Study on the Genetic Diversity of Vetiver grass (*Vetiveria zizanioides*)*

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Abstract: Using the materials of 13 ecotypes of vetiver grass (*Vetiveria zizanioides*) from 8 countries, the genetic relationships of them were analyzed by means of RAPD molecular makers. The results showed that a total of 220 reproducible RAPD fragments were produced using 18 random primers. 186 fragments (84.55% of the total observed) were polymorphic, which indicated that there were very high genetic diversity and conspicuous genetic differentiation within 13 ecotypes of vetiver grass. Through the results of Neighbor-Joining（NJ）cluster analysis, 13 ecotypes of vetiver grass were mainly divided into 2 groups. One included 7 ecotypes, i.e. Sunshine, Zomba, Domesticated type, Wild type, Capitol, Lilongwe and Malaysia, which was strongly supported by bootstrap value (100%), reflecting very close relationships of these ecotypes. Except Capitol, this group shared an earlier earing trait to some extent, in which the relationships between Domesticated type and Wild type were closer, and their bootstrap value was 82%. In addition, It was recorded that Domesticated type was an introduced ecotype from India or Indonesia 50 years ago, and Wild type was a natural population distributed in Wuchuan town of Guangdong province in China. Based on the result of RAPD analysis, we know that Domesticated type and Wild type have closer relationships and nearer genetic distance (0.018). Therefore, we speculate that Wild type of China was probably derived from India or Indonesia through natural or introduced approaches.

Another group included Huffman, Parit buntar, Kandy and Karnataka, which was weakly supported by bootstrap value (58%). These ecotypes all shared the trait of lower earing rate in earing stage. Meanwhile, the NJ dendrogram also showed that Monto and Sabak beinam respectively formed different group by itself, and there was an evident genetic differentiation between these 2 ecotypes and others. However, the reasons are not clear now and the further studies should be performed afterward. It is clear that the study will be able to provide a theoretical evidence for the selection and the breeding of vetiver grass varieties with excellent characters.

Key words: *Vetiveria zizanioides*; ecotype; RAPD; genetic diversity

1 INTRODUCTION

Vetiver grass (*Vetiveria zizanioides*) is a perennial grass of Gramineae, which is originated from

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India and Africa continent (Xia *et al.*, 1998). It has strong ecological adaptability and resistance to drought, wet, cold, heat, acidity and alkalinity. Meanwhile, it has somes other good traits such as strong root system, fast growth, easy planting, high survival rate, and it never turn into a kind of weed, because it cannot be pollinated and fertilized. In the past, the utilization of the vetiver grass was limited to the extraction of the fragrant oil from the root (Cheng, 1998; Cheng and Li, 1998). With the development of study and utilization of vetiver grass, planting technology of vetiver grass is payed more and more attentions in the world, and becomes one of the most valuable eco-engineering technologies, especially in water and soil conservation. In addition, it also can be used to recover the vegetation, to purify the polluted water and eutrophication water, to improve the soil quality, and to produce the forage, or be processed into paper and the crafts (Cheng and Li, 1998). In view of low investment, short period and quick effect in planting and growth, Vetiver grass will benefit the environment, economy and society.

With its wide application over the world, vetiver grass has been introduced to different countries and areas from the center of origin. Because of the differences in environment and management, gradually, it formes different ecotypes during the planting, adaptation and domestication. In this paper, 13 ecotypes of vetiver grass from 8 countries were analyzed by means of RAPD molecular marker. The results indicated that the genetic diversity of vetiver grass was abundant, and the genetic background was complicated, which gave the basic reference for the selection and the breeding of the vetiver grass varieties.

2 MATERIALS AND METHODS

2.1 Materials

13 ecotypes of Vetiver grass were employed to the experiment, which were all provided by Dr. Hanping Xia from South China Institute of Botany, Chinese Academy of Science. The codes, ecotype names and sources can be found in Table 1.

