Genetic Variation of *Zoysia* as Revealed by Random Amplified Polymorphic DNA (RAPD) and Isozyme Pattern

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**Abstract**: One hundred and thirty one clones of *Zoysia* spp. collected from 59 sites on the coasts of Taiwan and its neighboring islets were analyzed using both random amplified polymorphic DNA (RAPD) and isozymes to reveal their genetic variation. In the RAPD analysis with 12 primers, a total of 92 polymorphic bands which could distinguish 131 genotypes were found. There were 19 polymorphic bands and 81 zymogram patterns for esterase, and 9 bands and 10 patterns for acid phosphatase, respectively. Cluster analysis by the unweighted pair-group method with arithmetic means (UPGMA) of RAPD data indicated that clones collected from the same geographic region were clustered together. However, isozyme data showed discordant patterns. Interestingly, both RAPD data and isozyme fingerprinting revealed less correlation with the intuitive taxonomic classification of tested clones, but more related to the specific adaptation to the geographic or geologic aspects of their habitats.

**Keywords**: Genetic variation, Geographic distribution, Isozyme, RAPD, *Zoysia*.

*Zoysia* spp., perennial C\(_4\) grass with well-developed stolon and short culm, can form a dense lawn, and very good turf grass. They are found across China, Japan, and Tropical Asia to New Zealand. Previously we found that *Zoysia* spp. were commonly found in various environments of littoral regions in Taiwan, and showed a great variation in morphology, isozyme pattern and salt tolerance (Weng et al., 1995; Weng and Chen, 2001; Weng, 2002). For example, clones collected from rocky seashores had low-lying leaves and short (< 2.4 cm) internodes, and those from sandy seashores have erect leaves and long (> 6.5 cm) internodes, possibly to avoid being covered with sand (Weng et al., 1995). These morphological characteristics were retained even when they were potted in this work. Furthermore, clones collected from regions with high annual precipitation usually showed lower salinity tolerance than those from the regions with low precipitation. However, for the same level of rainfall, clones collected from the northeastern coast of Taiwan (EN region) showed lower salinity tolerance than those collected from other regions (Weng and Chen, 2001).

Previously, Weng (2002) found that *Zoysia* clones collected from the EN region showed a lower genetic diversity than and higher genetic distance to those from other regions, as revealed by esterase and acid phosphatase zymograms. This genetic differentiation in *Zoysia* spp., as examined by isozyme analysis, was not well related to their taxonomic status (Weng, 2002). Isozyme patterns have been suggested to link with some characteristics of plants, such as stress tolerance (Foolad and Jones, 1993; Basu et al., 1997; Gangopadhyay et al., 1997), disease resistance (Tanksley and Rick, 1980), and high photosynthetic ability (Weng and Chen, 1989). Since adding CaCl\(_2\) to the nutrient solution decreased the damage of *Zoysia* clones caused by NaCl (Weng and Chen, 2001), *Zoysia* clones collected from the EN region, which mainly consists of limestone, might have developed specific genetic adaptation to salinity and calcium (Weng, 2002). However, the genetic aspect of *Zoysia* spp., growing in Taiwan, is yet unclear.

Isozymes and DNA-based markers are frequently used as molecular markers for taxonomic and systematic analyses of plants (Bartish et al., 2000; Bessega et al., 2000; Garkava et al., 2000; Matos et al., 2001; Ochiai et al., 2001). Many researchers (Garkava et al., 2000; Matos et al., 2001; Ochiai et al., 2001) have pointed out that DNA-based markers, such as random amplified polymorphic DNA (RAPD), were superior to isozymes in detecting genetic diversity. The isozymes represent allelic expression of the same locus, but fragments produced by RAPD are independent genetic markers (Ochiai et al., 2001) with a lower proportion of non-neutral markers than the former (Bartish et al., 2000). Therefore, RAPD and isozyme analyses often give discordant patterns suggesting the importance of using multiple molecular marker systems in studies of population structure (Wendel and Doyle, 1998; Bartish et al., 2000; Lebot et al., 2003).

In this study, 131 clones of *Zoysia* spp. collected from the coastal regions of Taiwan and its neighboring

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islets were examined by RAPD and isozyme analysis. The results of these analyses were used to delineate the relationships of *Zoysia* spp., with geographical distribution, morphological characteristics and salinity tolerance reported previously (Weng et al., 1995; Weng and Chen, 2001; Weng, 2002).

