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SARAYUT THAIKUA

Science of Bioresource Production

The United Graduate School of Agricultural Sciences

Kagoshima University

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CHAPTER 1

General Introduction

Domestic ruminants are kept by humans to produce milk, meat, and wool from plant material, which, for the most part, is unsuitable for direct human consumption (Minson 1990). Jung and Allen (1995) mentioned that there are numerous reasons why forages should be maintained in ruminant diet at higher level although cattle and sheep can be raised on relatively low-forage diets. Firstly, ruminal function and animal health are best when forage based diets are fed. Moreover, the production costs for forages are lower than for grain crop. Finally, perennial forage crops are more environmentally friendly or sustainable. However, when forage is the sole source of nutrients, production is invariably much lower than the genetic potential of the animal (Minson 1990). The improvement of forage quality could account for a greater proportion of the diets fed to high-producing animals (Jung and Allen 1995).

Brachiaria is one of the most widely grown tropical grass genera in many areas, (Argel and Keller-Grein 1996; Ndikumana and de Leeuw 1996; Pizarro *et al*. 1996; Stür *et al*. 1996). This genus includes about 100 species, which occur in the tropical and subtropical regions of both eastern and western hemispheres, but mostly in Africa

(Renvoize *et al*. 1996). Six perennial species, including *B. arrecta*, *B. brizantha*, *B. decumbens*, *B. humidicola*, *B. mutica*, and *B. ruziziensis* (ruzigrass), and *B.* hybrids have been used as pasture (Miles *et al*. 2004; Cook *et al*. 2005). Species in this genus show rapid regrowth, good persistence under heavy or frequent defoliation, and drought tolerance (Fisher and Kerridge, 1996). Brachiariagrass also have high forage quality when they are fertilized with nitrogen and well managed (Lascano and Euclides, 1996).

Apomixis and ploidy differences delayed the initiation of brachiariagrass breeding programs until suitable sexual germplasm was developed (Miles *et al.* 2004). Until now, only 2 hybrid cultivars of *Brachiaria* spp. have been released (Cook *et al*. 2005), and they had been developed for utilizing in tropical America (Miles *et al.* 2004). For South-East Asia and tropical Japan, only few cultivars of brachiariagrass have been used and grown extensively in cultivated pastures. The risk to the extensive monoculture is obvious. Moreover, the higher quality brachiariagrass is also necessary as it can reduce the proportion of the expensive concentrate in diets. Hence, new superior cultivars of brachiariagrass having high quality are urgently needed for these area.

1. Reproductive system of *Brachiaria* spp.

Brachiariagrass reproduces both sexually and by apomixis (Valle and Savidan,

1996). All currently used cultivars of brachiriagrass, except *B. ruziziensis* cv. Kennedy, are apomicts (Cook *et al*. 2005). The type of apomixis of *Brachiaria* spp. is gametophytic apomixis, which is defined as asexual reproduction through the production of seed, and it is classified into aposporous form (Valle and Savidan 1996).

1.1 Sexual embryo sac development

A single cell, known as megaspore mother cell, in the hypodermal layer of a young ovule, enlarges and undergoes meiosis to produce a linear tetrad of megaspores. The three megaspores nearest to the micropyle degenerate, while the remaining chalazal megaspore, the one most distant from the micropyle, enlarges and undergoes three mitotic divisions to produce an eight-nucleate embryo sac of the *Polygonum* type. The nuclei in the mature embryo sac all reduced in chromosome number and differentiate as an egg cell, two synergid cells, two polar nuclei that form the central cell, and three or more antipodal cells (Burson and Young 2001). (Figure 1-1)

1.2 Aposporous embryo sac development

As in sexual embryo sac development, megaspore mother cell enlarges and undergoes meiosis to produce a linear tetrad of megaspore. However, at this stage or shortly thereafter, one or more of the adjacent nucellar cells in the ovule begin to enlarge and their nuclei become more dense. These enlarged nucellar cells begin to divide

mitotically. By this time, the functional megaspore, which had developed from meiosis, usually aborts and the enlarging nucellar cells occupy its area. As the nucellar cells continue to enlarge, they become vacuolated and their nuclei divide mitotically twice to produce aposporous embryo sacs of *Panicum* type. During the first mitotic division, the spindle is oriented crosswise to the long axis of the cell and the two nuclei produced are located in the micropylar end of the developing sac. At the same time, a vacuole forms in the chalazal end. These two nuclei divide again producing a mature four-nucleate embryo sac with all nuclei located in the micropylar end and a large vacuole in the chalazal end. The nuclei usually differentiate as an egg cell, one polar nucleus, and two synergids (Burson and Young, 2001). (Figure 1-1)

1.3 Degree of apomixis

Aposporous apomict is also classified by the degree or level of apomixis expressed. Plants that reproduce entirely by apomixis are obligate apomicts, whereas plants that reproduce by both apomixis and sexuality are facultative apomicts, and most apomictic species are facultative (Burson and Young, 2001).

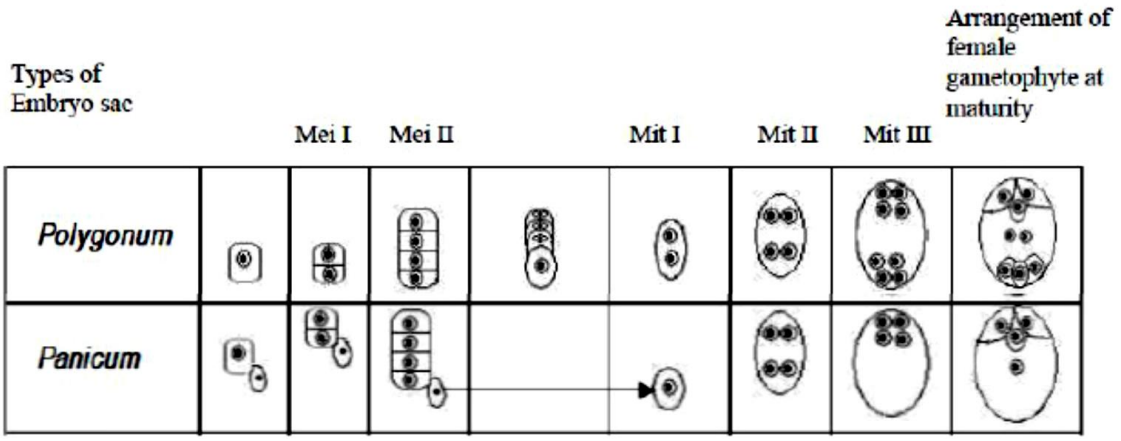

Figure 1-1 Schematic diagram of *Panicum* type of apospory embryo sac in comparison to *Polygonum* type (modified from Bhat *et al.* 2005).

2. Breeding of *Brachiaria* spp.

As mentioned previously that *Brachiaria* spp. reproduces both sexually and by apomixis, so that both open-pollinated and apomictic cultivars can be produced.

2.1 Open-pollinated cultivar

Up to date, there is only one open-pollinated cultivar of *Brachiaria* spp. that is *B. ruziziensis* cv. Kennedy (Cook *et al.* 2005). The usual strategy for open-pollinated cultivar breeding is recurrent selection (Simmonds 1979). Two basic forms of recurrent selection are used in forage grass breeding, including mass selection and the polycross methods (Burson and Young 2001). For the first method, it begins with the selection of superior plants from a base population. Seed is collected from the selected plants and

bulked to form the population for next selection cycle. For the latter method, it starts with the identification of superior plants from the base population. These selected plants are vegetatively propagated into several identical plants and recombined by planting the replicated genotypes in a randomized block design in isolated crossing block. Seed is then collected from the polycross and the cycle is repeated.

2.2 Apomictic cultivar

Both ecotype selection and hybridization can be adopted for developing apomictic cultivars (Vogel and Burson 2004). Most of currently used cultivars of brachiariagrass are selected from natural population (Cook *et al*. 2005), while hybridization breeding of brachiariagrass just began towards the end of the last century with the establishment of sexual tetraploid lines (Swenne *et al.* 1981; Lutts *et al.* 1991; Pinheiro *et al*. 2000; Argel *et al*. 2007; Ishigaki *et al*. 2009). In hybridization breeding, the sexual plant is used as the female parent and is pollinated with the apomict (Burson and Young 2001). Hybridization of *Brachiaria* spp. results in new genotypes that usually segregate for the proportion of sexual to apomictic progeny close to 1:1 (Valle and Savidan 1996). The advantage of apomixis is that superior heterozygous genotypes can be fixed and propagated faithfully by seed (Miles and Valle 1996). Recently developed hybrid apomictic cultivars of *Brachiaria* include ‘Mulato’ and ‘Mulato II’ (Cook *et al.*

2005). ‘Mulato’ was produced from a cross between a sexual tetraploid ruzigrass and apomictic *B. brizantha* cv. ‘Marandu’ , with the final selection based on tolerance of high soil aluminium, plant vigour, dry matter production and forage quality (Cook *et al.* 2005). ‘Mulato II’ was developed from an initial cross between sexual tetraploid ruzigrass and apomictic tetraploid ‘Basilisk’. Sexual progenies of this first cross were submitted to open pollination to generate a second generation of hybrids, from which a sexual genotype was selected for its superior agronomic characteristics and crossed again, using the same open pollination procedure, with a series of *B. brizantha* accessions and apomictic and sexual hybrids. A progeny clone, FM9503/S046/024, was selected visually for its vigor, productivity, and leafiness (Argel *et al*. 2007).

3. Assessment of reproductive mode in hybridization breeding of *Brachiaria* spp.

In hybridization breeding, excellent apomictic hybrid will be selected as the candidates of new cultivars. As described previously that the segregation ratio of sexual to apomictic genotype in first generation of the hybridization is about 1:1; hence, one of the most important task for *Brachiaria* breeder is to identify apomictic genotypes in the segregating population. Generally, two conventional methods for assessing reproductive mode of apomictic species include progeny test and cytological analysis (Burson and

Young 2001). For the former method, because apomixis is an asexual cloning process whereby the seeds are genetic copies of the maternal parent, the resulting progeny from an obligate apomictic or a highly apomictic plant are uniform and appear identical to that parent. Thus, uniform progeny from a heterozygous or cross-pollinated plants suggests apomixis. For the cytological method, this is usually accomplished by dehydrating the pistil, embedding it in paraffin, sectioning it with a microtome, staining it with the appropriate dyes, and examining megasporogenesis and subsequent embryo sac development in the serial sections of the ovule with light microscopy. Another method is to dehydrate the pistil, clear it with methyl salicylate, and observe the development inside the intact ovule using interference microscopy.

However, the efficiency of any breeding program in which apomixis is a goal will be improved by finding quicker, cheaper, and more reliable methods to assess the reproductive mode in a segregating population (Miles and do Valle 1996). Tohme *et al*. (1996) suggested that the reliable molecular marker that are tightly linked to the gene that confer apomixis would be useful in any breeding scheme based on apomixis. Molecular mapping studies of apomixis have been performed in various maize–*Tripsacum* hybrids (Grimanelli *et al*. 1998), *Panicum maximum* (Ebina *et al*. 2005), *Paspalum* spp. (Ortiz *et al*. 1997; Pupilli *et al*. 1997; Labombarda *et al*. 2002), *Pennisetum squamulatum* (Ozias-

Akins *et al*. 1998; Akiyama *et al.* 2004, 2011), *Poa pratensis* (Barcaccia *et al*. 1998), and *Citrus* and *Poncirus* species (Garcia *et al.* 1999, Nakano *et al.* 2012). In brachiariagrass, Pessino *et al*. (1997, 1998) conducted bulked segregant analysis using restriction-fragment length polymorphisms, randomly amplified polymorphic DNA, and amplified fragment-length polymorphisms (AFLPs) to identify molecular markers that co-segregated with apomixis in an F_1 population of *B. ruziziensis* × *B. brizantha*. Morever, Zorzatto *et al.* (2010) carried out bulked segregant analysis to identify Random Amplified Polymorphic DNA marker linked to apomixis in *B. humidicola*. However, because there are several genotypes of apomictic *Brachiaria* spp., which could be the appropriate paternal plants in the breeding program; hence, the studies on molecular markers linked to apomixis in other hybrid population of *Brachiaria* spp. are still needed.

4. *Brachiaria* breeding for increased forage quality

Forage quality is one of the main objectives of current brachiariagrass breeding program (Miles *et al.* 2004), and one of the most important traits for forage quality is dry matter digestibility (DMD) (Smith *et al*. 1997; Casler and Vogel 1999). Galyean and Goetsch (1993) noted that forages alone cannot meet the energy requirements of high-producing dairy cows or rapidly growing steers. The often low digestibility and high

concentration of cell walls in forages limit energy availability to animals fed diets with high proportion of forage. A reduction in the concentration of cell-wall material may improve both intake and energy density of forages, and increased digestibility of the cell wall would improve energy availability (Jung and Allen 1995). Many works showed that selection of grass cultivars with high DMD generally resulted in improved animal production (Utley *et al.* 1974; Utley *et al.* 1978; Casler and Vogel 1999); therefore, breeding for increased DMD can lead to rapid financial benefits to the agricultural community (Casler and Vogel 1999). Variation in DMD can occur from differences in both cell wall concentration and cell wall digestibility (Buxton and Casler, 1993). From the literature review of Miles *et al.* (2004), it was found that variation in DMD and fiber characteristics has been documented among accessions. Therefore, it is possible to develop new cultivars of brachiariagrass that have high DMD combining with the other important agronomic traits.

5. Bioassay for predicting DMD

Minson (1990) summarized that three bioassay techniques have been developed for predicting DMD.

5.1 Rumen fluid-pepsin technique

This technique is the *in vitro* digestibility using rumen microorganism. The common features of these methods are fermentation of a ground forage sample with rumen microoganisms in buffered medium under controlled conditions of anaerobiosis, temperature, and pH. Two-stage *in vitro* method was developed by Tilley and Terry (1963), by using acid-pepsin stage for removing the crude protein remaining after the microbial fermentation.

5.2 *In vitro* cellulase technique

The feature of this technique is the replacement of rumen fluid with cellulase, derived from *Trichoderma reesei,* in the rumen fluid-pepsin technique.

5.3 *In sacco* technique or nylon bag technique

The feature of this technique is fermentation of ground forage sample within the rumen using bags made from nylon cloth.

6. Screening method in the breeding for increased DMD

In the usual hybridization breeding program, it is necessary to work with a large number of progenies to identify some superior plants as the candidates for new cultivar. Evaluating DMD by bioassay techniques of large breeding population size is very

laborious and expensive; hence, development of the method for screening for promising high DMD plants with simple and reliable method could reduce the effort and cost in the breeding program.

Screening procedures based on predictors of forage quality are being developed at The Brazilian Agricultural Research Corporation's Beef Cattle Research Center (Miles *et al.* 2004). For example, Hughes *et al.* (2000) and Herrero *et al.* (2001) proposed the shearing strength as the selection criterion for quality in *Brachiaria* spp. They found that shearing strength positively correlated with cell wall component, and negatively correlated with leaf DMD. However, those works proposed only single selection criterion for DMD. For other species, Wilson *et al.* (1989) had ever mentioned about selection of buffel grass (*Cenchrus ciliaris*) for increased DMD with several selection criteria together.

7. Essential researches for *Brachiaria* breeding program

Burson and Young (2001) noted that 2 preliminary research activities, including germplasm acquisition and germplasm evaluation, are necessary for a plant improvement program. To date, there is a large diverse collection of *Brachiaria* germplasm (Keller-Grein *et al.* 1996; Miles *et al.* 2004), which could provide materials for plant breeders to develop new superior *Brachiaria* cultivars. The variation among these germplasm for

DMD and the other important traits must be identified and characterized to find out the appropriate materials in the breeding program. Moreover, as noted above that prediction of DMD of a large number of plants using bioassay techniques may demand considerable effort and budget. Therefore, the development of the simple selection methods is also necessary for the breeding for increased digestibility. In addition, the development of reliable molecular markers linked to apomixis is the other important work for *Brachiaria* breeding program as mentioned previously.