2.2 Methods

2.2.1 Extraction, amplification and electrophoresis detection of DNA

CTAB method (Doyle and Doyle, 1987) was used to extract the total DNA from fresh and young leaves, and each template came from 5 plants with equal weight. Amplifications were performed in 20 μl reaction mixture, including 1×buffer，MgCl 2mmol/L, each of dNTP 100μmol/L, TaqDNA polymerase 1U, primers 0.5μmol/L, DNA template 30ng. The process of amplification was as follows: After an initial heat denaturation at 94^{o}C for 4 minutes, 45 cycles were followed, and each cycle included 1 min at 94^{o}C, 1 min at 36^{o}C, 1.5min at 72^{o}C. All 45 cycles were in this system. And then further extension was 7 minutes at 72^{o}C (PTC-100^{TM}, thermocycler, USA). The amplified products were separated in 1.4% agarose gels with EB 0.5μg/ml in the 1× TAB buffer system, and the electrophoresis was undertook in the condition of 4.9 v/cm for 1-1.5 hours. At the same time, Lambda DNA/Ⅲ + EcoRI Marker was used to measure the molecular weight. The results were observed and pictured under UV transmission and reflection apparatus (ZF, Shanghai, China). All chemicals were purchased from Shanghai Sangon.

Table 1 Number, ecotype name and source of experiment material

Number	Ecotype name	Source
1	Huffman	USA
2	Capitol	USA
3	Kandy	Sri Lanka
4	Karnataka	India
5	Lilongwe	Malawi
6	Malaysia	Malaysia
7	Parit buntar	Malaysia
8	Sabak bernam	Malaysia
9	Sunshine	USA
10	Zomba	Malawi
11	Domesticated	Guangdong, China
12	Wiled	Guangdong, China
13	Monto	Australia

2.2.2 Data analysis

The software of Molecular Evolutionary Genetics Analysis (MEGA2) (Kumar *et al*. 1993) was applied to analyse the data. According to working requirements of the software, the amplified products of RAPD was recorded as 'a' (if absent) or ' t' (if present) in each ecotype. Based on the p-distance coefficient model in MEGA2, the cluster analysis was made. And the bootstrap test (1000 repeats) was used to determine the reliability of the different branches in the NJ dendrogram.

3 RESULTS AND DISCUSSION

Samples, Wild and Parit were chosen to select the appropriate primer. As a result, 27 out of 47 primers could obtain effective amplification. Then the 27 primers were used to amplify the all 13 samples. The better results were acquired in 18 primers with polymorphism (Appendix 1: the amplified results of S101, S102, S135 and S137). The number of amplified bands varied from 6 to 17 within different materials or different peimers, and percentage of polymorphic fragments varied from 37.50% to 100% among different primers (Table 2). In conclusion, all 18 primers produced 220 reproducible fragments, in which 186 ones were polymorphic, and the polymorphic percengtage was 84.55%. All these results indicated that different ecotypes of vetiver grass had high diversity and great genetic differentiation since they were planted and cultivated in different countries in the past years.

Table 2 Primers, sequences and amplified result

Primers	Sequences(5`-3`)	Number of amplified bands	Number of polymorphic bands
S101	GGTCGGAGAA	16	16(100%)
S104	GGAAGTCGCC	14	12(85.71%)
S107	CTGCATCGTG	14	13(92.86%)
S109	TGTAGCTGGG	10	7(70.00%)
S115	AATGGCGCAG	12	10(83.33%)
S117	CACTCTCCTC	11	10(90.91%)
S119	CTGACCAGCC	15	14(93.33%)
S122	GAGGATCCCT	14	11(78.57%)
S125	CCGAATTCCC	8	3(37.50%)
S127	CCGATATCCC	12	11(91.67%)
S129	CCAAGCTTCC	13	9(69.23%)
S134	TGCTGCAGGT	6	4(66.67%)
S135	CCAGTACTCC	13	11(84.62%)
S137	AACCCGGGAA	9	9(100%)
S141	CCCAAGGTCC	17	15(88.24%)
S142	GGTGCGGGAA	12	10(83.33%)
S145	TCAGGGAGGT	10	9(90.00%)
S148	TCACCACGGT	14	12(85.71%)
Total		220	186(84.55%)