**Materials and Methods**

1. **Plant materials**

In 1993-1994, 131 clones of *Zoysia* were collected from 59 sites on the coasts of Taiwan, Penghu Islands (located in Taiwan Strait), and Lanyu (a small island located in the Pacific Ocean) (Fig. 1). Most clones collected from the western coast have longer (>4 mm) spikelets, and are classified as *Z. sinica* according to Flora of Taiwan (Hsu et al., 2000). On the contrary, most clones collected from the northern and eastern coasts as well as from Penghu Island have shorter (<4 mm) spikelets and are classified as *Z. matrella*. Only one clone, collected from Lanyu islet, with short spikelets and narrow leaf blades, was classified as *Z. tenuifolia*.

Collected rhizomes were transplanted in pots (15 cm in diameter, 13 cm in depth) filled with loam and sand (1:1), and placed outdoors on the campus of the National Chung-Hsing University, Taichung, Taiwan (24°10’ N, 78 m).

2. **RAPD analysis**

DNA was extracted from about 500 mg of fresh young leaves from each *Zoysia* clone according to the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1990). Leaves were ground with sea sand and liquid nitrogen and extracted with 1.5 ml of CTAB extraction buffer. DNA precipitated with isopropanol was washed with 76% ethanol-washing solution and dissolved in TE buffer. The resulting DNA extract was quantified using a spectrophotometer (U-2000, Hitachi, Japan), and diluted to 5 ng µl⁻¹.

Each 25 µl of PCR reaction volume contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.1% (w/v) gelatin, 1% Triton X-100, 200 µM dNTP, 0.2 µM primer, 1 U of Taq Polymerase (Promega) and 25 ng of genomic DNA. Amplification was performed.
in a thermocycler (FGEN05TP, Techne, UK) for 40 cycles after initial denaturation at 94°C for 2 min. Each cycle consisted of 94°C for 30 s, 34°C for 1 min and 72°C for 30 s. This was followed by a 10-min final extension at 72°C. Amplification products were resolved on a 2% agarose gel in 0.5X TBE buffer and stained with ethidium bromide solution.

Among 200 deca-nucleotide primers from kits A-J of Operon Technology (USA) screened for the suitability for RAPD amplification, 12 (A1-A5, A9, A13, A18, D2, D7, F14 and G5) with polymorphic bands were selected (Table 1).

3. Isozyme analysis

The analyses of esterase and acid phosphatase from leaves have been reported previously (Weng, 2002). The esterase zymogram was determined by a vertical slab polyacrylamide gel electrophoresis (AE 6220, ATTO, Japan). The final acrylamide concentration in the resolving gel was 7.5%, and sucrose was added at 18%. Gel preparation and buffers (high pH discontinuous) were as described by Hames and Rickwood (1981), except that the Tris-HCl solution for the resolving gel was pH 7.1, instead of 8.8. The developed gel was stained with Fast Blue RR salt and α-naphthyl acetate in phosphate buffer (pH 6) (Wetter and Dyck, 1983). Acid phosphatase zymogram was resolved by the same method as that for the esterase zymogram, except that the developed gel was stained with Fast Garnet GBC salt and α-naphthyl acid phosphate in acetate buffer (pH 5.5).

4. Data analysis

The presence or absence of high intensity and highly reproducible polymorphic RAPD and isozyme bands was used as the character to elucidate variations of the clones (Garkava et al., 2000; Ochiai et al., 2001).

The genetic distance between two clones was calculated according to the formula proposed by Excoffier et al. (1992): 

\[ D = N(1 - (N_{ii}/N)) \]

where N is the total number of bands and N_{ii} is the number of bands shared by two samples. The resulting distance matrix among clones was used for cluster analysis by an unweighted pair-group method with arithmetic means (UPGMA cluster analysis) (Sneath and Sokal, 1973) using NTSSYS-PC software (version 1.8, Rohlf, 1993).