Hence, this Ph.D. dissertation, which focused on *Brachiaria* breeding for increased DMD, was undertaken with the following objectives:

1) To evaluate digestibility and its related traits of *Brachiaria* germplasm for identifying the appropriate materials in the breeding for increased DMD.
2) To develop selection index for digestibility as the simple selection method in the breeding for increased DMD.
3) To develop molecular markers associated with the apomixis locus in *Brachiaria* spp. as the rapid and reliable method for identifying apomictic progeny in hybridization breeding.

CHAPTER 2

Evaluation on dry matter digestibility and the relation to morphology and water content of *Brachiaria* spp. and their heritability

Abstract

The objectives of this study were to evaluate dry matter digestibility (DMD) of brachiariagrass germplasm as material in breeding program, and also to determine the appropriate selection criteria for DMD. *In vitro* dry matter digestibility (IVDMD), morphological traits and water content of 17 genotypes of *Brachiaria* spp. were evaluated. The correlations among traits were determined, and their heritability were also estimated. For morphological traits, leaf-stem index (ratio of leaf width/stem diameter) showed the highest correlation with whole plant IVDMD followed by leaf shape ratio (leaf width/leaf length). Leaf water content also highly correlated with whole plant IVDMD. The broad sense heritability of IVDMD and the related traits of 2 harvests combination were high. The results demonstrate that leaf-stem index, leaf shape ratio, and water content could be the useful selection criteria for the breeding of brachiariagrass for high digestibility.

Introduction

As mentioned in Chapter 1 that dry matter digestibility (DMD) is one of the crucial traits for grass breeding. To date, there is a large diverse collection of *Brachiaria* germplasm (Keller-Grein *et al.* 1996; Miles *et al.* 2004), which could provide materials for plant breeders to develop new superior *Brachiaria* cultivars. Some promising accessions have been selected as the candidates for new cultivars (Cook *et al.* 2005). These accessions could also be the materials in the breeding for increased DMD; therefore, the variation among these germplasm for DMD and their related traits must be identified and characterized to find out the appropriate materials in the breeding program.

Furthermore, the study on relationship between DMD and the simple traits is also important as it can clarify the easy and inexpensive selection criteria for increased digestibility rather than the laborious and expensive bioassay methods. The relationships between physical strength and quality trait of brachiariagrass have been reported (Hughes *et al*. 2000; Herrero *et al*. 2001). Those studies focused on the relation between cell wall and digestibility; however, there has been little knowledge in the relation between DMD and cell water content. Also, the relationships between some morphological traits and digestibility in *Brachiaria* spp. are still not well known, whereas, there were some reports of these relations in the other grasses (Lentz and Buxton 1991; Batistoti *et al*. 2012)

In addition, the heritability of DMD related traits are also essential for plant improvement as it reveals the effectiveness of selection for the traits. The broad sense heritability of the traits is the proportion of the genetic variance to the phenotypic total variance (Burton and DeVane 1953). Heritability in broad sense would have meaning for asexual reproduced plants since all genetic variability is usable (Hanson 1963).

The objectives of this study were to evaluate DMD and the correlation with the possible related traits, including morphological traits and cell water content, and to estimate the broad sense heritability of each trait of *Brachiaria* germplasm. The information from this study could clarify appropriate materials and selection criteria for *Brachiaria* breeding for increased DMD.

Materials and methods

Plant material

Seventeen genotypes of *Brachiaria* spp. (Table 2-1) were used in this experiment. Each genotype was clonally propagated with 3–4 tillers and transplanted in Wagner pot (0.02 m^2) containing 3 kg of air-dried soil (calcaric dark red soil, pH 7.7) in November 2012. The experimental unit was 1 plant per pot, and they were arranged in a completely randomized design with 4 replications and density of 16 pots m^{-2} in the greenhouse in the

Field Science Center of the University of the Ryukyus, Okinawa, Japan. Plants were clipped at 5-cm height after the establishment to make the uniformity in January 2013, and fertilized with 70 kg N ha^{-1}, 39 kg P_2O_5 ha^{-1} and 54 kg K_2O ha^{-1}.

Sampling, measurement and chemical analysis

Each plant was harvested at two harvesting times by clipping at 5-cm height, the first was performed after 12 week-regrowth in early April 2013 (the average temperature was 19.2±5.2 ℃), and the second was performed after 8 week-regrowth in late May 2013 (the average temperature was 23.7±3.5 ℃). The fertilizer was applied with the rate of 70 kg N ha^{-1}, 39 kg P_2O_5 ha^{-1} and 54 kg K_2O ha^{-1} after the harvest. At the first harvest, width and length of three first fully expanded leaves were measured per pot. Stem diameter were also measured at the position just below first fully expanded leaves. All samples were kept in the iceboxes after the harvest to maintain moisture content. Each sample was separated into stem (including leaf sheath) and leaf (blade), and fresh weight were measured and oven-dried at 70 ^oC for 48 hours. The dry weight of each part was measured, and leaf/stem ratio was calculated. Water content of each part was determined as ((fresh weight – dry weight)/fresh weight) x100.

All samples were ground to pass through a 1-mm screen. The samples were analyzed for *in vitro* DMD (IVDMD) by the pepsin-cellulase assay (Goto and Minson

1977). Leaf part of each genotype was analyzed for neutral detergent fiber (NDF) by the method of Goering and Van Soest (1970).

Statistical analysis

Analysis of variance was conducted on each data set within a harvest, and combined analysis of 2 harvests was performed. Correlation analysis (Pearson) was conducted to evaluate the relationships between traits.

Broad sense heritability was calculated as outlined and discussed by Burton and DeVane (1953) and Hanson (1963). Estimation of total genetic variance (σ^2_G), genetic x harvest covariance (σ^2_{GE}) and error variance (σ^2) were calculated by equating the mean square expectations to the mean squares in the analysis of variance. For a single harvest, mean square of genotype is equal to $\sigma^2+r\sigma^2_G$, where r is replication, and mean square of residual is equal to σ^2. For combined analysis of 2 harvests, mean square of genotype is equal to $\sigma^2 + r\,\sigma^2_{GE} + re\,\sigma^2_G$, mean square of genetic x harvest is equal to $\sigma^2 + r\,\sigma^2_{GE,}$ mean square of residual is equal to σ^2, where r and e are the number of replication and harvest, respectively.

Phenotypic variance (σ^2_P) of single plant was calculated as $\sigma^2_G + \sigma^2$ and $\sigma^2_G + \sigma^2_{GE} + \sigma^2$ for a single harvest and combination of 2 harvests, respectively. Phenotypic variance of the mean was calculated as $\sigma^2_G + \sigma^2/r$ and $\sigma^2_G + \sigma^2_{GE}/e + \sigma^2/re$ for

single harvest and combination of 2 harvests, respectively. Broad sense heritability was determined by σ^2_G/σ^2_P.

Table 2-1 Seventeen genotypes of *Brachiaria* spp.

Species	Cultivar	Accession†
B. brizantha	Marandu	CIAT6294
B. brizantha	Piata	-
B. brizantha	Xaraes	CIAT26110
B. brizantha	-	CIAT16113
B. brizantha	-	CIAT16306
B. brizantha	-	CIAT16315
B. brizantha	-	CIAT16316
B. brizantha	-	CIAT16467
B. brizantha	-	CIAT16488
B. brizantha	-	CIAT26124
B. brizantha	-	CIAT26318
B. brizantha	-	CIAT26990
B. decumbens	Basilisk	CIAT606
B. ruziziensis‡	Kennedy	CIAT605
B. ruziziensis§	Miyaokikoku	-
B. hybrid	Mulato	CIAT36061
B. hybrid	Mulato II	CIAT36087

† Genetic resources of International Center for Tropical Agriculture (CIAT).

‡ Diploid ruzigrass.

§ Tetraploid ruzigrass. The genotype of 'Miyaokikoku' in this study is No. 1-42.

Results

IVDMD

IVDMD of 17 genotypes are showed in Table 2-2. At the first harvest, ‘Kennedy’ had the highest whole plant IVDMD, while *B. brizantha* CIAT16113 and CIAT16316 had the lowest value. Any significant differences between leaf IVDMD and stem IVDMD were not found among 8 genotypes. Seven genotypes had higher IVDMD in leaves than in stems, while *B. brizantha* CIAT16113 and ‘Xaraes’ had higher IVDMD in stems than in leaves. At the second harvest, ‘Mulato II’ had the highest whole plant IVDMD, while *B. brizantha* CIAT26318 had the lowest value. Leaf and stem IVDMD were not significantly different in Mulato II and 3 genotypes of *B. brizantha*, whereas the other genotypes had higher IVDMD in leaves than in stems.

Dry matter weight (DMW), leaf/stem ratio and water content

DMW, leaf/stem ratio and water content of all genotypes are shown in Table 2-3. ‘Basilisk’ had the highest DMW at first harvest, whereas *B. brizantha* CIAT16113 had the lowest DMW. At the second harvest, *B. brizantha* CIAT26318 had the highest DMW and *B. brizantha* CIAT16316 had the lowest DMW. For both harvests, the highest leaf/stem ratio cultivar was ‘MulatoII’, while the lowest leaf/stem ratio cultivar was ‘Basilisk’. The whole plant water content of ‘Miyaokikoku’ was the highest

at both harvests, while *B. brizantha* CIAT26318 showed the lowest content at both harvests.

Leaf NDF

Mean values of leaf NDF of all genotypes are shown in Table 2-4. *B. brizantha* CIAT16113 had the highest leaf NDF content at the first harvest, while 'Kennedy' had lowest content. At the second harvest, *B. brizantha* CIAT16316 had the highest leaf NDF content, and *B. brizantha* CIAT16315 had the lowest content.

Morphological traits

Mean values of morphological traits of all genotypes are shown in Table 2-5. 'Miyaokikoku' had the widest leaf, while B. *brizantha* CIAT16113 had the narrowest. The longest leaf genotype was *B. brizantha* CIAT16113, while the shortest leaf genotype was 'Basilisk'. Mulato' had the highest leaf shape ratio (leaf width / leaf length), whereas *B. brizantha* CIAT16113 had the lowest ratio. *B. brizantha* CIAT16306 had the largest stem diameter, and *B. brizantha* CIAT26990 and 'Basilisk' had the smallest stem diameter. 'Miyaokikoku' had the highest leaf-stem index (leaf width / stem diameter), whereas *B. brizantha* CIAT16306 had the lowest index.

Estimate of broad sense heritability

Table 6 shows the broad heritability for IVDMD, dry matter weight, leaf/stem ratio,

water content and NDF. At the first harvest, stem IVDMD showed highest heritability, and leaf water content showed the lowest heritability. At the second harvest, leaf NDF showed highest heritability, whereas dry matter showed the lowest heritability. The combine analysis of 2 harvests showed the lower heritability for all traits, with the highest values of stem IVDMD and leaf NDF, and the lowest value of dry matter weight. Table 2-7 shows that broad sense heritability for all morphological traits was relatively high with the range from 0.72–0.91 for a single plant, and 0.91–0.98 for the mean of replications. Leaf width showed highest heritability, and leaf length showed the lowest heritability.

Relationships between traits

Table 2-8 shows the correlation coefficients between traits. Leaf width positively correlated with whole plant IVDMD and stem IVDMD. Leaf length negatively correlated with whole plant IVDMD and leaf IVDMD. Leaf shape ratio and leaf-stem index positively correlated with whole plant IVDMD, leaf IVDMD and stem IVDMD. Leaf/stem ratio positively correlated with whole plant at the 2nd harvest, while it positively correlated with stem IVDMD at both harvests. Whole plant water content, leaf water content and stem water content positively correlated with IVDMD at both harvests. Dry matter weight negatively correlated with stem IVDMD at only the 1st harvest.

Table 2-2 *In vitro* dry matter digestibility (IVDMD) of 17 genotypes of brachiariagrass

Species	Cultivar/ Accession	1st harvest				2nd harvest			
		IVDMD (%)			Difference	IVDMD (%)			Difference
		Whole plant	Leaf	Stem	between leaf-stem	Whole plant	Leaf	Stem	between leaf-stem
B. brizantha	Marandu	52.4±0.4†	52.3±0.3	52.7±0.3	ns	51.5±0.7	52.7±1.0	49.2±0.8	*
B. brizantha	Piata	51.4±0.5	51.2±0.7	52.1±0.7	ns	50.7±0.6	51.6±0.4	49.4±1.1	ns
B. brizantha	Xaraes	49.3±0.3	48.4±1.0	52.3±0.3	*	48.8±0.6	49.6±0.8	47.5±0.4	ns
B. brizantha	CIAT16113	48.9±0.5	47.8±0.9	51.5±0.4	**	45.4±0.4	47.9±1.1	42.3±0.6	**
B. brizantha	CIAT16306	53.5±0.9	53.1±1.1	55.0±0.4	ns	49.9±0.3	50.0±0.6	50.0 ±0.2	ns
B. brizantha	CIAT16315	50.2±0.7	52.4±0.9	47.1±0.6	**	47.5±1.7	53.5±1.3	42.7±1.9	**
B. brizantha	CIAT16316	48.9±0.2	49.7±0.2	47.8±0.6	*	44.2±0.9	47.6±1.7	40.5±0.8	*
B. brizantha	CIAT16467 ‡	50.1±0.9	54.6±0.7	44.5±1.3	**	48.6±1.3	54.7±0.2	43.3±1.8	**
B. brizantha	CIAT16488 ‡	50.4±1.0	54.5±1.3	45.1±1.4	**	48.4±0.5	55.5±0.9	42.7±0.5	**
B. brizantha	CIAT26124	50.6±1.0	51.8±0.7	48.2±1.7	ns	49.7±0.3	53.1±0.7	45.3 ±0.4	**
B. brizantha	CIAT26318 ‡	51.1±0.6	52.6±0.3	48.5±0.8	**	42.9±0.7	48.3±1.6	38.6±1.5	**
B. brizantha	CIAT26990	50.5±1.4	51.9±1.5	48.5±1.6	ns	48.6±1.6	52.0±1.6	45.1±1.8	*
B. decumbens	Basilisk ‡	49.7±0.3	54.5±0.7	44.0±0.4	**	44.8±0.6	52.4±0.5	40.3±0.7	**
B. ruziziensis	Kennedy	59.0±1.1	61.8±1.1	54.8±0.7	**	53.0±1.1	60.5±0.9	47.9±1.4	**
B. ruziziensis	Miyaokikoku	58.6±0.6	58.7±0.7	58.7±0.9	ns	52.6±0.8	55.6±0.7	50.0 ±0.9	**
B. hybrid	Mulato	55.3±0.6	53.5±1.3	57.0±0.7	ns	51.6±0.7	53.6±0.7	48.7 ±0.1	*
B. hybrid	Mulato II	56.2±2.0	56.2±2.1	59.5±0.9	ns	54.4±0.3	54.6±0.4	53.7±0.2	ns

† Standard error of mean.

‡ These genotypes were in flowering stage at the 2nd harvest.

* $P < 0.05$; ** $P < 0.01$; ns = no significant difference ($P > 0.05$).