Table 3 Matrix of genetic distance coefficient based on p-distance model

	1	2	3	4	5	6	7	8	9	10	11	12
2	0.318											
3	0.345	0.282										
4	0.350	0.314	0.041									
5	0.336	0.055	0.255	0.286								
6	0.327	0.064	0.273	0.305	0.027							
7	0.323	0.368	0.286	0.291	0.341	0.350						
8	0.455	0.391	0.409	0.405	0.373	0.382	0.459					
9	0.359	0.059	0.305	0.327	0.059	0.068	0.373	0.368				
10	0.355	0.055	0.300	0.323	0.055	0.064	0.368	0.373	0.005			
11	0.364	0.082	0.318	0.332	0.082	0.091	0.386	0.382	0.032	0.027		
12	0.355	0.064	0.309	0.323	0.064	0.073	0.377	0.382	0.014	0.009	0.018	
13	0.400	0.373	0.427	0.423	0.373	0.364	0.377	0.436	0.368	0.364	0.355	0.355

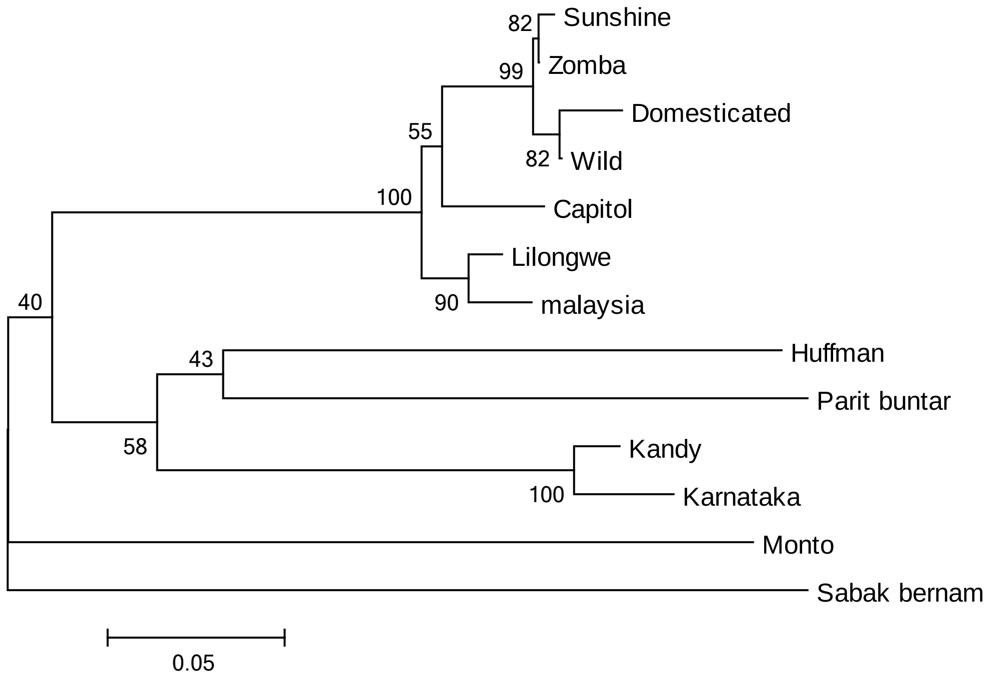

Fig. 1 NJ dendrogram based on p-distance model of MEGA2. The numbers in figure represent bootstrap values

According to the NJ dendrogram (Fig. 1), 13 ecotypes of vetiver grass were mainly divided into 2 groups. One group included Sunshine (USA), Zomba (Malawi), Domesticated type (Guangdong, China), Wild type (Guangdong, China), Capitol (USA), Lilongwe (Malawi) and Malaysia (Malaysia), which was strongly supported by bootstrap value (100%), indicating that these 7 ecotypes had lower genetic differentiation and closer relationships. From biological feature, this group shared an earlier earing trait to some extent except Capitol (Xia and Liu, 2003), so Capitol constituted a branch by itself in this group. As for as Domesticated type and Wild type were concerned, the genetic distance (Table 3) between them was 0.018, which indicated that they had a closer genetic relationship. As a sub-branch, the bootstrap value was 82%. It was recorded that Domesticated type was an introduced ecotype frome India or Indonesia 50 years ago, and Wild type was a natural population distributed in Wuchuan town of Guangdong province in China (Xia and Liu, 2003). Based on the closer genetic relationships between them, we speculate that the Wild type of China was probably derived from India or Indonesia through natural or introduced approaches. Lilongwe and Malaysia came from Malawi and Malaysia respectively, but the relationships between them were close, and commonly formed a sub-branch, strongly supported by bootstrap value 90%. Moreover, they had a similar biological characteristic that was they did not appear as the frozen and wilted phenomenon on the leaf tips in winter in Guangzhou (Xia and Liu, 2003). In addition, the NJ dendrogram showed that the 2 ecotypes from Malawi assembled together, which indicated that there was not too much genetic differentiation in the process of planting and management, and the relationships were closer between them.

