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DNA fingerprinting reveals clonal nature of *Vetiveria zizanioides* (L.) Nash, Gramineae and sources of potential new germplasm

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Abstract

Random amplified polymorphic DNAs (RAPDs) were used to examine accessions of vetiver (*Vetiveria zizanioides* (L.) Nash, Gramineae) from around the world. It appears that only one genotype, 'Sunshine', accounts for almost all the germplasm utilized. Screening of numerous accessions of vetiver revealed only a few nonfertile germlines. Analysis of *V. zizanioides* and other *Vetiveria* species, *Chrysopogon fulvus* (Spreng.) Chiov., *C. gryllus* (L.) Trin., *Sorghum bicolour* (L.) Moench., and *S. halepense* (L.) Pers., revealed that *Vetiveria* and *Chrysopogon* are not separable by their RAPDs. This lends support to merging the genus *Vetiveria* with *Chrysopogon*. The lack of genetic diversity in this important cultivated plant is a critical factor and additional, suitable germplasm must be collected and introduced into erosion control projects around the world.

Keywords: Chrysopogon, clones, erosion control, RAPD, *Sorghum*, *Vetiveria*

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Introduction

The introduction of new plants into the environment by humans is often in the form of monocultures. These monoculture crops are extremely inbred for factors such as yield, uniform flowering and height, and cosmetic features of the products. This narrow genetic base has resulted in several disastrous crop failures. For example, Ireland's potato (*Solanum tuberosum* L.) famine of 1846 resulted in famine and the emigration of a quarter of the country's population. This was due to the fact that the potatoes had no resistance to *Phytophthora infestans*, the late blight fungus (Plucknett *et al.* 1987). The lack of resistance can be traced to the lack of genetic diversity in Irish potatoes, which had been multiplied using clonal materials from just **two** separate South American introductions to Spain in 1570 and thence to England in 1590 (Hawkes 1979). When the late blight fungus attacked the Irish potatoes there were no individuals with resistance genes among these two potato lines.

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A more recent example is the southern corn leaf blight (fungus, *Helminthosporium maydis*) in 1970 in the USA. Because almost all of the corn (*Zea mays* L.) in the USA was of hybrid origin and contained the Texas cytoplasmic male sterile line, the fields of corn presented an unlimited extremely narrow gene-base habitat for the fungus. By the late summer of 1970, plant breeders were scouring corn germplasm collections in Argentina, Hungary, Yugoslavia and the USA for resistant sources (Plucknett *et al.* 1987). Nurseries and seed fields were used in Hawaii, Florida, the Caribbean, and Central and South America to incorporate the resistance into hybrid corn in time for planting in the spring of 1971 (Ullstrup 1972). Without these genetic resources this technological feat would not have been possible. Both the potato and corn examples show the susceptibility of a very narrow genetic base to an ecological disaster.

During the past 10 years, a tall, pantropical grass has been utilized in many parts of the world to control soil erosion: vetiver (*Vetiveria zizanioides* (L.) Nash). Hedges of the nonseeding vetiver provide an effective living dam against erosion (NRC 1993) and this technique is now in use in more than 160 countries. The exact origin of the

nonseeding vetiver is not known. However, *V. zizanioides* seems to have originated in the area from India to Vietnam and its fragrant roots have been used for centuries for mats and perfumes (NRC 1993).

Kresovich *et al.* (1994) reported on clonal variation of vetiver using RAPDs. They found that RAPD patterns were very stable within clones and were able to distinguish between 'Huffman' and 'Boucard' plants, and the USDA PI 196257 introductions. In addition, they found that each of the three USDA PI 196257 accessions (nos 1, 2, and 3) were genetically different. They concluded that RAPDs would be very useful for identifying truly new and/or different sources of diversity.

One of the most desirable features of hedgerow vetiver is that it is nonfertile and must be grown from cuttings (clumps of rootstock). Thus, because it does not reproduce by seeds, it is a very well-behaved grass throughout the tropics and subtropics. It has not escaped cultivation and become a weed. However, the mere fact that it is always distributed by cuttings could lead to the widespread cultivation of a single clone. This would be extremely dangerous. If an insect or disease became adapted to a clone, the adaptation could spread and decimate millions of erosion-control terraces of vetiver. In order to investigate this matter, we assembled leaf materials from cultivated vetiver from around the world and analysed these accessions using RAPDs (DNA fingerprints). In addition, other *Vetiveria* species and two putatively related genera, *Chrysopogon* Trin. and *Sorghum* Moench., were analysed in an effort to begin to understand the potential germplasm pool for future selections.