Table 2-3 Dry matter weight (DMW), leaf/stem ratio and water content of 17 genotypes of brachiariagrass

Species	Cultivar/	1st harvest					2nd harvest				
	Accession	DMW	Leaf	Water content **(%)**			DMW	Leaf	Water content **(%)**		
		(g pot^{-1})	ratio	Whole	Leaf	Stem	(g pot^{-1})	ratio	Whole	Leaf	Stem
B. brizantha	Marandu	7.2 ±0.6†	3.8±0.41	80.2±0.6	79.8±0.6	81.8±0.5	14.1±0.9	1.8±0.09	78.9±0.8	78.6±0.8	79.6±0.8
B. brizantha	Piata	8.6±0.2	2.8±0.14	79.4±0.7	78.7±0.7	81.2±0.8	15.2±1.0	1.6±0.04	79.2±0.7	78.8±0.6	79.1±0.8
B. brizantha	Xaraes	9.1±0.7	3.8±0.35	77.4±0.5	76.6±0.5	80.6±0.5	16.1±1.1	1.8±0.07	76.7±0.5	75.4±0.7	78.9±0.3
B. brizantha	CIAT16113	4.4±0.3	2.5±0.07	80.3±0.8	79.5±0.9	82.4±0.7	11.1±0.7	1.3±0.03	79.4±0.6	79.1±0.5	79.9±0.6
B. brizantha	CIAT16306	5.9±0.7	3.8±0.24	78.5±0.4	77.7±0.5	81.3±0.3	17.8±1.0	1.6±0.12	75.4±0.4	74.3±0.5	78.0±0.3
B. brizantha	CIAT16315	10.3±0.4	1.3±0.04	79.4±0.4	78.7±0.5	80.4±0.4	10.5±1.6	0.8±0.04	80.5±1.0	80.5±1.0	80.5±1.0
B. brizantha	CIAT16316	5.0 ±0.1	1.3±0.22	79.6±0.5	78.2±0.5	81.4±0.5	9.0±1.0	1.1±0.09	76.6±1.4	75.1±1.2	78.0±1.4
B. brizantha	CIAT16467	9.4±1.1	1.3±0.09	77.4±1.0	77.2±0.9	77.7±1.3	12.4±2.0	1.0±0.17	79.1±0.9	79.3±1.0	78.9±0.8
B. brizantha	CIAT16488	12.2±0.8	1.3±0.11	76.7±0.1	76.8±0.2	76.7±0.5	15.4±1.2	0.8±0.03	77.0±0.6	77.6±0.6	76.6±0.6
B. brizantha	CIAT26124	8.8 ±0.6	2.5±0.40	79.5±0.2	78.5±0.2	81.9±0.4	15.9±0.9	1.4±0.09	78.4±0.7	77.8±0.8	79.3±0.7
B. brizantha	CIAT26318	10.1±0.5	1.8±0.19	75.4±0.7	75.0±1.0	76.2±0.3	19.6±0.8	0.8±0.03	74.8±0.5	74.5±0.6	75.0±0.4
B. brizantha	CIAT26990	7.7±1.4	1.6±0.10	76.7±1.4	75.7±1.8	78.3±0.9	10.5±2.1	1.1±0.07	78.5±0.5	79.2±0.6	77.9±0.5
B. decumbens	Basilisk ‡	12.8±0.6	1.2±0.09	75.6±1.1	74.9±1.9	76.5±0.3	16.5±0.3	0.6±0.02	77.5±0.3	78.4±0.5	77.0±0.2
B. ruziziensis	Kennedy	5.7±0.8	1.6±0.30	82.3±0.4	82.5±0.3	82.1±0.7	16.8±1.7	0.7±0.03	78.4 ±0.7	79.5±0.6	77.7±0.8
B. ruziziensis	Miyaokiko	5.6±1.0	2.9±0.48	85.2±0.3	84.6±0.3	86.8±0.3	16.3±0.6	0.9±0.04	81.9±0.2	81.7±0.3	81.9±0.3
B. hybrid	Mulato	5.9±0.9	7.2±1.78	79.1±1.1	79.9±0.8	82.5±0.8	17.0±0.3	1.4±0.07	77.9±0.3	78.3±0.4	77.6±0.1
B. hybrid	Mulato II	6.5±0.4	7.4±0.04	81.6±0.6	81.0±0.5	83.6±0.6	12.8±0.7	4.9±0.72	80.7±0.5	80.6±0.6	81.5±0.2

† Standard error of mean.

‡ These genotypes were in flowering stage at the 2nd harvest.

Table 2-4 Leaf neutral detergent fiber (NDF) of 17 genotypes of brachiariagrass

Species	Cultivar/ Accession	Leaf NDF (% dry matter)	
		1st harvest	2nd harvest
B. brizantha	Marandu	61.5±0.5†	63.7±0.4
B. brizantha	Piata	64.3±0.2	63.7±0.4
B. brizantha	Xaraes	66.1±0.9	65.0±0.4
B. brizantha	CIAT16113	68.3±0.5	68.3±0.5
B. brizantha	CIAT16306	62.3±0.5	64.1±0.4
B. brizantha	CIAT16315	63.0±0.6	59.9±0.4
B. brizantha	CIAT16316	67.7±0.9	69.3±0.1
B. brizantha	CIAT16467 ‡	62.8±1.0	63.5±0.3
B. brizantha	CIAT16488 ‡	63.7±1.1	64.6±0.3
B. brizantha	CIAT26124	63.6±0.9	62.9±0.9
B. brizantha	CIAT26318 ‡	67.8±0.2	69.1±0.4
B. brizantha	CIAT26990	64.2±1.1	64.5±0.6
B. decumbens	Basilisk ‡	62.1±0.4	62.5±0.5
B. ruziziensis	Kennedy	58.0±0.5	61.2±0.4
B. ruziziensis	Miyaokikoku	59.1±0.5	61.4±0.7
B. hybrid	Mulato	60.9±0.7	61.3±0.4
B. hybrid	Mulato II	62.0±0.5	66.1±0.4

† Standard error of mean.

‡ These genotypes were in flowering stage at the 2nd harvest

Table 2-5 Leaf width, leaf length, leaf shape ratio, stem diameter and leaf-stem index of 17 genotypes of brachiariagrass

Species	Cultivar/ Accession	Leaf width (cm)	Leaf length (cm)	Leaf shape ratio†	Stem diameter (mm)	Leaf-stem index‡
B. brizantha	Marandu	1.7±0.03§	29.5±1.7	0.057±0.0021	2.7±0.12	6.3±0.25
B. brizantha	Piata	1.7±0.06	36.8±2.3	0.046±0.0014	2.7±0.11	6.3±0.21
B. brizantha	Xaraes	1.9±0.04	37.3±2.0	0.050±0.0023	3.3±0.18	5.7±0.25
B. brizantha	CIAT16113	1.1±0.05	41.9±3.1	0.028±0.0012	2.2±0.06	5.3±0.20
B. brizantha	CIAT16306	1.6±0.04	30.1±1.8	0.054±0.0022	3.6±0.16	4.5±0.19
B. brizantha	CIAT16315	1.3±0.05	38.6±2.2	0.035±0.0014	2.7±0.15	4.9±0.12
B. brizantha	CIAT16316	1.3±0.02	37.6±3.9	0.036±0.0033	2.5±0.08	5.4±0.31
B. brizantha	CIAT16467	1.5±0.03	35.4±0.6	0.043±0.0010	2.5±0.14	6.0±0.28
B. brizantha	CIAT16488	1.5±0.03	31.6±1.3	0.048±0.0005	2.6±0.19	5.7±0.33
B. brizantha	CIAT26124	1.6±0.05	35.7±1.8	0.045±0.0007	2.5±0.16	6.4±0.29
B. brizantha	CIAT26318	1.2±0.02	30.8±1.0	0.039±0.0006	2.3±0.07	5.2±0.18
B. brizantha	CIAT26990	1.2±0.03	23.5±1.2	0.051±0.0029	1.9±0.05	6.3±0.22
B. decumbens	Basilisk	1.3±0.04	20.4±0.8	0.065±0.0017	1.9±0.05	6.9±0.26
B. ruziziensis	Kennedy	1.6±0.07	28.6±2.5	0.057±0.0031	2.0±0.11	7.8±0.49
B. ruziziensis	Miyaokikoku	2.0±0.04	28.1±1.5	0.074±0.0027	2.3±0.10	8.8±0.25
B. hybrid	Mulato	1.8±0.02	23.5±0.7	0.081±0.0123	2.4±0.06	7.4±0.23
B. hybrid	Mulato II	1.7±0.03	24.1±1.5	0.073±0.0052	2.3±0.16	7.7±0.43

† Leaf-shape ratio = leaf width/leaf length.

‡ Leaf-stem index = leaf width/stem diameter.

§ Standard error of mean.

Table 2-6 Mean, range among genotypes, broad sense heritability, genetic coefficients of variation (CV_G), and significance of differences (*P*) between genotypes for *in vitro* dry matter digestibility (IVDMD) , dry matter weight, leaf/stem ratio, water content (WC) and leaf neutral detergent fiber (NDF) of 17 genotypes of brachiariagrass

Characters	Mean	Range†	h^2s‡	h^2m§	CV_G ¶	P
Whole plant IVDMD (%)	51.9	48.9–59.0	0.76	0.93	5.7	***
	49.0	42.9–54.4	0.77	0.93	5.9	***
	50.6	46.6–56.0	0.60	0.85	5.3	***
Leaf IVDMD (%)	53.3	47.8–61.8	0.72	0.91	6.2	***
	52.6	47.6–60.5	0.72	0.91	5.9	***
	52.9	47.9–61.2	0.69	0.93	5.9	***
Stem IVDMD (%)	51.1	44.0–59.5	0.87	0.96	9.2	***
	45.8	38.6–53.7	0.78	0.94	8.8	***
	48.4	42.2–56.6	0.72	0.91	8.4	***
Dry matter weight (g pot^{-1})	8.1	4.4–12.8	0.71	0.91	28.3	***
	14.6	9.0–19.6	0.56	0.84	18.1	***
	11.2	7.0–14.9	0.14	0.33	10.5	***
Leaf/stem ratio	2.6	1.2–7.4	0.85	0.96	58.1	***
	1.4	0.6–4.9	0.86	0.96	70.3	***
	2.1	0.9–6.2	0.56	0.77	49.6	***
Whole plant WC (%)	79.0	75.4–85.2	0.74	0.92	1.8	***
	78.4	74.8–81.8	0.63	0.87	2.1	***
	78.7	75.2–83.6	0.51	0.80	2.2	***
Leaf WC (%)	78.5	75.0–84.6	0.67	0.89	3.1	***
	78.2	74.3–81.8	0.68	0.90	2.6	***
	78.4	74.8–83.2	0.45	0.75	2.3	***
Stem WC (%)	80.6	76.2–86.8	0.83	0.95	3.4	***
	78.7	75.0–81.9	0.62	0.87	2.1	***
	79.7	75.6–84.4	0.56	0.81	2.4	***
Leaf NDF (%DM)	63.3	58.0–68.3	0.78	0.93	4.2	***
	64.2	59.9–69.3	0.89	0.97	4.0	***
	63.8	59.6–68.5	0.72	0.90	3.8	***

†Range of mean of each genotype.
‡ h^2_s is the broad sense heritability for single plant.
§ h^2_m is the broad sense heritability for mean.
¶ $CV_G = (\sqrt{\sigma^2_G} \times 100) / \text{mean}$.
*** Genotypes are significantly different at $P < 0.001$.
The first, second and third rows of each character are the values of the 1st harvest, 2nd harvest and combined of two harvests, respectively.

Table 2-7 Mean, range among genotypes, broad sense heritability, genetic coefficients of variation (CV_G), and significance of differences (*P*) between genotypes for morphological traits of 17 genotypes of brachiariagrass at the first harvest

Characters	Mean	Range†	h^2_s‡	h^2_m§	CV_G¶	*P*
Leaf width (cm)	1.5	1.1–2.0	0.91	0.98	16.4	***
Leaf length (cm)	31.3	20.4–41.9	0.72	0.91	18.9	***
Leaf shape ratio	0.052	0.028–0.081	0.79	0.94	27.3	***
Stem diameter (mm)	2.5	1.9–3.6	0.74	0.92	16.9	***
Leaf-stem index	6.3	4.5–8.8	0.80	0.94	17.8	***

Leaf shape ratio = leaf width/leaf length; Leaf-stem index = leaf width/stem diameter.

†Range of mean of each genotype.

‡ h^2_s is the broad sense heritability for single plant.

§ h^2_m is the broad sense heritability for mean.

¶ $CV_G = (\sqrt{\sigma^2_G} \times 100) / \text{mean}$.

*** Genotypes are significantly different at $P < 0.001$.

Table 2-8 correlation coefficients between traits of 17 genotypes of brachiariagrass

	Leaf† width	Leaf† length	Leaf† -shape ratio	Stem† diameter	Leaf† -stem index	Leaf/ stem ratio	Whole plant WC	Leaf WC	Stem WC	Leaf NDF	Dry matter weight	Whole plant IVDMD	Leaf IVDMD	Stem IVDMD
Leaf width		-0.17^{ns}	0.66^{**}	0.39^{ns}	0.55^{*}	0.56^{*}	0.51^{*}	0.56^{*}	0.59^{*}	-0.58^{*}	-0.19^{ns}	0.59^{*}	0.34^{ns}	0.64^{**}
Leaf length	-		-0.81^{***}	0.41^{ns}	-0.58^{*}	-0.34^{ns}	0.06^{ns}	-0.02^{ns}	0.07^{ns}	0.57^{*}	-0.10^{ns}	-0.49^{*}	-0.55^{*}	-0.26^{ns}
Leaf-shape ratio	-	-		-0.14^{ns}	0.78^{***}	0.66^{**}	0.34^{ns}	0.43^{ns}	0.39^{ns}	-0.73^{***}	-0.14^{ns}	0.72^{**}	0.57^{*}	0.62^{**}
Stem diameter	-	-	-		-0.53^{*}	0.21^{ns}	-0.08^{ns}	-0.11^{ns}	0.10^{ns}	0.13^{ns}	-0.03^{ns}	-0.16^{ns}	-0.36^{ns}	0.14^{ns}
Leaf-stem index	-	-	-	-		0.34^{ns}	0.59^{*}	0.65^{**}	0.50^{*}	-0.70^{**}	-0.20^{ns}	0.74^{***}	0.69^{**}	0.52^{*}
Leaf/stem ratio	-	-	-	-	-		0.31^{ns}	0.36^{ns}	0.52^{*}	-0.25^{ns}	-0.41^{ns}	0.45^{ns}	0.03^{ns}	0.77^{***}
Whole plant WC	-	-	-	-	-	0.28^{ns}		0.98^{***}	0.93^{***}	-0.50^{*}	-0.68^{**}	0.71^{**}	0.44^{ns}	0.72^{**}
Leaf WC	-	-	-	-	-	0.16^{ns}	0.95^{***}		0.89^{***}	-0.60^{*}	-0.65^{**}	0.79^{***}	0.54^{*}	0.74^{***}
Stem WC	-	-	-	-	-	0.48^{*}	0.86^{***}	0.67^{**}		-0.37^{ns}	-0.77^{***}	0.61^{**}	0.19^{ns}	0.82^{***}
Leaf NDF	-	-	-	-	-	0.19^{ns}	-0.46^{ns}	-0.55^{*}	-0.28^{ns}		0.08^{ns}	-0.80^{***}	-0.84^{***}	-0.43^{ns}
Dry matter weight	-	-	-	-	-	-0.11^{ns}	-0.40^{ns}	-0.32^{ns}	-0.40^{ns}	-0.20^{ns}		-0.45^{ns}	-0.03^{ns}	-0.70^{**}
Whole plant IVDMD	-	-	-	-	-	0.53^{*}	0.54^{*}	0.52^{*}	0.55^{*}	-0.55^{*}	0.15^{ns}		0.83^{***}	0.79^{***}
Leaf IVDMD	-	-	-	-	-	0.02^{ns}	0.47^{ns}	0.63^{**}	0.19^{ns}	-0.72^{**}	0.17^{ns}	0.69^{**}		0.32^{ns}
Stem IVDMD	-	-	-	-	-	0.66^{**}	0.43^{ns}	0.34^{ns}	0.58^{*}	-0.36^{ns}	0.17^{ns}	0.93^{***}	0.39^{ns}	

Leaf-shape ratio = leaf width/leaf length; Leaf-stem index = leaf width/stem diameter; WC = water content; NDF = neutral detergent fiber; IVDMD = *in vitro* dry matter digestibility.

† The morphological data were determined at the first harvest only.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = no significant difference ($P > 0.05$).

Values above diagonal are for the first harvest, and values below diagonal are for second harvest.

