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Allotetraploidy of *Zoysia* species with 2n = 40 based on a RFLP genetic map

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Abstract A RFLP linkage map of *Zoysia* spp. (2n = 40), a warm-season turfgrass, was constructed by using the self-pollinated progenies obtained from an interspecific hybrid. Out of 115 DNA clones tested, 100 (87.0%), including 55 genomic clones, 38 cDNA clones, and seven gene clones encoding photosynthetic enzymes showed allelic-RFLP banding patterns among the parental accessions. We found that 26 probes detected two or more loci segregating in the self-pollinated progenies independently. The RFLP linkage map of *Zoysia* spp. consists of 115 loci in 22 linkage groups ranging in size from 12.5 cM to 141.3 cM with a total map distance of 1506 cM. Six RFLP loci (5.4%) showed significant segregation distortion ($P < 0.01$). Two loci out of six were mapped to linkage group II, and another two loci were mapped to group VII. In the RFLP linkage map of zoysiagrass, five pairs of linkage groups sharing a series of duplicated loci with approximately the same order were identified. Therefore, we conclude that *Zoysia* spp. with 2n = 40 should be considered as allotetraploids, which might have evolved from progenitors with a basic chromosome number of ten ($x = 10$).

Key words Zoysiagrass· Turfgrasses · RFLP map · Allotetraploid

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Introduction

Zoysia species are one of the native grasses distributed along sea coasts and in natural grasslands of Pacific Rim countries (Shoji 1983) and have been popularly used as a turf in gardens in Japan since the 1700s (Nakamura 1980). *Zoysia* spp. are also utilized in sports fields because of their stoloniferious habit. With increasing interest in recreational activities, there is a demand for the development of new varieties possessing fine winter color-retention and tolerance to the diseases caused by soil-borne pathogens. Wide variation in desirable traits, including cold–tolerance (Matumura et al. 1990), the growth speed of stolons (Ikeda and Hoshino 1978), and photosynthetic ability at low temperature (Okawara and Kaneko 1997) among and within *Zoysia* species, will facilitate the development of new varieties.

As for genetic studies of *Zoysia* spp., Forbes (1952) reported the chromosome constitution of three species of *Zoysia*, i.e. *Z. japonica* Steud., *Z. matrella* Merr., and *Z. tenuifolia* Willd., to be 2n = 40 without any irregularities of meiosis. He also suggested genetic similarities between these species by producing interspecific hybrids among all combinations of them. Interspecific hybrids of *Zoysia* were identified in natural populations distributed in Japan, based on both morphological characteristics (Fukuoka 1989) and DNA markers (Yaneshita et al. 1997). Kitamura (1970) pointed out that *Zoysia* spp. growing in Japanese islands have the same chromosome number (2n = 40) with no variation of chromosome constitution, and that their morphological characteristics varied continuously within the genera. These data suggest that gene introduction by interspecific hybridization would be an efficient

procedure for the improvement of zoysiagrass. However, no conventional genetic markers of *Zoysia* spp. have been established, because the genetic information obtained from cytogenetical studies is restricted to karyotypes.

In the present study, we constructed a basic RFLP linkage map of zoysiagrass by using self-pollinated progenies obtained from an interspecific hybrid with the aim of establishing informative genetic markers for the improvement of zoysiagrass. Additionally, the genomic constitution of zoysiagrass revealed from the RFLP linkage map is discussed in this paper.

Materials and methods

Plant material

Three accessions of *Z. japonica*, JTZJ-1, JTZJ-17 and JTZJ-24, one accession (JTZM-1) of *Z. matrella*, and an interspecific hybrid (JTZJ-13) of zoysiagrass used as the parent of the mapping population, were surveyed for their RFLP. The genetic characteristics of these accessions were described in Yaneshita et al. (1997), i.e. JTZJ-1 for accession no. 2, JTZJ-13 for accession no. 7, JTZJ-17 for accession no. 6, JTZJ-24 for accession no. 1, and JTZM-1 for accession no. 9. One-hundred-and-five self-pollinated progenies of JTZJ-13 were employed in the linkage analysis.