Materials and methods

Specimens were collected as shown in Tables 1 and 2. Leaves were shipped fresh, air-dried, or desiccated in silica gel (Adams *et al.* 1992). The DNA from vetiver was not preserved very well in either fresh or air-dried leaves. Interim preservation of the leaves in silica gel was necessary. Upon receipt, all the materials were frozen until analysed. DNA was extracted using the hot CTAB protocol (Doyle & Doyle 1987) with the addition of 1% (w/v) PVP and Pronase E (150 μg). Grinding in hot CTAB (60 °C) in a hot mortar and pestle was somewhat effective for some accessions, but most accessions yielded larger-molecular-weight DNA and greater yields when the tissue was ground in liquid nitrogen and then placed in hot CTAB. Yellowed leaves frequently yielded degraded DNA when ground directly in hot CTAB, but yielded more high-molecular-weight DNA (20–50 kbp) when ground in liquid nitrogen and then incubated in hot CTAB.

Table 1 Vetiver accessions with the Sunshine (= Haiti, Huffman, Monto & Vallonia types) DNA profile based on the fact that no band variation was seen with primers 184, 239, 249, 268, 327, and 346. Lane no. refers to the gel lane no. in Fig. 1

Accession no.	Laboratory no.	Source	Fertile?	Lane no.
VET-RPA-7655	7655	Haiti	no	27
VET-RPA-7659	7659	Haiti	no	26
VET-RPA-7660	7660	Haiti	no	25
VET-RPA-7661	7661	Haiti	no	24
VET-RPA-7663	7663	Haiti	no	23
VET-PT-1 A	7711	cv. Monto, Australia	no	22
VET-PT-1B	7712	cv. Fiji, Australia	no	21
VET-PT-1D	7714	Australia	no	20
VET-PR-1E	7715	New Guinea	no	19
VET-RGG-CR-A	7721	Costa Rica	no	18
VET-MR-VAL1	7722	South Africa	no	17
VET-OSR-1	7729	Venezuela	no	16
VET-DEKN-1001	7730	Aneityum Isl., (New Zealand)	no	15
VET-DEKN-1003	7731	Efate Isl., (New Zealand)	no	14
VET-DEKN-1002	7732	Atiu Isl., (New Zealand)	no	13
VET-DEKN-1004	7733	Mangaia Isl., (New Zealand)	no	12
VET-GVB-001	7742	Texas, USA	no	11
VET-MJ-F1	7747	Ft Bragg, NC, USA	no	10
VET-MJ-F2	7748	Ft Bragg, NC, USA	no	9
VET-MRL-0001	7749	cv. Sunshine, Louisiana, USA	no	8
VET-MRD-0001	7750	cv. Sunshine, Wash., DC, USA	no	7
VET-MRD-0002	7751	Huffman, Cult. Wash, DC, USA	no	6
VET-RDH-0001	7767	Hong Kong, (Thailand?)	no	5
VET-RDH-0002	7768	South China Botanical Institute	no	4
VET-RGG-PA-A	7719	Panama, site A	no	3

Table 2 Eighteen accessions of *Vetiveria*, *Chrysopogon* and *Sorghum* analysed using primers: 134, 184, 212, 218, 234, 239, 244, 250, 265, 268, 327, 346 and 347