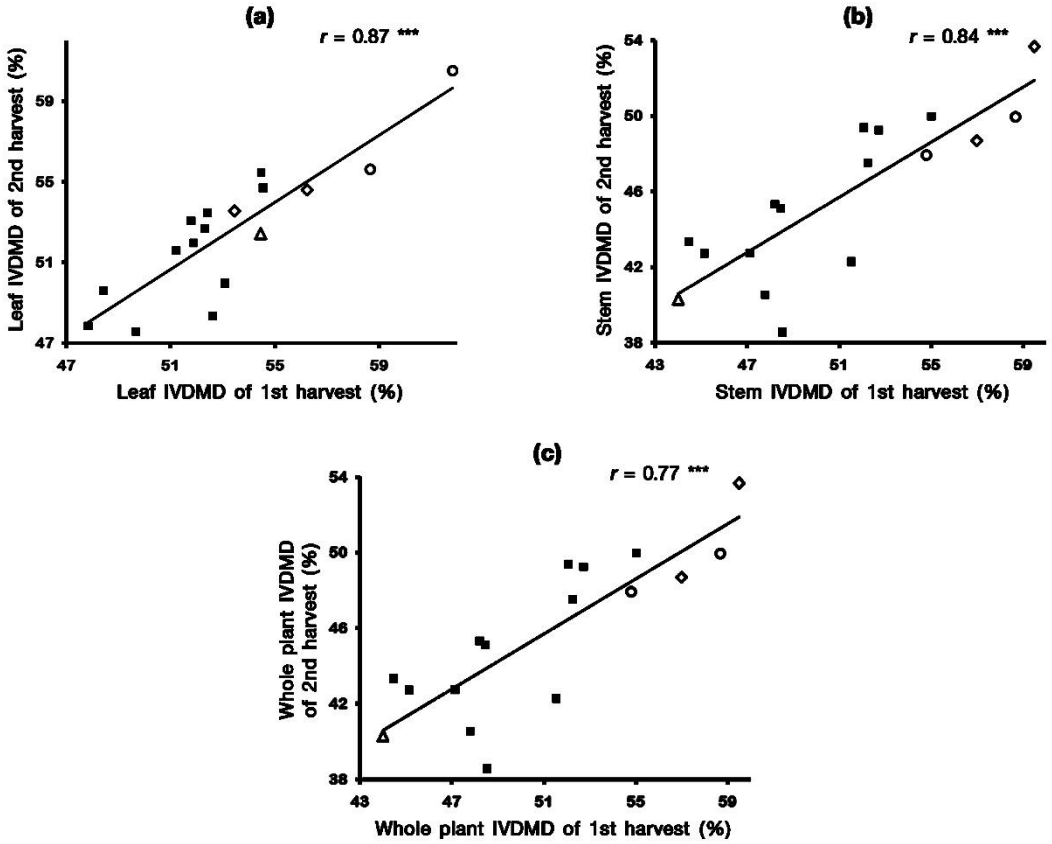

Figure 2-1 Correlations between *in vitro* dry matter digestibility (IVDMD) of first and second harvest of 17 genotypes of brachiariagrass [*B. brizantha* (■), *B. decumbens* (△), *B. ruziziensis* (○) and *B.* hybrid (◇)]: (a) leaf IVDMD; (b) stem IVDMD; (c) whole plant IVDMD. Significance: *** $P<0.00$

Discussion

Seventeen *Brachiaria* spp. genotypes were significantly different in all traits. IVDMD technique based on Pepsin-cellulase assay (Goto and Minson 1977) was used to determine DMD. It was found that both *B. ruziziensis* tended to have highest IVDMD especially 'Kennedy', and this result was similar to the works of Hughes *et al*. (2000) and Herrero *et al.* (2001). It demonstrates that *B. ruziziensis* is the most appropriate material for the breeding for increased IVDMD. However, although 'Kennedy', sexual diploid *B. ruziziensis*, showed the highest IVDMD, it could not be easily used as maternal plant in the hybridization breeding program due to the ploidy barrier between sexual diploid and apomictic tetrapolid genotypes (Miles *et al*. 2004). 'Miyaokikoku', a newly developed sexual tetraploid brachiariagrass (Ishigaki *et al*. 2009), should be more preferable in the conventional breeding through genetic recombination because it can be crossed with several apomictic tetraploid cultivars and accessions of *Brachiaria* spp.

The relationships between IVDMD and morphological characteristics, and water content were evident. The positive correlation between leaf width and IVDMD was similar to the study by Lentz and Buxton (1991) in orchardgrass (*Dactylis glomerata* L.). They reported that leaf and stem digestibility of the wide-blade clones were higher than those of narrow-blade clones, and the former group had fewer tissues that stained for

lignin as a percentage of cross-sectional area than did the latter group. Because lignin has been identified as primarily responsible for limiting digestibility of fiber (Jung 1989; Buxton and Redfearn, 1997); thus, the genotypes that show high concentration of lignin should have low DMD. However, in this present work, it was noticeable that leaf width did not correlate with leaf IVDMD. It was also found that leaf length correlated negatively with leaf IVDMD, and this finding was similar to the study in *Panicum maximum* by Batistoti *et al*. (2012). Buxton and Redfearn (1997) noted that grass leaves develop lignified midrib to provide mechanical support, therefore, the long-blade leaves genotypes may need higher lignin concentration to support their leaves. As it was noticed previously that leaf width had no relationship with leaf IVDMD in this study, it may be due to the effect of leaf shape ratio that is ratio of width to length of leaf blade. Because of the positive correlation between leaf shape ratio and leaf IVDMD, genotypes that even had wide leaf may had low IVDMD in leaf if their leaf shape ratio were too low. Furthermore, it was found that leaf NDF had relationship with leaf morphology especially leaf shape ratio (Table 2-5). Since grass cell wall is measured as NDF (Van Soest 1967; Theander and Westerlund 1993); thus, the shape of leaf blade may affect the concentration of cell wall. We also developed leaf-stem index that is the ratio of leaf width and stem diameter. This index showed high correlation with whole plant IVDMD; therefore, it

should be one of the promising selection criteria in *Brachiaria* breeding for increased DMD. Leaf-stem index was developed due to the positive correlation between leaf width and whole plant IVDMD and the trend of negative correlation between stem diameter and leaf IVDMD. Although these morphological traits were measured at only first harvest, they could be applicable to second harvest as the selection criteria because we found the high correlations between IVDMD of two harvests for leaf, stem and whole plant (Figure 2-1).

Leaf/stem ratio showed the positive correlation with whole plant IVDMD at only the second harvest in early summer season. This phenomenon was caused by greater difference between leaf and stem IVDMD in summer season than in winter season. Buxton (1996) noted that the development of plant in summer season is more than that of winter season. It indicates that leaf/stem ratio can be used as the selection criterion for DMD at the later maturity.

Water content was the other potential selection criterion because it showed high positive correlation with IVDMD at both harvests. Buckner *et al.* (1979) also reported the relation between these 2 traits in *Lolium-Festuca* hybrids. This relationship may due to the differences of the plant cell wall thickness. Cytoplasm of the cell consists of various organelles distributed in the cytosol, a matrix consisting of large amounts of water, protein,

other organic molecules, and ions (Levetin and McMahon 2008). Cell wall of higher digestibility genotypes may be thinner than those of lower digestibility group, and it leads to the larger fraction of cytosol including water in cell of the higher digestibility group. It was also found that leaf NDF negatively correlated with leaf water content (Table 2-5). Because NDF analysis is the estimation of cell wall content as we mentioned earlier, this result must support the above assumption that the thickness of cell wall influences cell water content.

IVDMD and all promising related traits showed high heritability. These results were different from the results obtained by Senanayake (1994), broad sense heritability of quality traits were moderate to low among narrow genetic variation of 10 genotypes of *B. brizantha*. The high heritability in this study could be resulted from highly controlled and uniform environment in greenhouse and pot-soil conditions. The promising related traits with high heritability such as leaf-stem index, leaf shape ratio and water content, demonstrates that the breeding of brachiariagrass for increased digestibility via indirect selection with the simple methods is possible.

Hughes *et al*. (2000) suggested that the ease, cost effectiveness, reproducibility, speed and available resources are the important factors to decide the appropriate selection criteria in breeding program. In this study, the morphological traits including leaf shape

ratio and leaf-stem index, and water content are the promising selection criteria because of the high correlation with IVDMD and high heritability. Both criteria are the simple and inexpensive method, and they need no way-out technique and equipment. The judgment of breeding achievement is enabled at high speed and considerable accuracy by applying one criterion of morphological traits, while, the analysis of water content takes slightly more time than that of morphology traits. However, these are different measurement methods, morphological traits are based on the length, whereas the water content is based on the weight. It was also found that leaf shape ratio, one of promising criteria, had no correlation with water content (Table 2-5). This result showed the tendency of the independence between these 2 types of traits; therefore, combined selection with these 2 different criteria should be considered as it could provide us more powerful selection method. In addition, the analysis of correlation of this experiment was performed with 4 species of *Brachiaria* spp. with different genetic background though they seem to be the same agamic complex (Valle and Savidan 1996; Jungmann *et al*. 2009). Therefore, in the further selection of hybrids with these different parents, the preliminary analysis of correlation between these promising traits and IVDMD should be conducted to clarify the appropriate selection criteria for the hybrids of each parent.

Conclusion

Sexual tetraploid ‘Miyaokikoku’ could be the most appropriate material in the hybridization breeding for increased DMD as it can cross with many tetraploid apomictic. It was also found that plant water content, leaf-stem index, and leaf shape ratio could be the promising simple selection criteria in *Brachiaria* breeding for increased DMD.

CHAPTER 3

Evaluation on dry matter digestibility stability of *Brachiaria* spp.

Abstract

The objectives of this study were to estimate the dry matter digestibility (DMD) stability, ability of plants to maintain DMD with advanced age, of *Brachiaria* germplasm. *In vitro* DMD (IVDMD), morphological traits and water content of 17 genotypes of *Brachiaria* spp. were evaluated in 5 harvesting times. 'Miyaokikoku', tetraploid ruzigrass (*B. ruziziensis*) showed the high leaf IVDMD stability with high IVDMD. *B. brizantha* CIAT 16306 showed the highest stem IVDMD stability with high IVDMD. 'Miyaokikoku' showed the highest whole plant IVDMD stability with high IVDMD, whereas 'Kennedy', diploid ruzigrass, showed very low whole plant IVDMD stability with high IVDMD. Leaf width positively correlated with stem and whole plant IVDMD stability. Stem diameter positively correlated with stem IVDMD stability. Leaf/stem ratio positively correlated with whole plant IVDMD stability. This work demonstrated that variation of IVDMD stability in both leaves and stem is available within *Brachiaria* germplasm; therefore, the development of new superior *Brachiaria* hybrids for high DMD stability is possible.

Introduction

The fact of dry matter digestibility (DMD) is that it decreases with age, and this effect causes the failure of pasture practices, which decreases animal production (Cowan *et al.* 1986; Humphreys 1991). Therefore, the selection and improvement of pasture cultivars for low DMD deterioration rate is quite important for the stable of animal production. It was found that pasture species is the significant factor affecting rate of DMD deterioration (Norton 1982). Minson (1990) calculated the mean rate of DMD deterioration of temperate and tropical grasses from many works, and it was found that DMD deterioration rate of temperate grasses was higher than that of tropical grasses. Humphrey (1991) stated that the rate of deterioration with age is greater for plants of high DMD than for plants of low DMD, but for any level of DMD there is considerable variation available in the rate of deterioration.

In this study, stability analysis was used to estimate the ability of plant to maintain its DMD with advanced age in various seasons. Becker and Léon (1988) concluded that stability composed of 2 concepts, static concept and dynamic concept. A stable genotype according to the first concept has constant performance over different environments. While dynamic concept permits predictable response to environments, and stable genotype according to this concept has no deviation from this response to environments.

For the present work, the static concept is more preferable to analyze the stability of DMD because it can reveal the constancy of DMD over the various plant ages. Regression analysis is one of the methods that can estimate stability of static concept (Becker and Léon 1988). Probably this technique was introduced by Stringfield and Salter (1934) firstly. Subsequently, it was developed by Finlay and Wilkinson (1963) and Eberhart and Russell (1966). The principle of the method is that a linear regression of individual genotype performance on the mean performance of all genotypes at each environment is computed (Finlay and Wilkinson 1963).

Norton (1982) found that *Brachiaria* spp. was one of the tropical grasses that had low rate of digestibility deterioration. However, any literature on DMD stability, among *Brachiaria* genotypes has not been available yet; therefore, the main objective of the study was to analyze DMD stability among commercial cultivars and promising accessions of *Brachiaria* spp. Moreover the study aimed to identify the plant characteristics that affect DMD stability of *Brachiaria* spp.

Materials and methods

Plant material

This experiment had continued from the experiment in Chapter 2. Seventeen

genotypes of *Brachiaria* spp. (Table 3-1) were used. Each genotype was clonally propagated with 3–4 tillers and transplanted in Wagner pot (0.02 m^2) containing 3 kg of air-dried soil (calcaric dark red soil, pH 7.7) in November 2012. The experimental unit was 1 plant per pot, and they was arranged in a completely randomized design with 4 replications and density of 16 pots m^{-2} in the greenhouse in the Field Science Center of University of the Ryukyus, Okinawa, Japan. Plants were clipped at 5-cm height after the establishment to make the uniformity in January 2013, and fertilized with 70 kg N ha^{-1}, 39 kg P_2O_5 ha^{-1} and 54 kg K_2O ha^{-1} as the basal application.

Sampling, measurement and chemical analysis

Each plant was harvested at 5 occasions by clipping at 5-cm height (see Table 3-2). The fertilizer was applied with the rate of 70 kg N ha^{-1}, 39 kg P_2O_5 ha^{-1} and 54 kg K_2O ha^{-1} after each harvest. At the first harvest, width at the widest position and length of three first fully expanded leaves were measured per pot. Stem diameter were also measured at the position just below first fully expanded leaves. Each sample was separated into stems (including leaf sheath and inflorescence) and leaves (blade), and fresh weight were measured, and oven-dried at 70 ^oC for 48 hours. The dry weight of each part was measured, and leaf/stem ratio was calculated. All samples were ground to pass through a 1-mm screen. The samples were analyzed for *in vitro* dry matter digestibility

(IVDMD) by the pepsin-cellulase assay (Goto and Minson, 1977).

Statistical analysis

The plant genotypes were random grouped for 4 replications, with all genotypes for each replication. In order to compare IVDMD stability in leaves and stems for each genotype, a linear regression of individual leaf and stem IVDMD on the mean whole plant IVDMD of all genotypes at each harvest was computed for each replication. The different between regression coefficient (*b*) of leaf and stem part of each genotype were calculated by one-way analysis of variance. For comparison of IVDMD stability of each part (leaves, stems) and whole plant among 17 genotypes, a linear regression of individual leaves, stems and whole plant IVDMD on the mean leaf, stem and whole plant IVDMD of all genotypes at each harvest was computed, respectively, for each replication. The interpretations of the data are as follows: Regression coefficient (*b*) approximating to 1.0 indicates average IVDMD stability. Regression coefficient increasing above 1.0 indicates low IVDMD stability. Regression coefficient decreasing below 1.0 indicates high IVDMD stability (Finlay and Wilkinson 1963).

The correlation (Pearson) between *b* value and leaf width, leaf length, stem diameter, and leaf/stem ratio was analyzed. The negative correlation between *b* value and each morphological trait was interpreted as positive correlation between IVDMD stability

and each morphological trait.

Table 3-1 Seventeen genotypes of *Brachiaria* spp.

Species	Cultivar	Accession†
B. brizantha	Marandu	CIAT6294
B. brizantha	Piata	-
B. brizantha	Xaraes	CIAT26110
B. brizantha	-	CIAT16113
B. brizantha	-	CIAT16306
B. brizantha	-	CIAT16315
B. brizantha	-	CIAT16316
B. brizantha	-	CIAT16467
B. brizantha	-	CIAT16488
B. brizantha	-	CIAT26124
B. brizantha	-	CIAT26318
B. brizantha	-	CIAT26990
B. decumbens	Basilisk	CIAT606
B. ruziziensis‡	Kennedy	CIAT605
B. ruziziensis§	Miyaokikoku	-
B. hybrid	Mulato	CIAT36061
B. hybrid	Mulato II	CIAT36087

† Genetic resources of International Center for Tropical Agriculture (CIAT).