DNA extraction, restriction digestion, and hybridization

Total DNA was extracted from mature leaves of each plant by the CTAB method (Murray and Thompson 1980). About 5 μg of DNA per plant, digested with restriction enzymes, was electrophoresed in 0.85% agarose gels with 1 × TAE buffer. Restriction fragments were transferred onto a nylon membrane, Hybond N^+ (Amersham Ltd.), following Southern (1975). The labelling of probe DNAs and the detection of hybridized fragments were both conducted with an ECL system (Amersham Ltd.) according to the supplier's protocol.

DNA clones

*Pst*I-digests (ZG clone), *Taq*I-digests (ZGT clone), and *Sau*3AI-digests (ZGS clone) from *Z. japonica* JTZJ-24 were cloned into the pUC19 vector and used as DNA probes. Complementary DNA clones were constructed with mRNAs extracted from mature leaves (ZCL clone) and rhizomes (ZCS clone) of *Z. japonica* JTZJ-24. Seven genes isolated from JTZJ-24 [actin, light-harvesting chlorophyll a/b-binding protein (LHC), the small subunit of riblose-biphosphate carboxylase (SSU), phenylalanine ammonia-lyase (PAL), phosphoenolpyruvate carboxykinase (PCK), phosphoenolpyruvate carboxylase (PEPC), and pyruvate, orthophosphate dikinase (PPDK)] were also used as probes.

RFLP mapping

In order to survey suitable combinations between probes and restriction enzymes for the detection of allelic RFLP patterns in the interspecific hybrids, we hybridized genomic clones and cDNA clones with membrane filters containing the DNA of five zoysiagrass accessions digested with *Dra*I, *Eco*RV, *Hin*dIII, and *Xba*I. In this experiment, the allelic-band pattern involves two different hybridized fragments, one of which is common to the three accessions of *Z. japonica* while the other is detected specifically in the *Z. matrella* accession. Combinations of DNA clones and restriction enzymes showing the allelic patterns were applied to the RFLP analysis of self-pollinated progenies. Genotypes of the individuals were identified by comparing their patterns to those of accessions used for the survey of DNA clones. Individuals possessing hybridized fragments shared by *Z. japonica* accessions were identified as 'J', those shared by *Z. matrella* as 'M', and others with both fragments as 'H'. Duplicated loci detected with the common probes were distinguished by a suffix (a, b, or c) depending on the molecular size of hybridized fragments and the intensity of hybridization signals. The linkage analysis among RFLP loci was carried out with MAPMAKER ver.3 computer software (Lander et al. 1987). Linkages were determined with a recombination value of 0.3 and a LOD score of 3.0.

Results

RFLP probes

DNA clones detecting allelic-banding patterns in the parental accessions were selected as RFLP probes. Of the total of 115 clones tested, 87.0% of them (100 clones) showed allelic variation with at least one of the four restriction enzymes employed (Table 1). All

Table 1 Number of DNA clones detecting polymorphism in the interspecific hybrid

Source	Tested	Restriction enzyme				Any enzyme
		*Hin*dIII	*Xba*I	*Dra*I	*Eco*RV	
Genomic	66	23 (34.8%)[a]	37 (56.1%)	21 (31.8%)	31 (47.0%)	55 (83.3%)
cDNA						
ZCL	20	9 (45.0%)	13 (65.0%)	12 (60.0%)	12 (60.0%)	17 (85.0%)
ZCS	22	13 (59.1%)	12 (54.5%)	10 (45.5%)	10 (45.5%)	21 (95.5%)
Gene	7	7 (100%)	3 42.9%)	4 (57.1%)	3 (42.9%)	7 (100%)
Total	115	52 (45.2%)	65 (56.5%)	47 (40.9%)	56 (48.7%)	100 (87.0%)