Code	Accession no.	Laboratory no.	Material, origin, collector	Fertile?
SS	VET-RPA-7655	7655	*V. zizanioides*, Haiti	N
PB	VET-RGG-PA-B	7720	*Vetiveria* spp?, Panama, Western site B (Costa Rica)	?
GR	VET-PT-1C	7713	*V. zizanioides* cv. 'Grafton', Australia, Queensland	YL
EN	VET-PT-2 A	7716	*V. elongata* (R. Br.) Stapf (narrow leaf), Australia	YF
EB	VET-PT-2B	7717	*V. elongata* (R. Br.) Stapf (broad leaf), Australia	YF
FP	VET-PT-2C	7718	*V. filipes* (Benth.) C.E.Hubb., Australia	YF
BG	VET-BANG-B001	7723	*V. zizanioides*, Bangladesh	YF
InP	VET-USDA-196257	7735	*V. zizanioides*, Simla, Punjab, India, USDA PI 196257	YF
In1	VET-K-Dtp-1	7752	*V. zizanioides*, Orissa, India	YF
In2	VET-K-Pnb-2	7753	*V. zizanioides*, Orissa, India	YF
In8	VET-K-Brk-8	7759	*V. zizanioides*, Orissa, India	YF
In10	VET-U-Nlg-10	7761	*V. zizanioides*, Orissa, India	YF
NP	VET-CWDS-01	7764	*V. zizanioides*, Kathmandu, Nepal (lowlands)	?
NG	VET-ISV-AGA-	7766	*V. nigritana* (Benth.) Stapf, Lilongwe, Malawi, Africa	YL?
CF	VET-CFP-219579	7769	*C. fulvus* (Spreng.) Chiov., Pakistan, USDA PI 219579	YF
CG	VET-CGP-383762	7771	*C. gryllus* (L.) Trin., Turkey, USDA PI 383762	YF
SH	VET-AW-01	8030	*S. halepense* (L.) Pers. Texas, USA, commercial	YF
SB	VET-RPA-8031	8031	*S. bicolour* (L.) Moench., Texas, USA, commercial	YF

Codes for fertility: N = no; Y = yes, F = fully; L = low.

PCR was performed in a volume of 15 μL containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 μM primers, 0.3 ng of genomic DNA, and 0.6 units of *Taq* DNA polymerase (Promega). A control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination.

The following 13 10-mer primers (numbers from the University of British Columbia project) were used: 134, 5'-AACACACGAG-3'; 184, 5'-CAAACGGACC-3'; 212, 5'-GCTGCGTGAC-3'; 218, 5'-CTCAGCCCAG-3'; 234, 5'-TCCACGGACG-3'; 239, 5'-CTGAAGCGGA-3'; 244, 5'-CAGCCAACCG-3'; 250, 5'-CGACAGTCCC-3'; 265, 5'-CAGCTGTTCA-3'; 268, 5'-AGGCCGCTTA-3'; 327, 5'-ATACGGCGTC-3'; 346, 5'-TAGGCGAACG-3'; 347, 5'-TTGCTTGGCG-3'. These primers gave several bright bands, did not have any false bands (in the controls) and were proven to be reproducible in replicated analyses. DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). The thermal cycle was: 94 °C (1.5 min) for initial strand separation, then 40 cycles of 38 °C (2 min), 72 °C (2 min), 91 °C (1 min). Two additional steps were used: 38 °C (2 min) and 72 °C (5 min) for final extension. Amplification products were analysed by electrophoresis on 1.5% agarose gels and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. pGEM DNA (Promega) was used as a molecular-weight marker. The RAPD bands were scored by molecular weight and assigned a code based on primer-number prefix and molecular-weight category. In addition, the RAPD band intensity was scored as: 0 = no band; 4 = faint; 5 = medium; 6 = bright band, in reference to a grey tone standard (Adams & Demeke 1993). It might be noted that analyses using simple scoring, such as 0 = absent and 1 = present, gave very similar results, except that the eigenroots were not as strongly loaded on the first few axes, implying that the information content was less than when the 0–6 scale was used. In replicated analysis, we have found that the relative band intensity is very reproducible in our laboratory. In our RAPD analyses, every primer generated at least one very bright band (level 6). Over the past several years we have screened over 250 primers and selected about 15 primers that we use routinely Any primer that did not generate at least one level 6 band (very bright) was not used in the analyses. In addition, if the PCR amplification did not result in at least one level 6 band, the sample was re-analysed in triplicate. Invariably, upon re-amplification all three re-analyses resulted in at least one level 6 band. The brightest of the triplicate samples was then re-electrophoresed with the other samples. This iterative approach results in a set of very similar amplifications for each sample. Thus, the relative intensities are preserved.

Several factors may be responsible for the presence of faint bands: single-copy DNA for faint bands vs. multiple DNA copies for bright bands; tertiary folding of DNA

with cross-bonding making the DNA less amenable to PCR amplification; and competitive interactions between bands for *Taq* enzymes and substrates during amplification.

These data were coded into a matrix of taxa by character values. Similarity measures were computed using absolute character state differences (Manhattan metric), divided by the maximum observed value for that character over all taxa (= Gower metric; Gower 1971; Adams 1975). Division by the character state range was tried and found to be less informative than using the maximum observed character value (i.e. including zero in the range). Principal coordinate analysis (PCO) of the similarity matrix follows Gower (1966) by the program PCO3D (available for PC computers from RPA).