‡ Diploid ruzigrass.

§ Tetraploid ruzigrass. The genotype of 'Miyaokikoku' in this study is No. 1-42.

Results

Mean IVDMD of all genotypes

Table 3-2 shows the mean IVDMD of 17 *Brachiaria* genotypes for each harvest. It was found that the first harvest showed the highest mean IVDMD for both leaves and stems, and whole plant, while the forth harvest showed the lowest mean IVDMD for whole plant.

Difference of IVDMD stability between leaves and stems

Seven genotypes had no difference of IVDMD stability between leaves and stems (Table 3-3). Eight genotypes showed higher IVDMD stability in leaves than in stems, with the largest difference in *B. brizantha* CIAT16113. Whereas, 'Marandu' *and* *B. brizantha* CIAT16306 showed higher IVDMD stability in stems than in leaves, with the largest difference in *B. brizantha* CIAT16306.

IVDMD stability among genotypes

Seventeen genotypes of brachiariagrass were classified in 4 groups for each plant part and whole plant (Figures 3-1, 3-1 and 3-3) as follows:

Group 1 was assigned as above-average IVDMD and above-average stability ($b < 1.0$).

Group 2 was assigned as above-average IVDMD and below-average stability ($b > 1.0$).

Group 3 was assigned as below-average IVDMD and above-average stability.

Group 4 was assigned as below-average IVDMD and below-average stability.

Stability of leaf IVDMD

Figure 3-1 shows four groups of *Brachiaria* spp. based on leaf IVDMD and their stability. In group 1, only 'Miyaokikoku' clearly showed high stability ($b = 0.67$) with high IVDMD (58.7%). 'Kennedy' showed the highest IVDMD (61.8%), but its stability was almost average ($b = 0.97$). In group 2, *B. brizantha* CIAT 16467 showed the lowest stability ($b = 1.24$) with slight above-average IVDMD (54.6%). In group 3, *B. brizantha* CIAT 26124 showed the highest stability ($b = 0.72$) with slight below-average IVDMD (51.8%). In group 4, *B. brizantha* CIAT 26318 showed the lowest stability ($b = 1.37$) with almost average IVDMD (52.6%).

Stability of stem IVDMD

Figure 3-2 shows four groups of *Brachiaria* spp. based on stem IVDMD and their stability. In group 1, *B. brizantha* CIAT 16306 showed the highest stability ($b = 0.58$) followed by 'Marandu' ($b = 0.67$) and 'Piata' ($b = 0.68$). IVDMD of *B. brizantha* CIAT 16306 was slightly high (55.0%), while those of 'Marandu' and 'Piata' were slightly

above average (52.7 and 52.1%, respectively). In group 2, *B. brizantha* CIAT 16113 showed the lowest stability (*b* = 1.45) with slight above-average IVDMD (51.5%). In group 3, *B. brizantha* CIAT 26124 showed the highest stability (*b* = 0.76) with slightly low IVDMD (48.2%). In group 4, *B. brizantha* CIAT 26318 showed the lowest stability (*b* = 1.49) with slightly low IVDMD (48.5%).

Stability of whole plant IVDMD

Figure 3-3 shows four groups of *Brachiaria* spp. based on whole plant IVDMD and their stability. In group 1, 'Miyaokikoku' showed the highest stability (*b* = 0.74) followed by 'Mulato II' (*b* = 0.73), *B. brizantha* CIAT 16306 (*b* = 0.81), 'Marandu' (*b* = 0.84) and 'Mulato' (*b* = 0.92), respectively. 'Miyaokikoku' and 'Mulato II' showed clearly high IVDMD (58.6% and 56.2%, respectively), whereas 'Marandu' showed slightly high IVDMD. Only 'Kennedy' was classified to group 2. It showed very low stability (*b* = 1.27) with high IVDMD (59.0%). In group 3, *B. brizantha* CIAT 26124 showed the highest stability (*b* = 0.76) with slight below- average IVDMD (50.6%). In group 4, *B. brizantha* CIAT 26318 showed the lowest stability (*b* = 1.46) with slight below-average IVDMD (51.1%).

Correlations between morphological traits and IVDMD stability

Figure 3-4 shows relationships between some morphological traits and IVDMD

stability. Leaf width positively correlated with stem and whole plant IVDMD stability ($r = -0.63$, $P < 0.01$; $r = -0.62$, $P < 0.01$, respectively). Stem diameter positively correlated with stem IVDMD stability ($r = -0.51$, $P < 0.05$). Leaf/stem ratio positively correlated with whole plant IVDMD stability ($r = -0.56$, $P < 0.05$).

Table 3-2 Mean *in vitro* dry matter digestibility (IVDMD) of leaf, stem and whole plant of 17 *Brachiaria* genotypes

	Date of harvest	Ages after regrowth (Weeks)	Average temperature ± S.D. (°C)	Mean IVDMD ± S.D. (%)		
				Leaf	Stem	Whole plant
Harvest 1	4 April 2013	12	19.2±5.2	53.3±3.3	50.8±4.7	52.0±3.1
Harvest 2†	30 May 2013	8	23.7±3.5	52.6±3.1	45.6±4.1	48.8±3.2
Harvest 3‡	25 July 2013	8	29.1±3.7	45.5±3.8	40.8±5.6	42.3±4.0
Harvest 4§	19 September 2013	8	29.7±3.9	45.6±3.6	39.4±5.1	42.1±3.6
Harvest 5¶	21 November 2013	9	25.1±4.4	52.7±3.2	49.2±3.0	51.3±2.7

S.D. = Standard deviation.

† *B. brizantha* CIAT 16467, CIAT 16488, CIAT 26318 and 'Basilisk' were at early flowering stage.

‡ *B. brizantha* CIAT 16315, CIAT 16488, CIAT 26318 and 'Basilisk' were at early flowering stage.

§ *B. brizantha* CIAT 16315, CIAT 16467, and 'Basilisk' were at early flowering stage.

¶ Kennedy' and 'Mulato' were at early flowering stage. S.D. = Standard deviation.

Table 3-3 Comparison of *in vitro* dry matter digestibility (IVDMD) stability in leaves and stems

Species	Cultivar/ Accession	IVDMD stability		
		Leaves†	Stems‡	Difference between leaves and stems
B. brizantha	Marandu	0.92	0.68	0.24 *
B. brizantha	Piata	0.81	0.69	0.13 ns
B. brizantha	Xaraes	0.86	0.76	0.10 ns
B. brizantha	CIAT16113	0.59	1.52	– 0.93 ***
B. brizantha	CIAT16306	0.98	0.57	0.40 *
B. brizantha	CIAT16315	0.73	1.23	– 0.49 ***
B. brizantha	CIAT16316	0.99	1.44	– 0.45 **
B. brizantha	CIAT16467	1.00	1.20	– 0.19 ns
B. brizantha	CIAT16488	0.95	1.05	– 0.10 ns
B. brizantha	CIAT26124	0.56	0.80	– 0.24 ns
B. brizantha	CIAT26318	1.15	1.54	– 0.39 ***
B. brizantha	CIAT26990	0.70	1.12	– 0.42 *
B. decumbens	Basilisk	0.80	0.91	– 0.12 ns
B. ruziziensis	Kennedy	0.79	1.27	– 0.48 ***
B. ruziziensis	Miyaokikoku	0.55	0.89	– 0.34 **
B. hybrid	Mulato	0.67	1.18	– 0.51 **
B. hybrid	Mulato II	0.79	0.92	– 0.13 ns

†Leaf IVDMD stability is the regression coefficient that was calculated by a linear regression of individual leaf IVDMD on the mean whole plant IVDMD of all genotypes for each harvest.

‡Stem IVDMD stability is regression coefficient that was calculated by a linear regression of individual stem IVDMD on the mean whole plant IVDMD of all genotypes for each harvest.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = no significant difference ($P > 0.05$).

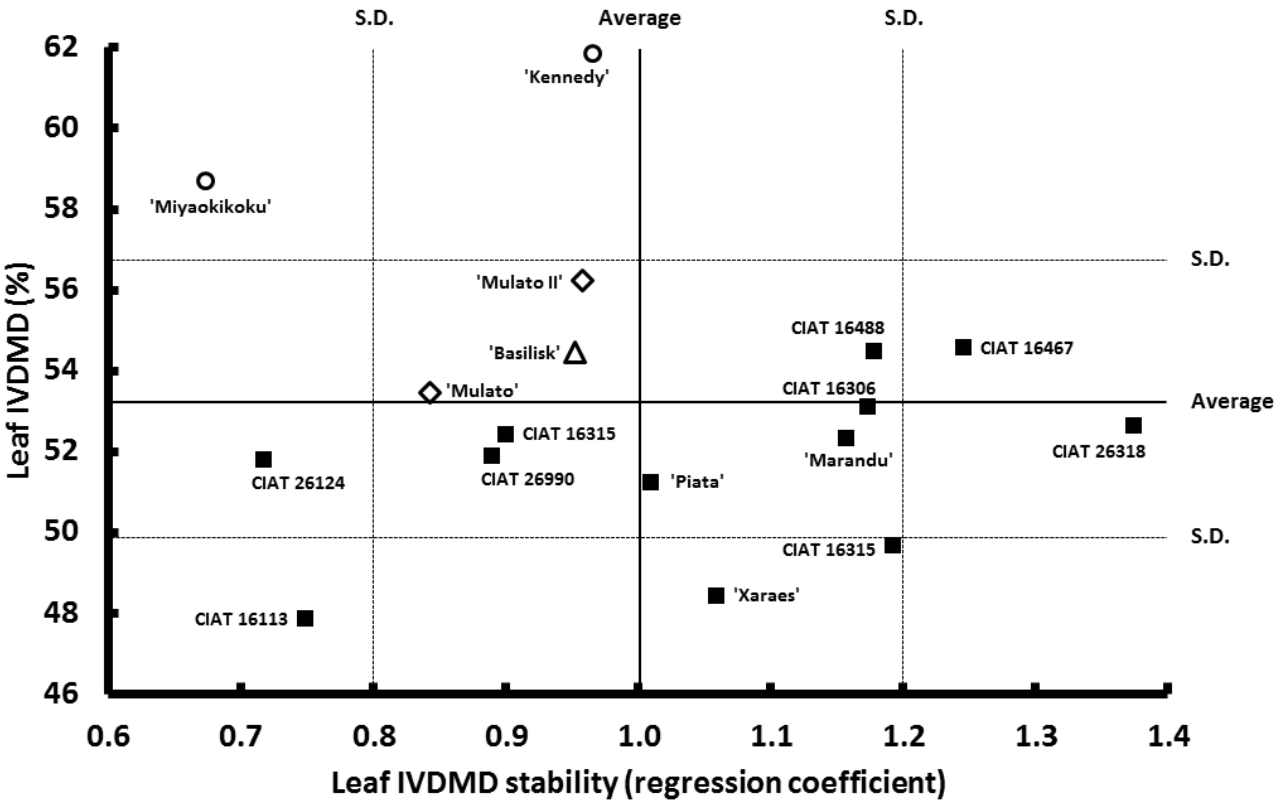

Figure 3-1 Relationship of leaf *in vitro* dry matter digestibility (IVDMD) stability (regression coefficient) and leaf IVDMD of the first harvest of 17 genotypes of brachiariagrass [*B. brizantha* (■), *B. decumbens* (Δ), *B. ruziziensis* (○) and *B.* hybrid (◇)].

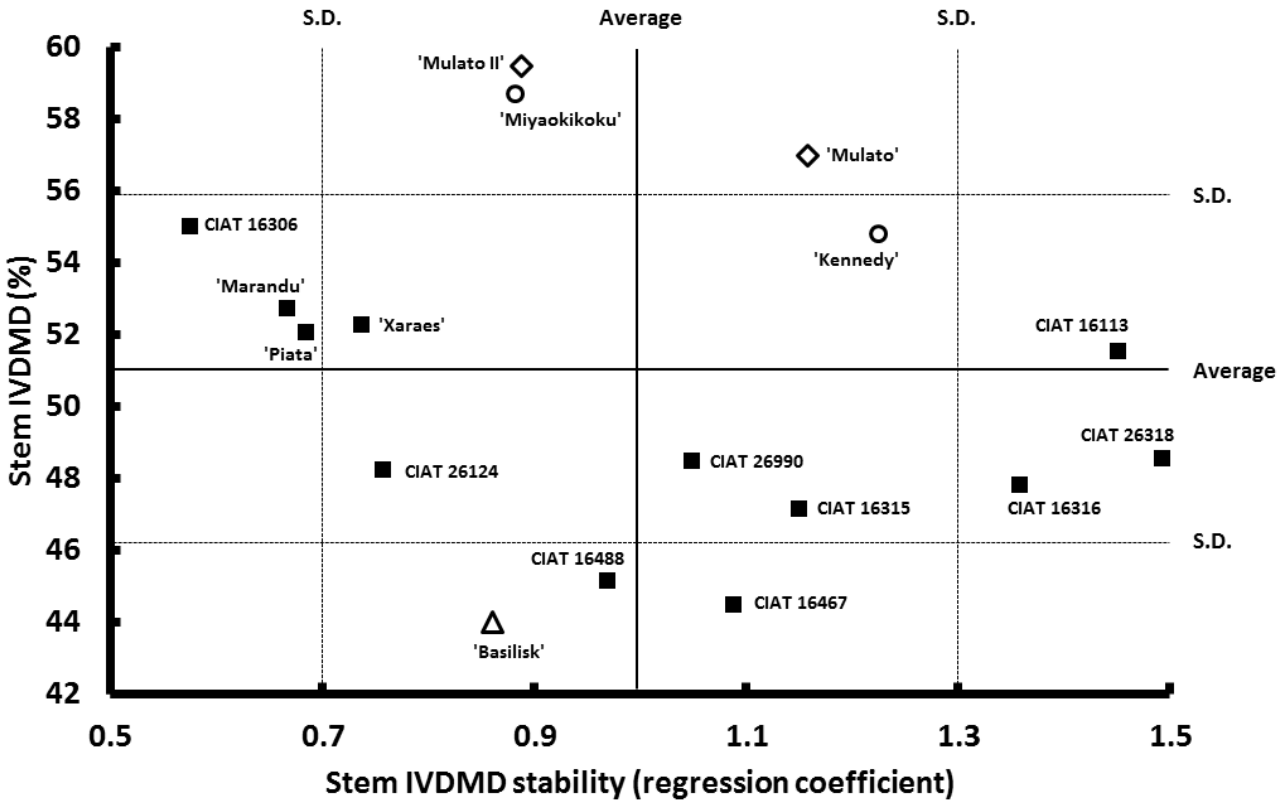

Figure 3-2 Relationship of stem *in vitro* dry matter digestibility (IVDMD) stability (regression coefficient) and stem IVDMD of the first harvest of 17 genotypes of brachiariagrass [*B. brizantha* (■), *B. decumbens* (Δ), *B. ruziziensis* (○) and *B.* hybrid (◇)].