[a] Numbers in parantheses indicate the frequencies (%) in the tested clones

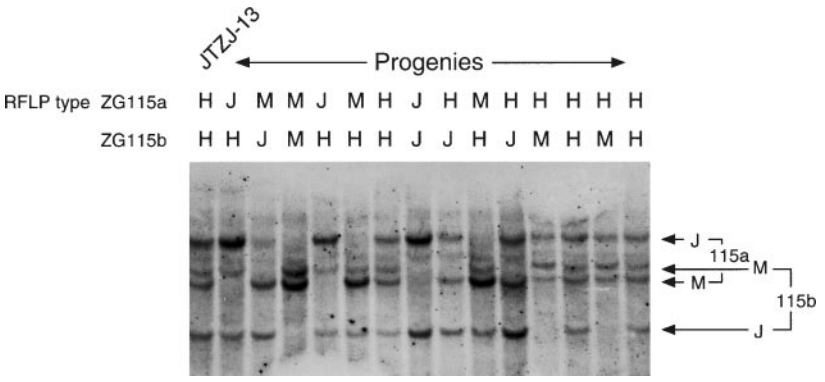


Fig. 1 A RFLP profile of duplicate loci in self-pollinated progenies of the interspecific hybrid JTZJ-13 detected with ZG115 as a probe. RFLP types *H*, *J*, and *M* indicate hetrozygous, japonica and matrella types, respectively. The ZG115 probe detected duplicated loci, *a* and *b*, both showing RFLPs

seven gene clones exhibited RFLPs between the two species. The frequency of RFLP with *Xba*I was higher than those with other restriction enzymes. No significant difference for the frequency of RFLP probes due to the DNA source of clones was observed.

Duplication of RFLP loci

Of the 100 RFLP probes selected, 26 (26.0%) detected two or more segregating loci in the self-pollinated progenies (Fig. 1). The frequency of RFLP probes detecting duplicate loci was constant among the DNA sources used as clones (Table 2). Eleven probes showed almost equal hybridization intensity, whereas 14 hybridized differently between two loci. One clone (ZCL2) detected three loci. Segregation ratios indicated independence between all combinations of duplicated loci detected with the respective probes. In total, 127 RFLP loci were identified in the mapping population of zoysiagrass with 100 RFLP probes.

Segregation of RFLP loci

Twelve out of the one hundred and twenty seven RFLP loci (9.4%) showed a significant segregation distortion ($P < 0.05$). For RFLP loci *ZG60a*, *ZGT156a*, *Actin-b*, *ZCS43*, *ZCS45* and *ZCL12a*, the probability of observing the data under the null hypothesis of normal Mendelian segregation was less than 0.01. At RFLP loci *Actin-b*, *ZCS43*, *ZCS45* and *ZCL12a*, the numbers of progenies identified as heterozygous genotypes were higher than expected. At RFLP loci *ZG60a* and *ZGT156a*, the numbers of progenies identified with the japonica allele were higher than expected.

RFLP map

A zoysiagrass RFLP linkage map was constructed on the basis of segregation data for the progenies (Fig. 2),