Results and Discussion

As screening began, it became apparent that almost no genetic variation was present. The pattern obtained by primer 268 (Fig. 1) is typical of that obtained using primers 184, 239, 249, 327 and 346. Forty-one bands were obtained from these six primers. All 41 of these bands were found in each plant sample and no additional bands were found in any of the 27 accessions (Table 1), except for the accession from Malawi (lane 2, Fig. 1). We have shown in *Brassica* and *Juniperus* that individuals can readily be separated by RAPDs (Demeke *et al.* 1992; Adams *et al.* 1993). From our experiences over the past 7 years, it is clear to us that these 26 accessions (Table 1) are a single, clonal plant.

Furthermore, the principal classical usage of vetiver is for the production of oil from the roots. In order to maintain the same oil fragrance characteristics, it is a common practice to divide a clump and replant. Because vetiver was used as an essential oil plant (not for erosion control), clonal materials were distributed in many entrepreneurial schemes throughout the tropics to attempt to establish vetiver oil crops in many countries. And because vetiver is distributed (even today) by root cuttings, it appears that a single clone (which we are denoting as 'Sunshine' based on accession priority) has been planted around the world and essentially all of the vetiver used today for erosion control seems to be from the single Sunshine clone. This is very alarming when one considers the possibility of a disease or insect outbreak as we have seen in corn and potatoes.

These concerns led us to look for additional nonfertile germplasm to broaden the genetic base for erosion-control projects. A second solicitation for accessions of vetiver, related *Vetiveria* species and, presumably related, *Chrysopogon* and *Sorghum* species was made (Table 2).

Thirteen primers were run on the 18 accessions in Table 2. The RAPD analysis yielded 222 bands that were coded. Principal coordinate analysis (PCO) was run on these 18 Operational Taxonomic Units (OTUs) (Table 2). The first five eigenroots extracted 21.55, 11.22, 9.71, 7.47, and 7.00% of the variance among the 18 OTUs. The eigenroots appear to asymptote after the first five roots. The first principal coordinate separates *Sorghum* from *Vetiveria* and *Chrysopogon* (Fig. 2), supporting its taxonomic distinctiveness. The second axis separates the *V. elongata* (EB, EN), *V. filipes* (FP) and the Panama vetiver (PB) from the other vetiver accessions. The third axis separates the *Chrysopogon* taxa from all the other accessions. Notice that *C. fulvus* is most similar to *V. elongata* (0.72), whereas *C. gryllus* is most similar to *V. zizanioides* from Nepal (0.69, NP, Fig. 2). Note that the similarity between *C. fulvus* and *C. gryllus* is 0.67. The fact that these two *Chrysopogon* species are each more similar to *Vetiveria* taxa than to each other indicates that some taxonomic revision is warranted between *Chrysopogon* and *Vetiveria*.

The *Sorghum* taxa were added as an outgroup to *Vetiveria*, and that is exactly as they appear: similar to each other, but rather distant from *Vetiveria* (Fig. 2). The *Vetiveria* taxa cluster fairly tightly (similarities between 0.81 and 0.90). To examine subclustering among the *Vetiveria*, one can graph additional principal coordinates. However, because *Chrysopogon* and *Sorghum* principally accounted for coordinates 1 and 3 and part of coordinate 2, it is reasonable to remove these taxa and recompute the PCO using only the *Vetiveria* taxa.

After the *Chrysopogon* and *Sorghum* taxa were removed from the data set, PCO was performed using the remaining 14 *Vetiveria* OTUs. The first five eigenroots removed: 19.01, 12.82, 10.11, 9.34 and 8.78% of the variance among the *Vetiveria* OTUs, before appearing to asymptote. Most striking in the ordination (Fig. 3) was the distinctness of the vetiver from Panama, site B (PB). It is as dissimilar to

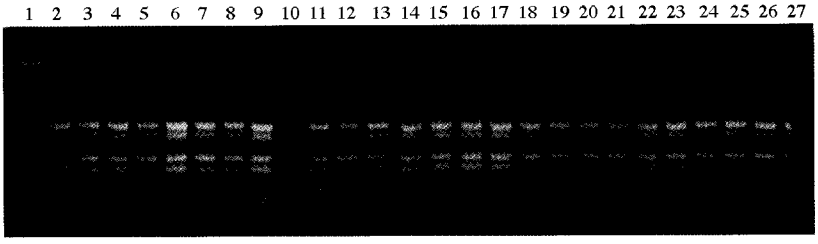

Fig. 1 RAPD banding pattern for primer 268 for vetiver accessions. Lanes: 1, pGEM markers; 2, vetiver, Malawi. Lanes 3–27 have the 'Sunshine' pattern (see Table 1 for accessions used in lanes 3–27).