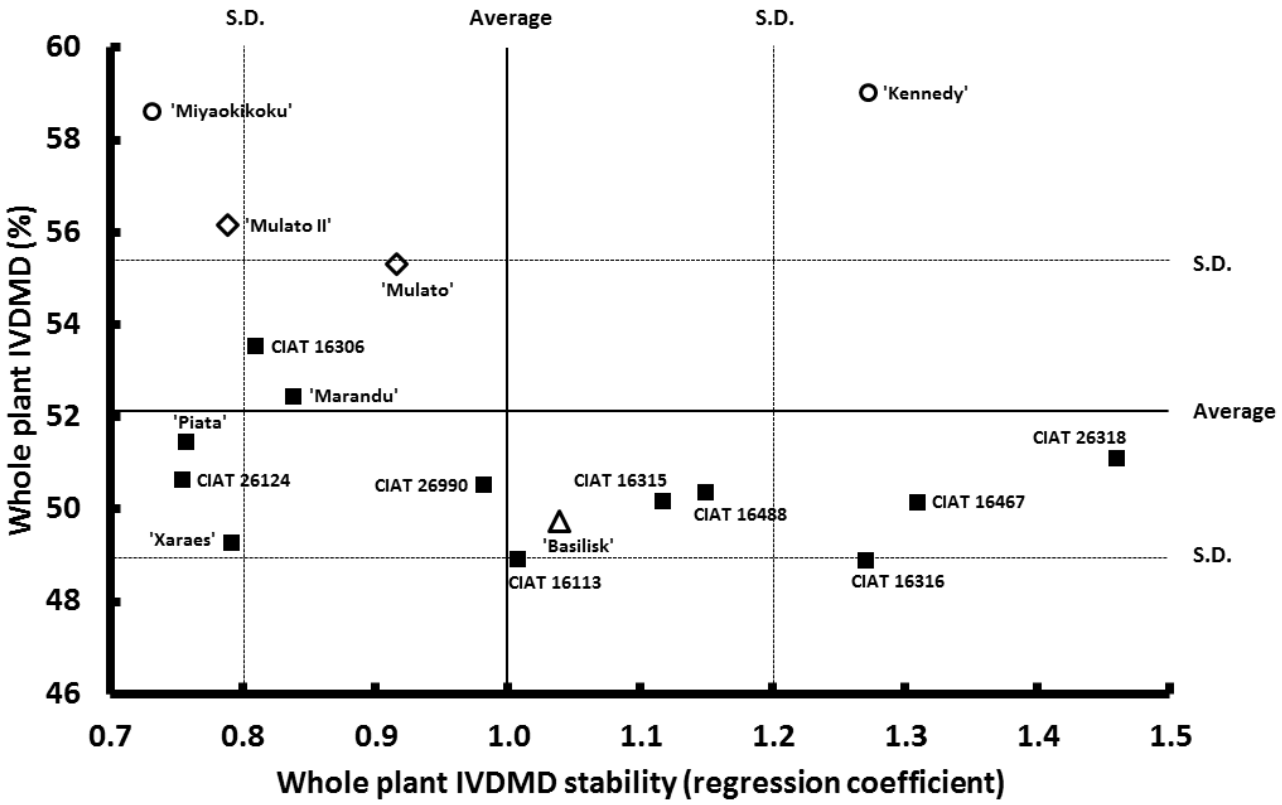

Figure 3-3 Relationship of whole plant *in vitro* dry matter digestibility (IVDMD) stability (regression coefficient) and whole plant IVDMD of the first harvest of 17 genotypes of brachiariagrass [*B. brizantha* (■), *B. decumbens* (Δ), *B. ruziziensis* (○) and *B.* hybrid (◇)].

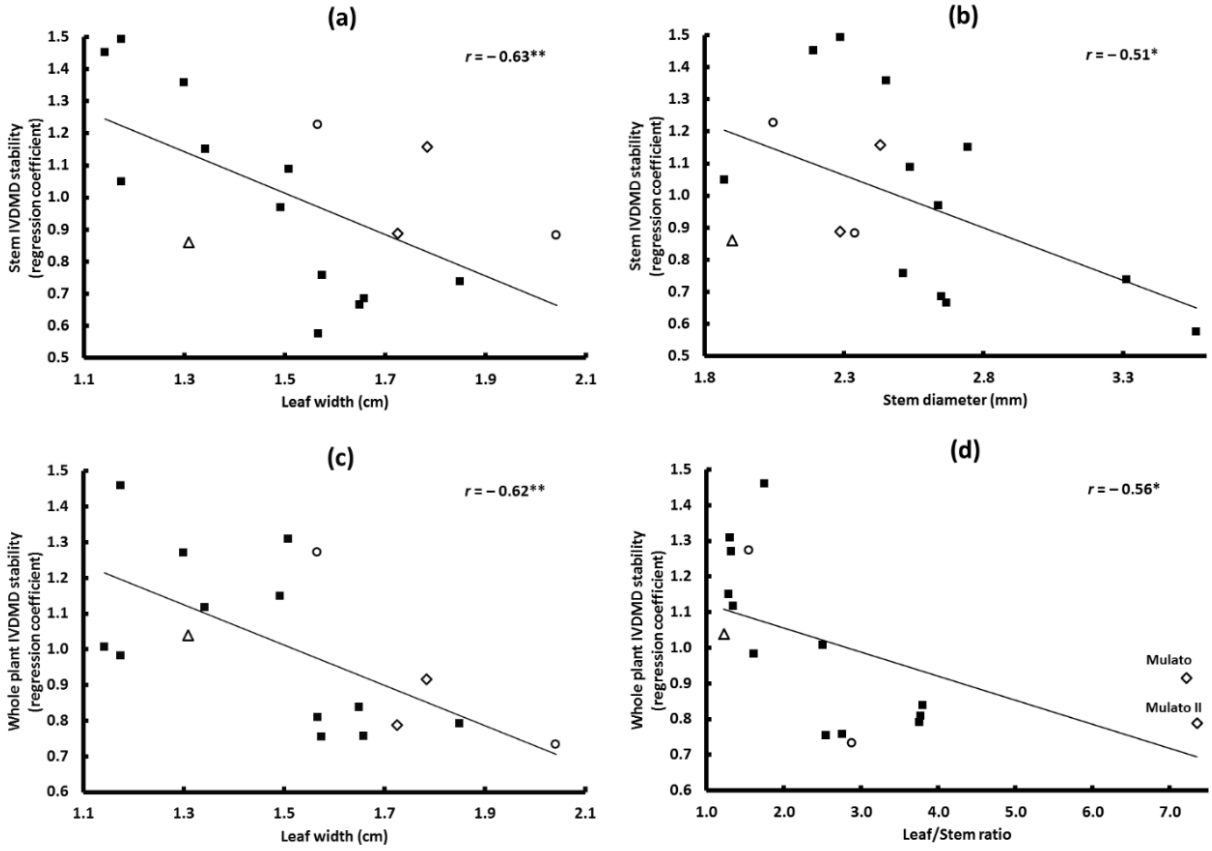

Figure 3-4 Relationship between *in vitro* dry matter digestibility (IVDMD) stability (regression coefficient) and related traits of 17 genotypes of brachiariagrass [*B. brizantha* (■), *B. decumbens* (Δ), *B. ruziziensis* (○) and *B.* hybrid (◇)]. Significance: * $P<0.05$; ** $P<0.01$. (a) Correlation between stem IVDMD stability and leaf width. (b) Correlation between stem IVDMD stability and stem diameter. (c) Correlation between whole plant IVDMD stability and leaf width. (d) Correlation between whole plant IVDMD stability and leaf/stem ratio of the first harvest. The negative correlation between regression coefficient and each morphological trait was interpreted as stability positively correlated with them.

Discussion

In this work, the term of 'DMD stability' has been proposed. High DMD stability is defined as low rate of DMD deterioration with maturity. Genotypes in group 1 could be the promising genotypes for high DMD with low deterioration rate. The graphs in Figures 3-1, 3-2 and 3-3 were drawn from the data between individual IVDMD stability and its IVDMD of the first harvest because it represented the youngest maturity stage due to highest mean IVDMD of all genotypes.

'Miyaokikoku', sexual tetraploid *B. ruziziensis* (ruzigrass), was classified to the superior group for both leaves and stems, and it clearly showed high whole plant IVDMD with high stability. While 'Kennedy', sexual diploid ruzigrass, was classified as high whole plant IVDMD with low stability. 'Miyaokikoku' genotype in this study had been developed by colchicines treatment of 'Kennedy' at Miyazaki University (Ishigaki *et al*. 2009) followed by one cycle of recurrent selection for high percentage of filled seeds at Okinawa Prefectural Livestock and Grassland Research Center. The large difference between these 2 relative ruzigrass in IVDMD stability is quite interesting, and 2 assumptions were hypothesized for this happening.

One may be the effect of ploidy on the DMD deterioration rate, since 'Kennedy' and 'Miyaokikoku' have difference ploidy level each other. In some works on temperate

grasses, they reported that tetraploids had higher DMD than their respective diploids (Kasperbauer *et al.* 1987; King *et al.* 1987); however, those works did not show the deterioration rate of DMD in their studies. The ploidy effect on the rate of DMD deterioration may be due to the slower growth rate in tetraploid, which may be caused by the disrupting effects of nuclear and cell enlargement, and the difficulty in mitosis process in polyploid organisms (Comai 2005). The effect of ploidy on growth rate of grasses has ever been reported by Evans (1967), who found that tetraploid Western Wolths ryegrass (*Lolium multiflorum*) had slower growth rate than respective diploid. As it is generally recognized that DMD of grasses decline as they mature (Skerman and Riveros 1990); hence, genotypes with slower growth rate should mature slowly, and its DMD should decline slowly as well. However, because 'Kennedy' is the open-pollinated cultivar (Cook *et al.* 2005), which consists of several genotypes, and the genotype that was used in this study was not the origin of 'Miyaokikoku'. Therefore, the further experiments are required to prove this assumption by comparing tetraploid ruzigrass with its respective diploid.

The second assumption is the effect of inbreeding depression on plant growth rate. Fehr (1987) defined inbreeding depression as the reduction in performance that is associated with an increase in homozygosity. Tamaki *et al*. (2007) showed that the

calculated breeding coefficients, parameter to predict degree of inbreeding depression, of synthetic strains of some forage species increased as the number of parental clones decreased. As described previously, the genotype of 'Miyaokikoku' that was used in our study was developed by recurrent selection with few genotypes of tetraploid ruzigrass; therefore, inbreeding depression may be occurred during the selection process. It was found that total dry matter weight of 5 harvests of 'Miyaokikoku' was lower than that of 'Kennedy', whereas Ishigaki *et al.* (2009) reported that the growth of tetraploid ruzigrass, the first generation of 'Miyaokikoku', was vigorous. This result could be the evidence of inbreeding depression in the advanced generation of 'Miyaokikoku'; however, it could not be concluded exactly whether low dry matter weight was due to slow growth rate or low dry matter weight potentiality. Moreover, the genotype of 'Kennedy' in this work was not the respective diploid of 'Miyaokikoku' as mentioned previously; hence, the further studies are also needed to prove this assumption. If it is proved to be true, the inbreeding method might be the alternative strategy to improve DMD stability, with the consideration of the decrease of dry matter yield.

However, if both assumptions are proved to be false, it means that the difference between these 2 genotypes in IVDMD stability may be due to the variation of this trait among 'Kennedy' population, so the opportunity to improve 'Kennedy' for high DMD

stability through recurrent selection is possible. Furthermore, it was found that IVDMD stability in leaves was higher than in stems for 'Kennedy', and leaf/stem ratio also correlated with stability of whole plant IVDMD; therefore, recurrent selection for leafiness could increase whole plant DMD stability of 'Kennedy' population.

The ideal cultivar having high DMD stability is that it has high DMD in both leaf and stem, with high stability in both parts. 'Miyaokikoku' showed the highest IVDMD stability in leaves with high IVDMD, but IVDMD stability in stems was slightly average, despite its high IVDMD; while 3 genotypes of *B. brizantha* including CIAT 16306, 'Marandu', and 'Piata' showed the highest IVDMD stability in stems, respectively. It was found that leaf and stem IVDMD stability had no relationship each other, and stem IVDMD stability had no relationship with stem IVDMD; thus, it revealed the independence between IVDMD stability in leaves and in stem, and between stem IVDMD stability and stem IVDMD. Therefore, genetic recombination between 'Miyaokikoku' and these 3 *B. brizantha* genotypes through conventional hybridization could provide the promising ideal hybrids with high DMD stability. However, the ploidy level of parents should be considered in the crossing. 'Marandu' is well-known tetraploid (Miles *et al.*, 2004), so it can be crossed with 'Miyaokikoku' successfully, whereas ploidy level of CIAT16306 and 'Piata' are still unknown to the best of our knowledge. It was also found

the positive correlation between leaf/stem ratio and whole plant IVDMD stability; hence, the introgression of leafiness trait to the excellent hybrids of 'Miyaokikoku' x 'Marandu' (or CIAT 16306 and 'Piata') may provide us even more excellent hybrid with high IVDMD stability. It was showed that 'Mulato II' and 'Mulato' had highest leaf/stem ratio, respectively (Figure 3-2), so they could be the appropriate sources of leafiness trait in the breeding program. It was also revealed that leaf width positively correlated with stem and whole plant IVDMD stability, and stem diameter positively correlated with stem IVDMD stability; therefore, they could be the promising selection criteria for DMD stability.

However, the development of cultivar for increased DMD stability may decrease dry matter yield, which would need to be considered for each pasture practice.

Conclusion

This work demonstrated that variation of DMD stability in both leaves and stem is available within *Brachiaria* germplasm; therefore, the development of new superior *Brachiaria* hybrids for increased DMD stability is possible. Leafiness, wide leaves and big stems could be the promising selection criteria for high DMD stability.

CHAPTER 4

Development of selection indexes for dry matter digestibility in *Brachiaria* breeding

Abstract

This study aimed to construct selection indexes for dry matter digestibility in *Brachiaria* hybrid population of 'Miyaokikoku' x 'Basilisk' using leaf water content and leaf morphological traits. Forty-nine individual hybrids were examined for *in vitro* dry matter digestibility (IVDMD), leaf water content (LWC), leaf width (LW), leaf length (LL) and leaf shape ratio (LR; LW/LL) in 5 leaves of the first fully expanded leaf. Values of LWC and leaf morphological traits were converted to be percentage of the maximum value of each trait. Selection indexes were constructed by combining adjusted values of each trait, together with different weight of trait according to the partial regression coefficient. Other 45 plants were sampled for validating selection index candidates using regression analysis of IVDMD values of validation set on selection index scores. The results showed that LWC-LW index (index value = 0.31 LWC + 0.10 LW) was the most appropriate selection index for DMD in population of 'Miyaokikoku' x 'Basilisk'.

Introduction

Other than dry matter digestibility (DMD), dry matter yield (DMY) is also important for animal production; hence, the development of new *Brachiaria* hybrids for increased DMD with high DMY should be more beneficial for livestock farmers. In Chapter 2, it was showed that both diploid and tetraploid *B. ruziziensis* had high DMD, but their DMW were slightly low; whereas, some genotypes such as *B. decumbens* cv. Basilisk had higher DMY with lower DMD. Thus, hybridization between sexual tetraploid *B. ruziziensis* and apomictic 'Basilisk' could provide new apomictic hybrids having high DMD with high DMY.

In the usual breeding programs, it is necessary to work with a large number of progenies to identify some superior plants as the candidates for new cultivar. Evaluating DMD of a large size of breeding population is very laborious and expensive; hence, the screening for promising high DMD plants with simple and reliable method could reduce the labor and cost in breeding program. For *Brachiaria* spp., Hughes *et al*. (2000) and Herrero *et al*. (2001) introduced physical plant strength including shearing strength and grinding resistance as selection criteria for pasture quality. In Chapter 2, it was found that some traits, such as leaf water content (LWC), leaf width (LW), leaf length (LL) and leaf shape ratio (LR; ratio of leaf width to length), could be the alternative potential selection

criteria for DMD, and it was suggested that the combination of plant water content and morphological traits could provide more powerful selection tool. The idea of the selection by the combination of several traits in our study was modified from the literatures of Hazel (1943) and Hazel and Lush (1943). Their concept is to select for all traits simultaneously by using a single selection index.

This study was examined for the construction of selection indexes for DMD in *Brachiaria* hybrids of 'Miyaokikoku' x 'Basilisk' population using LWC and leaf morphological traits as screening tool in the breeding for increased DMD.

