select the same type of roots. Root samples gathered in the field from living plants were dried at 80° for 8 h. Powdered dry roots were extracted for 6 hr with $CHCl_3$ in a Soxhlet. The extract was then evapd under red. pres. and analysed by HPLC with a photodiode-array detector. Analyses were conducted at 20°. Using a normal phase column, Si 60 Superspher (125 × 4 mm i.d., particle size 4 μm Merck) fitted with a pre-column (4 × 4 mm, same phase). The mobile phase was hexane–dioxane (4:1) at a flow rate of 1.5 ml min^{-1}. The inj. vol. was 10 μl with UV detection at 240 nm. R_ts (min) were: DMY = 8, DHK = 9.5, Y = 12.5, K = 14, DHM = 17 and M = 26.

Isozyme electrophoresis. Leaf tissues of 200 accessions were analysed for isozyme variation in 8 enzyme systems including aconitase (ACO), aldolase (ALD), DIA, isocitrate dehydrogenase (IDH), MDH, malic enzyme (ME), phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM). Leaf extracts were prepared as described in ref. [3] and loaded onto starch gels (12.5%). The buffer system was histidine citrate, pH 6.5 and samples were loaded onto starch gels (12.5%), electrophoresed at 4° during 6 hr at 15 V cm^{-1} and 40–50 mA. Because of the high ploidy level, no interpretation of the genetic significance of the banding patterns was attempted. The gels were scored for the presence or absence of electromorphs.

Field experiments. Experiment 1: 63 different cultivars of *P. methysticum* from varying origins, recognized as distinct morphotypes, were planted in a common garden (2.5 × 2.5 m) and harvested 2 years later to assess inter-cultivar variation of total kavalactone content and composition. These cultivars originated from the germplasm collection of the Dept of Agriculture in Vanuatu (Efate Island) and were all grown in rain-fed conditions without fertilizers or pesticides. *Experiment* 2: Different plants of the same clone grown under the same conditions (rain-fed, no fertilizers, no pesticides) were harvested at different ages in order to study variation with ontogeny. Clones from different cultivars were planted in a row (2.5 × 2.5 m) the same day and one plant of each was harvested every 5 months (after 13, 18, 23 and 28 months). Results obtained from this trial set up at the CIRAD station on Santo Island (altitude 140 m, average annual precipitation, 3200 mm), were compared with the same cultivar from the germplasm collection on Efate Island (altitude 20 m, average annual precipitation, 2400 mm). *Experiment* 3: Distinct clones of the same cultivar were planted in a row (2.5 × 2.5 m) on the same day and harvested together to assess interclonal variation (same conditions).

Data analysis. Data obtained from HPLC analyses of root samples were statistically appraised using cluster analysis, calculated using Euclidean distance and UPGMA, performed with NTSYS-pc software, version 1.21 (Applied Biostatistics Inc., U.S.A.). Multifactorial analysis was also used to confirm, by space projection, the groups defined by cluster analysis (data not presented herein).

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