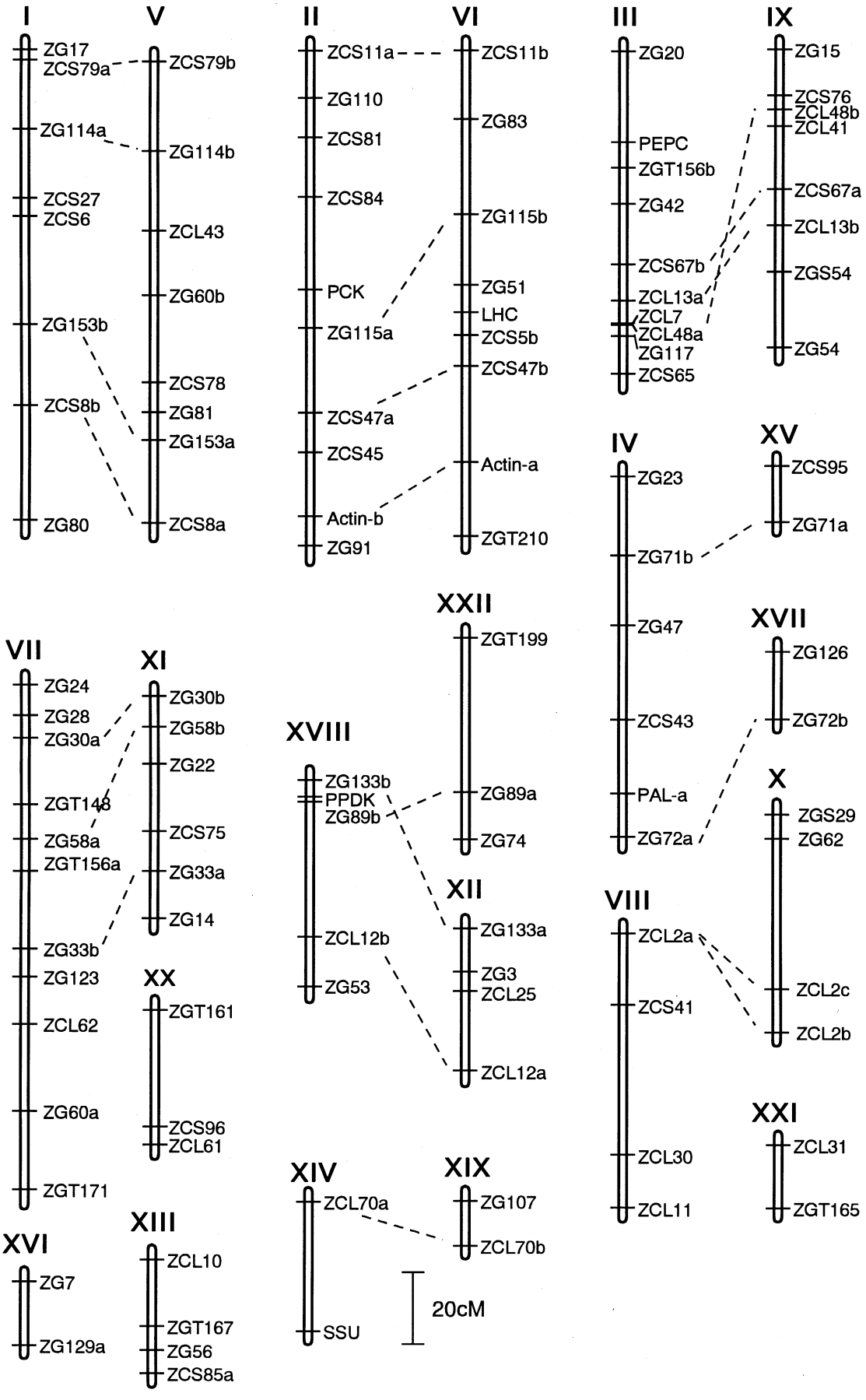
Table 2 Number of RFLP clones detecting duplicate loci

Source	Number of clones	
	Tested	Detecting duplicate loci[a]
Genomic	55	13 (23.6)
cDNA from leaf (ZCL)	17	5 (29.4)
cDNA from rhizome (ZCS)	21	6 (28.6)
Gene	7	2 (28.6)
Total	100	26 (26.0)

[a] Frequency (%) is indicated in parantheses

which consisted of 115 out of 127 loci in 22 linkage groups ranging in size from 12.5 cM to 141.3 cM with a total map distance of 1506.3 cM. The remaining 12 loci were not linked. Among the 22 linkage groups, six of them (XIV, XV, XVI, XVII, XIX and XXI) contained only two loci. The average distance between loci was 16.2 cM. Out of six RFLP loci showing significant segregation distortion, *ZCS45* and *Actin-b* were mapped to linkage group II, and *ZG600* and *ZGT156a* to group VII.