Materials and methods

Plant material

A total of 200 F_1 hybrid plants were developed from a cross between *Brachiaria* 'Miyaokikoku No.128' and 'Basilisk' in Okinawa Prefectural Livestock and Grassland Research Center, Okinawa, Japan in 2012. The plants were transplanted at the 3rd -5th leaf stage in the experimental field with spacing of 1.5 m x 1.5 m in Miyako Island, Japan on November 30, 2013. Fertilizer was applied with the rate of of 70 kg N ha^{-1}, 39 kg P_2O_5 ha^{-1} and 54 kg K_2O ha^{-1} as the basal application. On May 4, 2014, plants were cut to adjust the plant stage.

Sampling, measurement and analysis of IVDMD

On June 14, 2014, 5 leaves of the first fully expanded leaf were sampled for 49 plants. They were kept in plastic bags and put in the icebox immediately after the sampling. Fresh weight of leaves in each plant, width at the widest position (LW) and length (LL) of each leaf were measured, and LR was calculated as leaf width/leaf length. Then, they were oven-dried at 70^{o}C for 48 hours. The dry weight of each plant part was measured, and LWC of each leaf sample was determined as ((fresh weight – dry weight)/fresh weight) x 100. All samples were ground to pass through a 1-mm screen and analyzed for *in vitro* dry matter digestibility (IVDMD) by the pepsin-cellulase assay (Goto and Minson 1977).

Construction of selection indexes

The LWC and leaf morphological traits were used to construct selection indexes for leaf IVDMD. In this purpose, all traits were adjusted to be the same unit, which was the relative value to the maximum values as 100. Therefore, LWC of sample leaf at 60% was scored at 85.7 by the calculation of 60 x 100/70% of the maximum LWC lines. Selection indexes were constructed by combining adjusted values of each trait together, with different weight of trait according to partial regression coefficient from multiple regressions of IVDMD values on adjusted values of LWC and leaf morphological traits

analyzed by STATISTIX 10.0. The model of index is as follow.

Index value = $b_1X_1 + b_2X_2 + b_3X_3 \ldots + b_nX_n$,

where $X_{1\text{-}n}$ are adjusted values of traits, and $b_{1\text{-}n}$ are partial regression coefficient of traits.

Validation of selection indexes

Other 45 plants were sampled on November 8, 2014 after cut for adjustment on September 23, 2014. The measurement of leaf morphological traits and IVDMD analysis were performed with the same way as the construction of indexes. The validity of selection index candidates were determined by regression analysis of IVDMD values on selection index values using STATISTIX 10.0.

Statistical analysis

The Shapiro-Wilk statistic (*W*-test) was used to assess the normality of averaged data. Skewness was used to measure the degree of asymmetry of a distribution. Both statistics were analyzed by STATISTIX 10.0.

Results

Construction of selection indexes

Table 4-1 shows that LWC, LW and leaf IVDMD had normal distribution, whereas

LL and LR did not due to the significant *W* values. In Table 4-2, apart from LL, three traits of LWC, LW and LR showed significant association with leaf IVDMD. LWC showed the highest regression coefficient followed by LW and LR. Only LWC, LW and LR were used for constructing selection indexes due to the significant association with leaf IVDMD. Table 4-3 shows partial regression coefficient from multiple regression of leaf IVDMD on LWC and leaf morphological traits. In each set of multiple regression, it was found that LWC showed the highest partial regression coefficient. Then indexes for LWC and LW combination (LWC-LW index), LWC and LR combination (LWC-LR index), and LWC, LW and LR combination (LWC-LW-LR index) were constructed based on their partial regression coefficient as 0.31 LWC + 0.10 LW, 0.30 LWC + 0.06 LR and 0.30 LWC + 0.07 LW + 0.04 LR, respectively. LWC-LW-LR index showed the highest R^2 value followed by LWC-LW index and LWC-LR index (0.39, 0.35, and 0.35, respectively).

Validity of selection indexes

In 45 plants of validation population, LR and leaf IVDMD showed normal distribution, while LWC, LW and LL did not, with skew toward high numerical values for LWC and LW, and that toward low numerical values for LL (Table 4-4). Table 4-5 shows that LWC, LW and all selection index candidates, except for LR, were significantly

associated with leaf IVDMD, with the highest R^2 of LWC-LW index followed by LWC-LW-LR index and LWC-LR index (0.35, 0.31, and 0.20, respectively), whereas LR was not.

Efficiency of selection indexes

Table 4-6 shows that selection for plants having value of any of LWC, LW, LWC-LW index, LWC-LR index, and LWC-LW-LR index above mean plus standard deviation could pick at least one promising high leaf IVDMD plant (2 plants for LWC and 1 plant for the others). Selection for plants having value of any of LWC, LW and LWC-LW-LR index above mean could pick at most 6 promising high leaf IVDMD plants.

Table 4-1 Descriptive statistics for leaf water content, leaf morphological traits and leaf *in vitro* dry matter digestibility (IVDMD) in index construction population (49 plant)

Trait	n	Mean±SD	Range	*W*†	Skewness‡
Leaf water content (%)	49	67.0±2.4	62.5–71.5	0.97^{ns}	–0.26
Leaf width (cm)	49	2.3±0.2	2.0–2.8	0.97^{ns}	0.58
Leaf length (cm)	49	33.0±3.8	29.1–40.0	0.94^{*}	–0.83
Leaf shape ratio§	49	0.070±0.011	0.055–0.102	0.89^{***}	1.21
Leaf IVDMD (%)	49	65.7±2.2	60.5–70.3	0.99^{ns}	–0.32

n= number of plants; SD= standard deviation.

† Normality of averaged data was tested using the Shapiro-Wilk test.

‡ Skewness of the frequency distribution. Negative deviations indicate a skew towards high numerical values.

§ leaf shape ratio= leaf width/leaf length.

Table 4-2 Single regression of leaf *in vitro* dry matter digestibility with related traits in index construction population (49 plants)

Traits†	Regression coefficient	R^2	*P*-value
Leaf water content	0.34	0.27	<0.001
Leaf width	0.12	0.14	<0.01
Leaf length	–0.04	0.04	≥0.05
Leaf shape ratio‡	0.08	0.14	<0.01

†Values were adjusted to be percentage of maximum value of each trait.

‡Leaf shape ratio = leaf width/leaf length.

Table 4-3 Multiple regression of leaf *in vitro* dry matter digestibility (IVDMD) on leaf morphological trait and LWC in index construction population (49 plants)

Trait†	Constant	Partial regression coefficient			R^2	*P*-Value
		LWC	LW	LR		
LWC and LW	28.50	0.31	0.10		0.35	<0.001
LWC and LR	33.09	0.30		0.06	0.35	<0.001
LWC, LW, LR	29.75	0.30	0.07	0.04	0.39	<0.001

LWC = leaf water content; LW = leaf width; LR = leaf shape ratio (leaf width/leaf length).
†Values were adjusted to be the percentage of the maximum value.

Table 4-4 Descriptive statistics for leaf water content, leaf morphological traits and leaf *in vitro* dry matter digestibility (IVDMD) in index validation population (45 plants)

Traits	n	Mean±SD	Range	*W*†	Skewness‡
Leaf water content (%)	45	79.3±1.4	74.2–81.5	0.94*	–1.02
Leaf width (cm)	45	2.1±0.2	1.5–2.5	0.94*	–0.70
Leaf length (cm)	45	32.3±4.1	25.4–46.7	0.95*	0.92
Leaf shape ratio§	45	0.067±0.009	0.05–0.087	0.96ns	0.36
Leaf IVDMD (%)	45	69.2±1.8	64.5–72.4	0.96ns	–0.49

n= number of plants; SD = standard deviation.

† Normality of averaged data was tested using the Shapiro-Wilk test.

‡ Skewness of the frequency distribution. Negative deviations indicate a skew towards high numerical values.

§ leaf shape ratio= leaf width/leaf length.

Table 4-5 Validity of selection indexes by the single regression of leaf *in vitro* dry matter digestibility on trait values and selection index values in index validation population (45 plants)

Traits/Selection indexes†	R^2	*P*-value
LWC	0.20	<0.01
LW	0.24	<0.001
LR	0.04	≥0.05
LWC-LW index	0.35	<0.0001
LWC-LR index	0.20	<0.01
LWC-LW-LR index	0.31	<0.0001

LWC = leaf water content; LW = leaf width; LR = leaf shape ratio (leaf width/leaf length).

LWC-LW index = 0.31 LWC + 0.10 LW

LWC-LR index = 0.30 LWC + 0.06 LR

LWC-LW-LR index = 0.30 LWC + 0.07 LW + 0.04 LR

† All trait values were adjusted to be the percentage of the maximum value of the respective trait.

Table 6 Efficiency of selection indexes for high leaf *in vitro* dry matter digestibility (IVDMD) in index validation population (45 plants)

Trait/Selection index	Number of promising high IVDMD plants†	
	Above mean+SD selection	Above mean Selection
LWC	2	6
LW	1	4
LWC-LW index	1	6
LWC-LR index	1	3
LWC-LW-LR index	1	6

SD = standard deviation; LWC = leaf water content; LW = leaf width;

LR = leaf shape ratio (leaf width/leaf length).

LWC-LW index = 0.31 LWC + 0.10 LW

LWC-LR index = 0.30 LWC + 0.06 LR

LWC-LW-LR index = 0.30 LWC + 0.07 LW + 0.04 LR

†Plants with higher leaf IVDMD than mean IVDMD + SD (total of 6 plants).

Discussion

In this study, regression analysis, which is generally used in quantitative trait loci (QTL) analysis (Edwards *et al.* 1987; Yamada *et al.* 2004), was applied to clarify the association between leaf IVDMD and promising related traits. The positive association between leaf IVDMD and LWC, and LR matched with the previous study in Chapter 2. However, the present study, which study in segregated population of 'Miyaokikoku' x 'Basilik', showed the positive association between LW and leaf IVDMD, and non-association between LL and leaf IVDMD, which was different with the result in Chapter2. Nevertheless, the association between LW and leaf IVDMD was similar to the study by Lentz and Buxton (1991) in orchardgrass (*Dactylis glomerata* L.).

Three indexes were constructed from combination of LWC and leaf morphological traits, without LL due to non-association with leaf IVDMD. It was found that R^2 value of all index candidates was higher than that of any single trait (see Tables 4-2 and 4-3). This showed the powerful combination between LWC and leaf morphological traits for selecting high IVDMD plants. The highest correlation weight of LWC revealed that it could be the major IVDMD related trait in *Brachiaria* hybrids of 'Miyaokikoku' x 'Basilisk', whereas LW and LR could be the minors. The other previous works in *Brachiaria* spp. proposed only single selection criterion for digestibility (Hughes

et al. 2000; Herrero *et al*. 2001). For other species, Wilson *et al*. (1989) had ever mentioned about selection of buffel grass (*Cenchrus ciliaris*) for increased DMD with several selection criteria together. However, they did not present the combination as one index in their report.

When selection index candidates were validated, it was found that LWC-LW index had the highest R^2 value rather than LWC-LW-LR. This was caused by non-significant association between LR and leaf IVDMD in validation set. In this study, the sampling of leaves for index validation set was conducted at the different time from index construction set, and after another cut. These may affect the leaf morphology, especially the length, which caused non-significant association between LR and leaf IVDMD, whereas LW was still significantly associated with leaf IVDMD. It showed that LR data may not be reliable over different plant age or management; therefore, it is not the proper trait for constructing selection index. The highest R^2 value of LWC-LW index in the validity test indicates that it could be the most appropriate index for screening of high DMD hybrids in the population of *Brachiaria* 'Miyaokikoku' x 'Basilisk' in terms of simplicity, accuracy and reproducibility. However, it was noticed that the distribution of some traits, including LWC, LW and LR, were different between populations. This showed that the population size in this study may be slightly small, which caused the

different results between both populations. Therefore, the increase of plant number of both populations could make the normal distribution for all traits, which could provide more reliable results.

For the decision of screening intensity, in case of a very large size of breeding population, selection for plants having value of LWC-LW index above mean plus standard deviation could be enough for screening of hybrids for promising high DMD plants, which could greatly decrease the labor and cost in the further DMD bioassay. However, if we work with smaller population size, it is suggested that the selection for plants having value of LWC-LW index above mean should be more preferable as it could grab more promising high DMD plants, with consideration of available labor and budget.

This was the pioneer work for development of selection indexes as the screening tool in the breeding of *Brachiaria* for increased DMD, which could be the first report of combining several DMD related traits to be the single selection index. Although the experiment was conducted with small number of plants, and with only leaf part, the results could exhibit the potentiality of using plant water content and morphological traits simultaneously as a single index for screening high DMD *Brachiaria* hybrids as an alternative simple and inexpensive tool in the breeding for increased DMD. For further works, the increase of plant number and addition of other simple DMD related traits into

the index could improve the accuracy and efficiency of the selection index.

Conclusion

LWC-LW index, which is 0.31 LWC + 0.10 LW, was the most appropriate selection index for leaf DMD in *Brachiaria* hybrids of 'Miyaokikoku' x 'Basilisk' population.

CHAPTER 5

Tightly clustered markers linked to an apospory-related gene region in *Brachiaria* hybrids

Abstract

This study aimed to construct an Amplified Fragment Length Polymorphism (AFLP)-based linkage map using single-dose fragment, and identify markers linked to an apospory gene in segregated population of sexual autotetraploid *B. ruziziensis* cv. 'Miyaokikoku' and apomictic hybrid cultivar 'Mulato'. The map contained 29 linkage groups with 272 markers. The apospory apomixis locus co-segregated with 12 tightly clustered AFLP markers, and mapped near the center of the linkage group 2. The segregation for the mode of reproduction fit the 1:1 simplex inheritance model (Aaaa). These markers will be valuable tools for marker-assisted selection in brachiariagrass improvement programs.

Introduction

Two conventional methods for assessing reproductive mode of apomictic species include progeny test and cytological analysis (Burson and Young 2001). One of the disadvantages of the first method is that it takes quite long time because it needs one more season for planting the progenies. Moreover, it is very laborious to determine uniformity of progenies with several plant characteristics. For cytological or embryo sac analysis, it takes shorter time compared with progeny test; however, in practical, this method needs the experienced person, who can recognize the difference between sexual and apospory embryo sac very well. In addition, it is also quite laborious same as the first methods. Therefore, the reliable molecular markers that are tightly linked to the gene conferring apomixis could be more preferable as it is the quicker and more reliable method.

The identification of molecular markers linked to interested trait is usually conducted along with genetic linkage map construction (Ebina *et al.* 2005, Zorzatto *et al.* 2010) as it could use the data for finding the markers linked to other important traits, and for advanced molecular researches. Linkage mapping is putting markers in order, indicating the relative genetic distances between them, and assigning them to their linkage groups on basis of the recombination values from all their pairwise combinations (Jones *et al.* 1997). There are 4 important steps in the construction of linkage map and

identification of markers linked to the traits, including 1) construction of mapping population, 2) development of probes (or markers) and identification of polymorphism, 3) construction of map using segregation analysis and statistical analysis, and 4) putting traits on the map by identifying the traits that segregate together with particular markers (Jones *et al.* 1997; Kumar 1999). Construction of linkage maps directly on polyploids, including most of *Brachiaria* spp., is inherently more difficult than on diploids as mentioned by Wu *et al*. (1992). Hence, they proposed the general method for mapping polyploids based on segregation of single-dose restriction fragments. The principle of this method is that a fragment represented by a single dose (or one allele) in a heterozygous plants will segregate in a 1:1 ratio (presence : absence) in the gametes, and only these single-dose markers (or simplex markers) are used for constructing linkage map. The success of linkage map construction by this technique has been reported in other polyploidy warm season grass such as *Panicum maximum* Jacq. (Ebina *et al*. 2005).

In this study, Amplified Fragment Length Polymorphism (AFLP) was applied for constructing linkage map. This technique, developed by Zabeau and Vos (1993), is patented by Keygene NV (Wageningen, The Netherlands) (Blears *et al*. 1998). AFLP is the selective amplification of restriction fragments from a digest of total genomic DNA using the polymerase chain reaction (PCR). With AFLP, the polymorphisms are identified

by presence or absence of DNA fragments following restriction and amplification of genomic DNA (Blears *et al*. 1998).