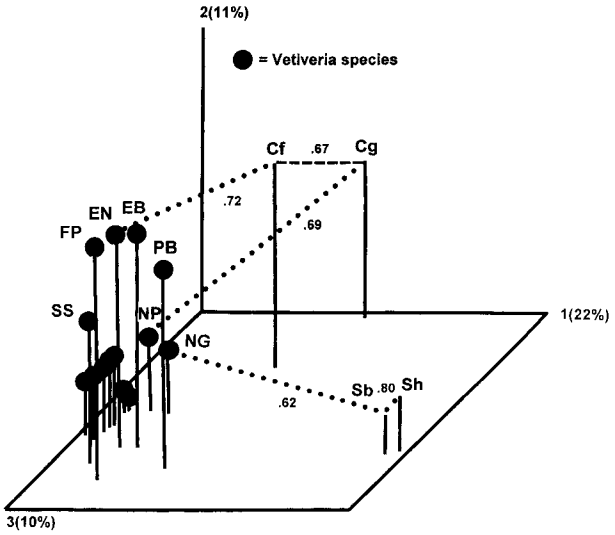

Fig. 2 Principal coordinate analysis of 14 *Vetiveria* accessions (closed circles), two *Chrysopogon* species (*C. fulvus*, Cf; *C. gryllus*, Cg) and two *Sorghum* species (*S. bicolour*, Sb; *S. halepense*, Sh) using 222 RAPD bands. Also highlighted are vetiver from Haiti (SS, cv. 'Sunshine'), Nepal (NP), Panama (PB), *V. filipes* from Australia (FP), two forms of *V. elongata* from Australia (narrow-leafed, EN; broad-leafed, EB), and putative *V. nigritana* from Malawi (NG). The unlabelled OTUs in the lower left are *V. zizanioides* from the Ganges plain. The nearest neighbour similarities of the outlying taxa (Cf, Cg, Sb, Sh) to the central cluster are indicated by the dotted lines and the decimal numbers. The similarity between the two *Chrysopogon* species (Cf, Cg) is denoted by the 0.67 above the dashed line. See the text for a discussion.

the other *V. zizanioides* (cf. SS, Fig. 3) as are the recognized species, *V. elongata* (EN, EB) and *V. filipes* (FP). As there are no recognized *Vetiveria* species native to the New World, the Panama accession may be an introduced plant from the old world, or perhaps *C. pauciflorus* (Chapm.) Vasey, which is reported from Cuba and Florida.

The vetiver OTUs from Bangladesh, India and Nepal formed a notably tight cluster. The putative *V. nigritana* from Malawi (NG) was loosely (0.81) associated with *V. zizanioides* (from northern India). Whereas, *V. zizanioides* cv. 'Grafton' (GR) from Australia was most similar to 'Sunshine' (SS), albeit at the same level of similarity as the putative *V. nigritana* is to vetiver from India (0.81).

PCO using only the nine putative *V. zizanioides* OTUs yielded eight eigenroots with no apparent asymptote. This indicates that there is little clustering among these OTUs. Ordination (Fig. 4) showed that three of the OTUs from India (In1, In2, In8) do form a tight cluster, but the other OTUs are fairly disjoint. There is some clustering of the Bangladesh (BG), India (In1, In2, In8, InP, In10), and Nepal (NP) OTUs (Fig. 4). 'Sunshine' (SS) is divergent from the main north India group, and 'Grafton' is even more divergent. It is interesting to note that, apparently, only 'Sunshine' is nonseeding, although 'Grafton' has low seed fertility (1–3%; P. Truong, personal communication). Several additional accessions had similar patterns to other OTUs (see Table 2).