The resulting distance matrix was also used for an analysis of molecular variance (AMOVA; Excoffier et al., 1992). In AMOVA analysis the sources of variation were divided into 2 nested levels: among group and among individuals (clones) within group. Mean squared deviation (MSD) was calculated by dividing sum of squared deviation (SSD) by the degrees of freedom (df). The variance components thus obtained were then expressed as percentages. The significance of each variance component was tested by the method of 1000 random permutations.

| Table 1. Nucleotide sequences of 12 selected primers and the number of polymorphic bands detected by RAPD analysis (A), and by isozyme analysis (B) of Zoysia clones. |
|---|
| **(A) Primer** | **Sequence (5’ to 3’)** | **No. of bands polymorphic** |
| OPA-01 | CAGGCCCTTC | 9 |
| OPA-02 | TGCCGAGCTG | 6 |
| OPA-03 | AATCCGGGCTTG | 8 |
| OPA-04 | AGGGGCTCTG | 8 |
| OPA-09 | GGGTAACGCC | 5 |
| OPA-13 | CAGCACCCAC | 7 |
| OPA-18 | AGGTGACGCT | 11 |
| OPD-02 | GACCCCAACC | 8 |
| OPD-07 | TTGGCAGGGG | 6 |
| OPF-14 | TCTGTCAGGT | 8 |
| OPG-05 | CTGAGACGGA | 11 |
| **(B) Isozyme** | **No. of bands polymorphic** |
| Esterase | 19 |
| Acid phosphatases | 9 |

Results

In the RAPD analysis, a total of 154 bands were recorded. Among them, 92 bands were high-intensity and highly reproducible polymorphic bands (Table 1), which showed 131 patterns (genotypes) in all 131 tested clones. On the contrary, a total of 26 bands in esterase zymogram and 9 bands in acid phosphatase zymogram were found. There were 19 high-intensity and highly reproducible polymorphic bands and 81 patterns in esterase zymogram, and for acid phosphatase zymograms there were 9 bands and 10 patterns.

Fig. 2 shows the result of UPGMA cluster analysis of RAPD data, based on genetic distance matrix (data not shown) among clones. Clones collected from the same geographic region formed a distinct cluster. At a distance value of 0.95, tested clones were divided into 3 main clusters (groups). Those were D1 group: collected from northern (clone No. 101-134) and eastern (No. 201-238) coasts, D2 group: collected from western coast (No. 301-325) and Penghu Island (No. 401-428), and D3 group: collected from southern part of eastern coast and Lanyu Islet (No. 239-244) (Fig. 1, Fig. 2 and Table 2). D1 cluster, at a distance value of 0.9, could be divided into 2 sub-clusters, i.e., D1a group: collected from northern coast and northern part of east coast (No. 101-203), and D1b group: collected from central to southern part of eastern coast (No. 204-238) (Fig. 1, Fig. 2 and Table 2). Based on UPGMA cluster analysis of isozymes (esterase + acid phosphatase) tested clones were divided into
3 main clusters (groups) (Fig. 3), i.e., I1 group: 47%, 25%, 68% and 54% of clones collected from northern coast, southern part of eastern coast, western coast and Penghu Island, respectively. I2 group: all clones collected from the northern part of the eastern coast (No. 201-220), one clone collected from the southern part of the eastern coast (No. 221), and 27% and 57% of the clones collected from the northern coast and southern part of eastern coast, respectively, and I3 group: 27%, 18%, 32% and 46% of clones collected from the northern coast, southern part of eastern coast, western coast and Penghu Island, respectively (Fig. 1, Fig. 3 and Table 2).

Table 3 shows the results of ANOVA based on RAPD analysis. Only 26.6% of the total variation was attributable to differences among the three groups, and 73.4% was accounted for by the differences among clones within each group.

Discussion

UPGMA cluster analysis of RAPD data indicated that clones collected from the same geographic region were clustered together (Fig. 2 and Table 2). However, this result is inconsistent with the morphological classification of Zoysia in conventional taxonomy. The genetic distance based on RAPD data indicated that the clones from Penhu islands (No. 401-428) were closely related to those from the western coast (No. 301-325), but estranged from those from the northern (clone No. 101-134) and eastern (No. 201-238) coasts (Fig. 2 and Table 2). Although Z. sinica is widely distributed in western coast, it is rarely spotted in northern coast, eastern coast and Penghu islands, where Z. matrella is the major species (Weng et al., 1995). The same tendency was also found in clones from the southeastern coast and Lanyu Islet (No. 239-244): among the six clones collected from these
sites, No. 243 with long spikelets (near 5 mm) is *Z. sinica*. On the contrary, No. 240-242 with short spikelets (< 2 mm), are *Z. matrella*, while No. 244 with short spikelets and narrow leaf blade is *Z. tenuifolia*. These clones still showed close genetic distance (< 0.69, Fig. 2), in spite of being classified into different species in conventional taxonomy.