In the RFLP linkage map of zoysiagrass, pairs of linkage groups sharing duplicated RFLP loci were identified (Fig. 2). For example, the series of duplicated loci detected with ZCS79, ZG114, ZG153 and ZCS8 was observed in both group I and group V. Other pairs of linkage groups that shared duplicate loci were found between groups II and VI, III and IX, VII and XI, and XII and XVIII, respectively. Furthermore, the duplicated loci that mapped on these pairs of linkage groups were arranged approximately in the same order. Between groups VIII and X, and XIV and XIX, only a pair of duplicated loci was mapped. Duplicated loci mapped on groups IV and XVIII were divided into two different linkage groups. Duplicated loci detected with ZGT156 and ZG60 were mapped on different linkage groups from pairs of groups sharing a series of duplicate loci. For example, the series of duplicate loci detected with ZG30, ZG58 and ZG33 were conserved


I
ZG17
ZCS79a
ZG114a
ZCS27
ZCS6
ZG153b
ZCS8b
ZG80
V
ZCS79b
ZG114b
ZCL43
ZG60b
ZCS78
ZG81
ZG153a
ZCS8a
II
ZCS11a
ZG110
ZCS81
ZCS84
PCK
ZG115a
ZCS47a
ZCS45
Actin-b
ZG91
VI
ZCS11b
ZG83
ZG115b
ZG51
LHC
ZCS5b
ZCS47b
Actin-a
ZGT210
III
ZG20
PEPC
ZGT156b
ZG42
ZCS67b
ZCL13a
ZCL7
ZCL48a
ZG117
ZCS65
IX
ZG15
ZCS76
ZCL48b
ZCL41
ZCS67a
ZCL13b
ZGS54
ZG54
IV
ZG23
ZG71b
ZG47
ZCS43
PAL-a
ZG72a
XV
ZCS95
ZG71a
XVII
ZG126
ZG72b
X
ZGS29
ZG62
ZCL2c
ZCL2b
VIII
ZCL2a
ZCS41
ZCL30
ZCL11
XXI
ZCL31
ZGT165
VII
ZG24
ZG28
ZG30a
ZGT148
ZG58a
ZGT156a
ZG33b
ZG123
ZCL62
ZG60a
ZGT171
XI
ZG30b
ZG58b
ZG22
ZCS75
ZG33a
ZG14
XX
ZGT161
ZCS96
ZCL61
XVI
ZG7
ZG129a
XIII
ZCL10
ZGT167
ZG56
ZCS85a
XXII
ZGT199
ZG89a
ZG74
XVIII
ZG133b
PPDK
ZG89b
ZCL12b
ZG53
XII
ZG133a
ZG3
ZCL25
ZCL12a
XIV
ZCL70a
SSU
XIX
ZG107
ZCL70b
20cM

between linkage groups VII and XI, whereas one of the duplicated loci, detected with ZG60, was mapped to linkage group V, while another, detected with ZGT156, was mapped to linkage group III. Out of 26 pairs of duplicated loci detected, *ZG129b*, *ZCS85b* and *PAL-b* remain unlinked.

Discussion

Genetic polymorphism in *Zoysia* species

The putative interspecific hybrid of zoysiagrass, identified from natural populations based on morphological characterization and RFLP analysis (Yaneshita et al. 1997), was used as the parental accession of the mapping population in the present study. In the survey of RFLP probes, the parental accession dispolayed more than two hybridized fragments including one of those shared with *Z. japonica* and another detected in *Z. matrella*, or none of four accessions, by hybridizing with DNA clones detecting RFLPs between two species. Genotypes of self-pollinated progenies, identified from RFLP patterns in the survey of RFLP probes, segregated according to normal Mendelian segregation at most of the RFLP loci identified. Therefore, the parental accession of the mapping population is concluded to be an interspecific hybrid between *Z. japonica* and another species. Fukuoka (1989) has also identified interspecific hybrids in natural populations distributed in Japan based on morphological analysis. Forbes (1952) reported that hand-pollinated interspecific hybrids between *Zoysia* species were completely fertile.