Conclusions

Based on DNA fingerprinting data, it appears that almost all the vetivers cultivated outside of South Asia have been derived from a single genotype which, based on accession priority, we have designated cv. 'Sunshine'. There is a critical need to screen additional, nonfertile vetivers to uncover additional germ lines to diversify the current and future plantings of this very important 'hedge against erosion' (NRC 1993). Common garden studies are planned using the divergent vetiver accessions uncovered in this study. In addition, more nonfertile vetiver lines will be analysed to assure the genetic diversity of cultivated vetiver.

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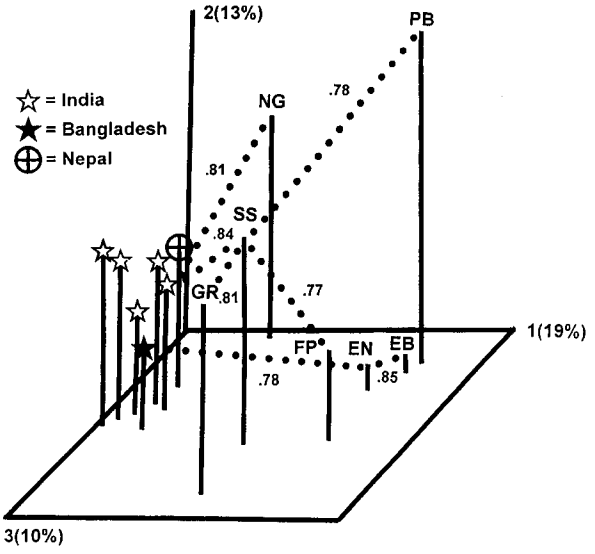

Fig. 3 Principal coordinate analysis of 14 *Vetiveria* OTUs using 197 RAPD bands. Open star, vetiver from India; closed star, vetiver from Bangladesh; crossed circle, accession from Nepal; GR, vetiver cv. 'Grafton', Australia; SS, cv. 'Sunshine' from Haiti; PB, vetiver from Panama (PB); FP, *V. filipes* from Australia; EN, EB, narrow- and broad-leafed forms of *V. elongata* from Australia; NG, putative *V. nigritana* from Malawi. The dotted lines indicate the most similar OTU to the outlying OTU, with the similarity denoted by the decimal numbers. See the text for a discussion.

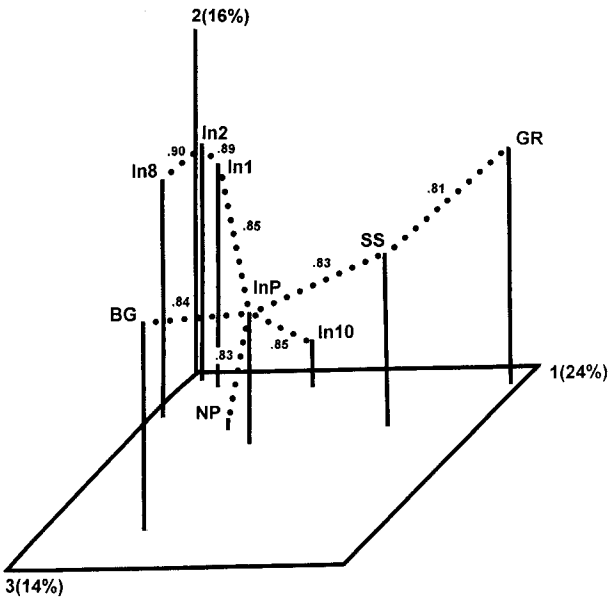

Fig. 4 Principal coordinate analysis of nine *Vetiveria zizanioides* OTUs using 197 RAPD bands. GR, cv. 'Grafton', Australia; SS, cv. 'Sunshine' from Haiti; InP, India, Punjab (USDA PI 196257); In1, In2, In8, India; NP, Nepal; BG, Bangladesh. The dotted lines indicate the most similar OTU to the outlying OTU, with the similarity denoted by the decimal numbers. See the text for a discussion.

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Dr Robert P. Adams is a Research Professor, interested in the application of RAPDs to problems of geographical variation and systematics of *Vetiveria*, *Juniperus* and other genera. Ming Zhong is a lecturer interested in the use of DNA markers for the study of species differences. Dr Yerlon Turuspekov is a populational geneticist, who has worked on geographical variation in several grass genera of Kazakstan. Mark Dafforn has been closely working with the Vetiver Network in vetiver development. Dr Jeff Veldkamp has a continuing interest in the systematics and evolution of *Vetiveria*, *Chrysopogon* and related genera.