Why is the result of UPGMA cluster analysis using RAPD data inconsistent with the classification by traditional taxonomy? The specific adaptation and high inter-species fertility between *Zoysia* spp. (Hong and Yean, 1985; Weng, unpublished data) might be the major factors. Different from other regions of Taiwan, the habitat of the six clones No. 239-244 is near the tropical coral reef areas, and these clones (grouped as D3, Fig. 2) showed a higher genetic distance (1.36) from the other two groups (D1 and D2). Gene flow seems to occur among species to adapt to a specific habitat. In addition, the spikelet length is a major morphological characteristic for the classification of *Zoysia* spp. (Hsu et al., 2000). The environment and non-neutral gene(s), which affect the expression of the spikelet length, probably are the other intrinsic factors for the inconsistency with the morphological classification. The spikelet length seems to relate to the propagation of *Zoysia* in different types of seashores. Because the western coast of Taiwan has sandy seashores, the seeds are covered easily by sand. Genotypes with large seeds (classified as *Z. sinica*) have the advantage of being capable of germinating from deeper soil (sand). On the contrary, the northern and eastern coasts as well as Penghu Islands consist mainly of rocky seashores; genotypes with a large number of smaller seeds (classification as *Z. matrella*) have better chance for propagation. Therefore, in spite of the fact that *Zoysia* clones from Penghu islands (No. 401-428) and from western coast (No. 301-325) have been classified into two individual species based on the morphological characteristics, gene flow might account for the short genetic distance observed in the molecular data.

The clustering of UPGMA based on RAPD distance (Fig. 2) is consistent with the geographical distribution of *Zoysia* clones. However, this congruence was not observed in the isozyme clustering analysis (Fig. 3 and Table 2). Clones collected from the same region or site are always divided into different groups. Discordance in genetic distance measured with two molecular

![Genealogy](image.png)

**Fig. 3.** The result of UPGMA cluster analysis of the combined data on esterase and acid phosphatase in *Zoysia* clones based on genetic distance matrix.

| Variance component | df  | SSD    | MSD    | Variance | %Total |
|--------------------|-----|--------|--------|----------|--------|
| Among group        | 2   | 409.10 | 204.55 | 5.44     | 26.58  |
| Within group       | 128 | 1923.36| 15.03  | 15.03    | 73.42  |

Table 3. The results of analysis of molecular variance (AMOVA) of *Zoysia* clones based on the RAPD analysis.
markers has been reported (Wendel and Doyle, 1998; Bartish et al., 2000; Lebot et al., 2003). It might be because the isozyme represents allelic expression of the same locus, and shows a higher proportion of non-neutral markers; while the fragments produced by a RAPD primer are independent genetic markers (Bartish et al., 2000; Ochiai et al., 2001).

In addition, the present study also showed that all clones collected from the EN region (clone No. 201-220) were placed in the same group (I2) in the UPGMA cluster analysis using isozyme data (Fig. 3 and Table 2). The same tendency was observed in our previous paper (Weng, 2002), in which the collection sites for Zoysia clones were divided into six regions according to the topography and climate, and clones collected from EN region showed a lower genetic diversity and longer genetic distance from those from other regions. This phenomenon might be due to the salinity tolerance of these clones and their specific adaptation to the geology (limestone) of their habitats (Weng, 2002).

The results of the present study have indicated that the genetic distance among Zoysia clones based on RAPD analysis was closely related to their geographic distribution. However, the isozyme analysis showed a discordant pattern, which was more related to the salinity tolerance and the specific adaptation to the geology (limestone) of their habitats. Furthermore, both RAPD and isozyme fingerprinting revealed less correlation with the intuitive taxonomic classification of the tested clones.

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