The frequency of DNA clones detecting RFLP loci in our population (87.0%) was comparable to that in a mapping population of rice obtained from a hybrid between *japonica* and *javanica* (McCouch et al. 1988) and to that in one of maize (Helentjaris et al. 1986). Helentjaris et al. (1985) suggested that species which are primarily out-crossing in nature yield a higher degree of RFLP compared with species that are self-pollinating. The high frequency of DNA clones detecting RFLP loci in zoysiagrass may have resulted both from the protogynous nature of flowering in *Zoysia* (Forbes 1952) and the mapping population, which originated from an interspecific hybrid.

Duplicated loci

Of the RFLP probes, 26.0% detected two or more RFLP loci in the mapping population of zoysiagrass. Even with the remaining 74.0% of RFLP probes, duplicated sequences with a segregating locus were observed. However, it was difficult to identify genotypes for duplicated sequences detected by the remaining probes, since their hybridization signals were very weak or else did not show polymorphism in combination with the four restriction enzymes employed. Therefore, duplicated sequences detected with DNA probes appear to be distributed throughout the whole genome of zoysiagrass.

Duplicated RFLP loci were reported not only in polyploid species but also in diploid species, including maize (Helentjaris et al. 1988) and sorghum (Whitkus et al. 1992). In the case of maize, 29% of the DNA probes hybridized to more than two RFLP loci. Furthermore, it was found in maize that duplicated loci on the pairs of chromosomes were arranged in approximately the same order. From these results, Helentjaris et al. (1988) suggested that numerous duplicated loci identified in the maize genome may have been involved in an allopolyploidization event. In the RFLP map of zoysiagrass, five pairs of linkage groups, including I and V, II and VI, III and IX, VII and XI, and XII and XVIII, shared more than two duplicated RFLP loci with approximately the same order. Therefore, duplicated RFLP loci detected in the *Zoysia* genome were arranged on a pair of respective linkage groups, suggesting a common or homoeologous origin.

Segmental allopolyploidy

Most reports on the cytological study of zoysiagrass (Forbes 1952; Tateoka 1954; Christopher and Abrahm 1974) indicated that the chromosome number of *Zoysia* species was $2n = 40$. Forbes (1952) suggested that $x = 20$ was the most likely basic chromosome number for *Zoysia* species, because no meiotic irregularities were observed in pollen mother cells of *Z. japonica*, *Z. matrella*, and *Z. tenuifolia* strains. However, from a study of the first meiotic division of microsporocytes, Gould and Soberstorm (1974) reported that the chromosome number of *Z. matrella* collected in Ceylon was $2n = 20$. Our RFLP map of zoysiagrass consists of 22 linkage groups including six small linkages mapped by only two loci. In these small linkages, groups XV and XVII may be parts of a common linkage group sharing duplicated loci mapped on linkage group IV. Also, groups XII and XXII may be linked together, based on the position of duplicated loci on group XVIII. From these inferences, we may conclude that the number of linkage group in zoysiagrass should be 20 in total, including pairs of linkage groups sharing duplicated RFLP loci. The co-segregation ratios of genotypes between duplicated loci detected with the common RFLP probes were coincident with those expected for an independence of homologous duplicated loci. For example, the chi-square value for the co-segregation ratio of genotypes at the duplicated loci of ZG115, mapped on the linkage groups II and VI (Figs. 1 and 2),

Fig. 2 RFLP linkage map of zoysiagrass produced from the self-pollinated progenies of the interspecific hybrid JTZJ-13

was 6.96 which indicated independence between ZG115a and ZG115b. The segregation independence of the duplicated loci for ZG115 also suggests that no chromosome pairing between linkage groups II and VI was involved at meiosis. Therefore, *Zoysia* species with $2n = 40$ seem to represent a segmental allotetraploid derived from progenitors with a basic chromosome number of ten ($x = 10$).

In conclusion, we have constructed the RFLP linkage map of zoysiagrass by use of the self-pollinated progenies derived from an interspecific hybrid. This RFLP map will be useful in order to facilitate the establishment of DNA markers tagging desirable traits for the improvement of zoysiagrass as well as for conducting a QTL analysis of environmental stress-response traits.

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