Another major group included Huffman (USA), Parit buntar (Malaysia), Kandy (Sri Lanka) and Karnataka (India). This group was weakly supported by bootstrap value (58%). The 4 ecotypes all shared the trait of lower earing rate in earing stage (Xia and Liu, 2003). For the sub-branch formed by Huffman and Parit buntar, the bootstrap value was 43%, and the genetic distance of them was 0.323. For the sub-branch formed by Kandy and Karnatake, the bootstrap value was 100% and the genetic distance was 0.041. Kandy and Karnatake came from Sri Lanka and India respectively. The two countries are adjacent in geographical place, and both have the alike tropical climate.

Monto and Sabak beinam were two special ecotypes outside above two magor groups in the NJ dendrogram, which came from Australia and Malaysia respectively. They had a distinct genetic differentiation from the other 11 ecotypes. The reasons are not clear now and the further studies should be performed afterward. As for Monto, it has some fine traits and extensive ecological adaptabilities so that it can adapt the extreme climate of semiarid region, as well as the temperate and wet climatic zone in Australia (Truong, 1998). Maybe the biological and ecological traits are the reason resulting in the grater genetic differentiation between Monto and other ecotypes.

The main distribution areas of vetiver grass were India, Sri Lanka, Burma, Indonesia, Fiji, Brazil, South China and so on, and they are all tropical and subtropical climate (He *et al*, 1998), indicating the vetiver grass is a kind of warm season grass. However, under the pressure of artificial selection, its genetic basis was changed, and the genetic differentiation took place so that some ecotypes could be cultivated in the temperate areas such as Maryland of America, Henan, Shandong in China (Xia and Liu, 2003; Cheng, 1998). 2 out of 3 ecotypes from USA fell in one group, and the other entered another group. The main reason may be as followers: 1) USA is not a natural distribution area of vetiver grass, and the 3 ecotypes originally had different genetic backgroud when they were introduced. 2) The 3 ecotypes had the similar genetic basis, but they genetic variations gradually occurred under the different conditions of climats, environments, cultivations and managements after they were introduced. So the 3 ecotypes from USA fell in different groups. The three ecotypes from Malaysia were separated in the NJ dendrogram. But the climate of Malaysia is single climate of tropical rain forest, so the ecotypes should have the closer relationships rather than high genetic differentiation. As a rule, the distribution center of species also is the original center, and the species diversity and genetic diversity are abundant too. Therefore, we guess that Malaysia may be a natural distribution area. Otherwise, the 3 ecotypes should go through introduction many times in other countries and areas before they came in Malaysia.

To sum up, the level and the structure of genetic diversity of plant have very close relations with the biological trait, the environment and the course of origin and evolution (Li, *et al*. 2003). The studies of plant genetic diversity need not only to analyze the effects of modern environment climate, but also to consider the profound effects of original environment on the plant. At the same time, the pressure of artifical selection also has some important influences on the genetic diversity of plant.

4 CONCLUSION

Recently, the global situation for the loss of the water and soil is becoming more and more serious, and the water from the daily life and the agricultural and industral production is also aggragating the eutrophication of water rapidly. The functions of vetiver grass are being studied widely, especially in the water and soil conservation and the water purification.

4.1 Vetiver grasses were introduced to many countries several centuries ago. Now different ecotypes havd been formed during cultivation and adaptation in the past years, and the evident genetic differertiaion happened within these ecotypes. The results showed that the genetic differertiaion of them was great at the DNA level and the percentage of polymorphic fragment could reach 84.55%, which were consistent with the study on selection of excellent ecotypes of *Vetiveria zizanioides* (Xia and Liu, 2003).

4.2 In the NJ dendrogram, Sunshine, Zomba, Domesticated type, Wild type, Capitol, Lilongwe and Malaysia were clustered into one group, which was strongly supported by bootstrap value 100%, indicating that they had close relationships. Huffman, Parit buntar, Kandy and Karnataka constituted another group supported by bootstrap value 58%, also showing the closer relationships of them. But Monto and Sabak beinam had conspicuous genetic differentiation from other 11 ecotypes. The study determined the genetic relationships and the genetic differentiation within 13 ecotypes by means of RAPD molecular makers, which provided the basis for the selection and breeding of vetiver grass.

4.3 The results of NJ cluster analysis showed that Domesticated type and Wild type had closer relationships. Therefore, we speculate that Wild type of China was probably derived from India or Indonesia through natural or introduced approaches.

4.4 The three ecotypes from Malaysia were separated in the NJ dendrogam. So, we guess that Malaysia may be a natural distribution area or vetiver grass was repeatedly introduced in many other countries and areas before it came in Malaysia.

5 ACKNOWLEDGMENTS

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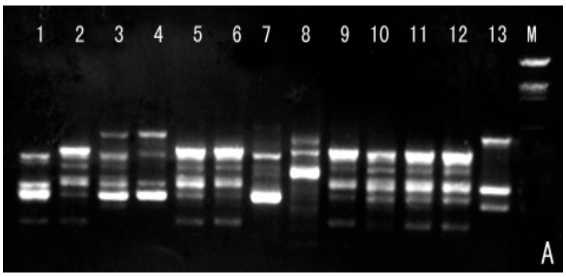
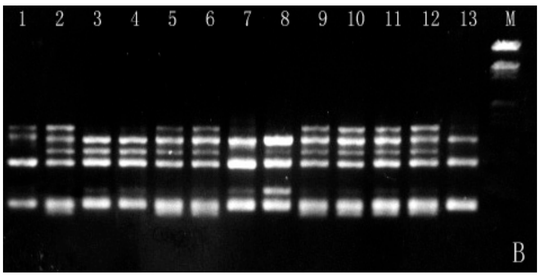
A Brief Introduction to the First Author

Zhaoxia Dong, a master, graduated College of Animal Science and Technology, Nanjing Agricultural University in 2002. Speciality in the period of master was pratacultural science. She is working at the South Pratacultural Center, South China Agricultural University. She mainly engaged in physiological study of forage and turf grass and in technique of RAPD molecular markers. **Email contact:** Zhaoxia Dong <dongzhaoxia106@163.com>

Appendix 1





DNA polymorphisms generated by primer S101, S125, S135 and S137 (A: S101, B: S125, C: S135, D: S137)

Statement of Merit:

So far, there has been a lack of wetlands treating oil-refined wastewater in China. This paper investigated the growth and tillering performance of 4 herbaceous plants in constructed wetlands filled with oil-refined wastewater, and the effects of the constructed wetlands in treating wastewater. The results indicated that the purifying rates of constructed wetlands for oil-refined wastewater were high. The efficiency of wetlands in removing the pollutants was always in order of ammonia N > oil > BOD > COD, but the net removal from plants was ranked as COD > BOD > oil and ammonia N. During the period of clean water cultivation, the new tiller-producing rate of *V. zizanioides* was the lowest among the four species, but it gradually rose during the period of waste water treatment, while the tiller-producing rates of the other three species were distinctly lowered. It was therefore suggested that *V. zizanioides* might have a stronger adaptation to the harsh environment than other tested species, especially in the situation of long time of adaptation to the harsh environment. At present, the further application and verification is ongoing; furthermore the preliminary application result has already shown that the performance of vetiver is becoming better and better, and seems to be far better than that of the other three species. It is therefore affirmative that vetiver is a good species for constructed wetlands to treat oil-refined wastewater, better than the 3 hydrophytes, *Phragmites australis*, *Typha latifolia*, and *Lepironia articutala*.