This study aimed to construct an AFLP-based linkage map in brachiariagrass by using a population developed from a cross between an obligate sexual autotetraploid *B. ruziziensis* cv. Miyaokikoku and the aposporous tetraploid *B.* hybrid cv. Mulato, and identify AFLP markers associated with the aposporous apomixis locus (*Apo*).

Materials and methods

Plant materials

Three plants of obligate sexual autotetraploid *B. ruziziensis* cv. 'Miyaokikoku' was crossed with one plant of apomictic hybrid cultivar 'Mulato' to generate a mapping population. The tetraploid maternal plants were obtained by means of *in vitro* colchicine treatment of multiple-shoot clumps and germinated seedlings of diploid *B. ruziziensis* (Ishigaki *et al.* 2009). 'Mulato' was used as the pollen parent for hybridization with 'Miyaokikoku' in a greenhouse at the Japan International Research Center for Agricultural Science (Tsukuba, Japan) in 2010. Plants from the mapping population were planted at a density of 1 plant per pot in the greenhouse at the National Institute of Livestock and Grassland Sciences (Tochigi, Japan) in 2011.

Assessment of the reproductive mode

The mode of reproduction (aposporous or sexual) was identified for each genotype by means of embryo sac analysis using methyl salicylate clearing and Nomarski differential interference contrast microscopy (Young *et al.* 1979; Nakagawa 1990). At least 20 embryo sacs were analyzed for each plant.

DNA isolation

Genomic DNA was extracted by a modified cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). Three to five g of fresh leaf tissue was ground into fine powder in liquid nitrogen and suspended the powder in 10 mL of boiled 2× CTAB buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0), with gentle shaking at 55℃ for 30 min. Then 10 mL of chloroform-isoamylalcohol (24:1 v/v) was added and gently shook the solution for 30 min at room temperature. After centrifugation (at 2500× g for 10 min at room temperature), The supernatant fraction was collected and mixed with 30 mL of precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, pH 8.0).The DNA was collected and resuspended in 5 mL of washing buffer (1 M NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0). After incubation at 55℃ for 16 h, three volumes of anhydrous ethanol were added. The precipitated DNA was collected and resuspended in 500 µL of Tris-EDTA(TE) buffer (10 mM Tris-HCl pH

8.0, 1 mM EDTA pH 8.0) containing RNase at 55°C for 16 h. The DNA concentration was determined spectrophotometrically by absorption of 320, 280 and 260 nm, and the DNA was stored at 4°C until use.

AFLP analysis

AFLP analysis was performed using the original protocol of Vos *et al.* (1995), with a slight modification. Five hundred ng of genomic DNA were digested with *Eco*RI/*Mse*I and ligated with the *Eco*RI and *Mse*I adaptors. Then preselective amplification by means of one-nucleotide extension of C for *MseI* site and A for *Eco*RI site was performed, with 25 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, with 72°C for 2 min of preliminary extension and 60°C for 30 min of final extension. Selective amplification was performed with 64 primer combinations of FAM-labeled *Eco*RI primers and nonlabeled *Mse*I primers, with three-nucleotide extensions as described in the AFLP Plant Mapping Protocol (Applied biosystems 2010). The selective amplification step was performed with a total of 35 cycles plus an initial denaturing step (20 s, 94°C) and a final extension step (2 min, 72°C). The duration of all annealing steps was kept constant at 30 s. The annealing temperature for the first cycle was 66°C, and the temperature was reduced by 1°C in each subsequent cycle to 57°C, after which a temperature of 56°C was maintained throughout the remaining 25 cycles, as described by

the AFLP Plant Mapping Protocol (Applied biosystems 2010). PCR was performed using a Gene Amp PCR System 9700 (Life Technologies Japan, Tokyo, Japan). Amplified DNA fragments were analyzed using the ABI-310 DNA sequencer and Gene mapper® software version 4.1 (Life Technologies Japan, Tokyo, Japan).

Linkage map construction

Informative AFLP fragments were scored as present (1) or absent (0) for the dominant marker. Only simplex markers that segregated at a 1:1 ratio from the 'Mulato' parental line were used for the linkage analysis. The χ^2 goodness-of-fit test was used to test whether the observed genetic segregation ratio fit the expected segregation ratio for tetrasomic monogenic inheritance. Individuals classified as an apomictic were labeled (H) for heterozygous, whereas those classified as a sexual were identified as (A), homozygous. Linkage analysis was performed for the complete set of AFLP markers using the F_2BC_1 model of MapMaker/EXP3.0 (Lander *et al.* 1987; Lincoln *et al.* 1992). Markers were grouped with an LOD (log-of-odds) score of 3.0, $\theta = 0.4$, and a maximum recombination fraction of 40%. Kosambi's mapping function was used to convert recombination units into genetic distances (Kosambi 1944).

Results

Evaluation of apospory

Eighty-four plants were generated from the crosses, and it was able to define the mode of reproduction of 69 plants. The segregation for the mode of reproduction in this population was 37 aposporous plants versus 32 sexual plants, which fits the model of a single dominant gene with tetrasomic monogenic inheritance (χ^2 =0.547, P>0.05). For the other 15 individuals, the mode of reproduction could not be identified based on embryo sac analysis with major reason of high sterility, but the plants were used in the subsequent linkage analysis, after being assigned a null score of apomixis locus. Individuals with aposporous embryo sacs were scored as exhibiting aposporous apomixis, despite the occasional appearance of a sexual embryo, as shown in Figure 5-1 (B4). In contrast, genotypes classified as sexual exhibited only sexual embryo sacs (Figure 5-1, A1 to A6). Polyembryony was frequently observed in the apomictic progeny (Figure 5-2, B5 and B6). No polyembryony was observed in the sexual progeny.

Linkage map construction

Twenty nine linkage groups were identified, which included 272 of the 274 possible markers (Figure 5-3). The linkage map had a total length of 1423.2 cM, with 272 loci and an average interval of 5.23 cM between markers. Two AFLP markers named M-

CTG/E-AAC_119 and M-CTG/E-AAG_263 could not be assigned to any linkage group.

Identification of molecular markers tightly linked to the apospory trait

Apo was successfully assigned to linkage group 2 (Figure 5-4). *Apo* co-segregated with 12 tightly clustered AFLP markers and mapped near the center of the linkage group. Two neighboring markers were located 2.4 cM downstream of *Apo*.

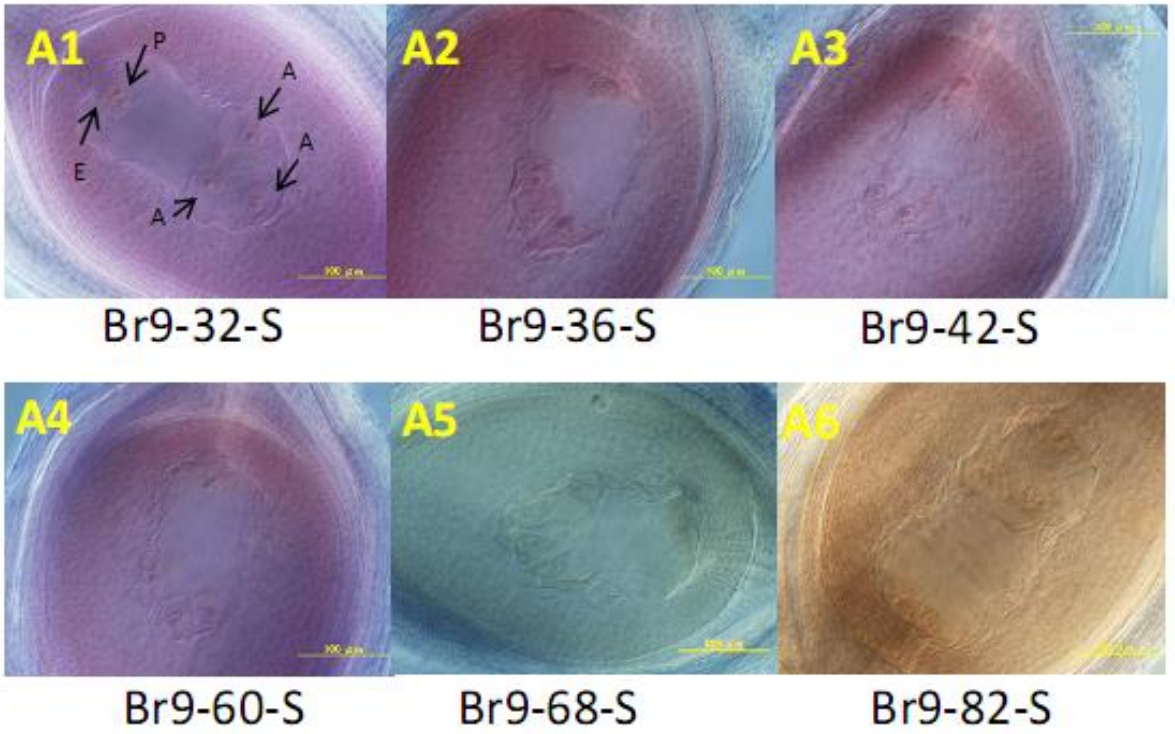

Figure 5-1 Embryo sac of sexual progeny. A, Antipodal; E, Egg cell; P, Polar nuclei.

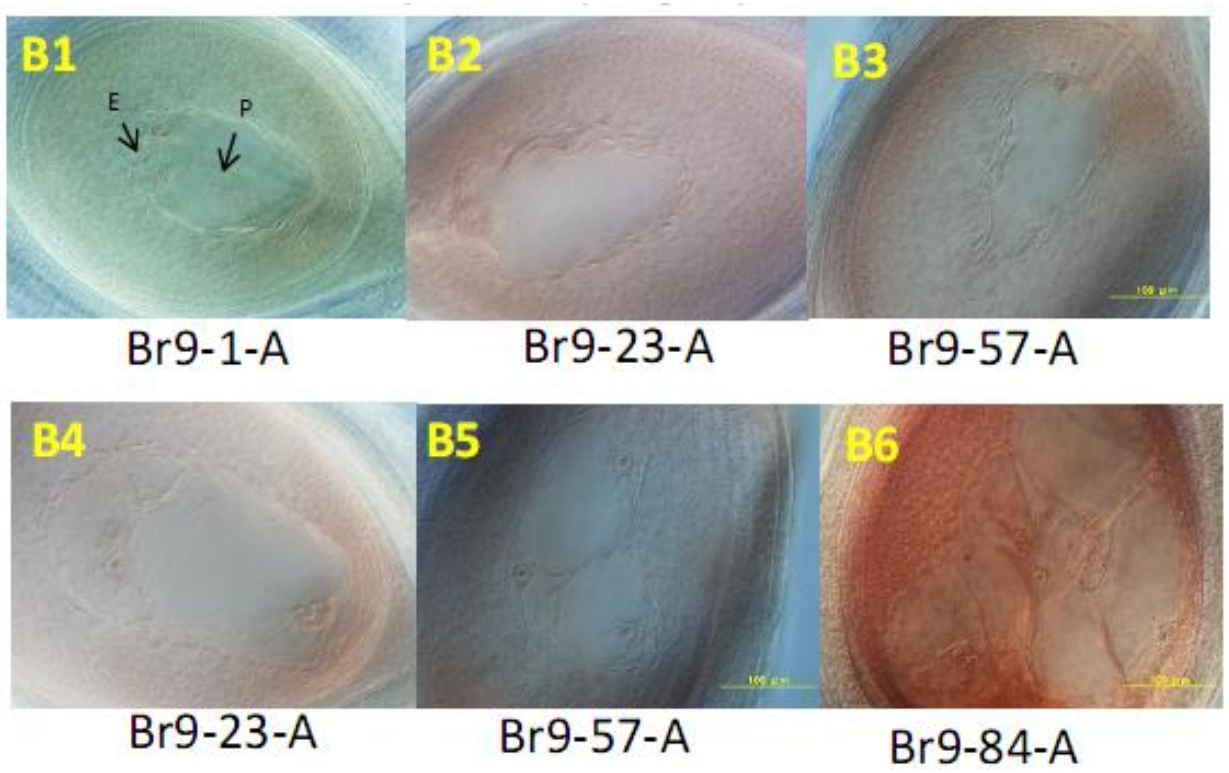

Figure 5-2 Embryo sac of apomictic progeny. B1–B3, apomictic embryo sacs ; B4, Sexual embryo sac; B5 and B6, Polyembryony embryo.

E, Egg cell; P, Polar nuclei.

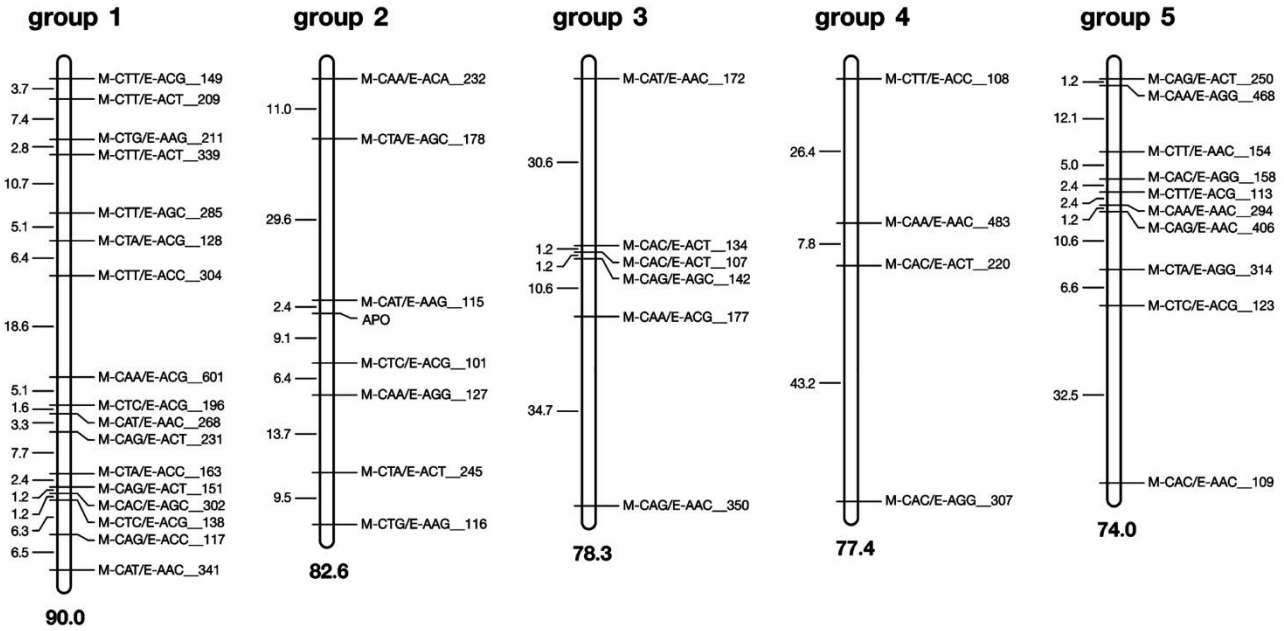

Figure 5-3 An AFLP linkage map of the aposporous brachiariagrass 'Mulato' pollen parent. Map distances in centimorgans (cM; Kosambi function) between each pair of markers appear on the left side of the linkage group. The marker loci are on the right side of the linkage group. The sizes of each linkage group are indicated at the bottom of the group. Marker nomenclature represents the name of the *Mse*I primer followed by the name of the *Eco*RI primer in the primer pair, followed by the primer size. For example, M-CTT/E-ACG_149 indicates an AFLP marker locus from the primer combination of *Mse*I-CTT and *Eco*RI-ACG that is 149 bp in size. The linkage groups (LG) are arranged according to their approximate size. Linkage analysis was performed using MapMaker/EXP3.0, with LOD (log-of-odds) = 3.0, $\theta = 0.4$, and maximum recombination fraction of 40%. The aposporous locus (*Apo*) was mapped to linkage group 2.

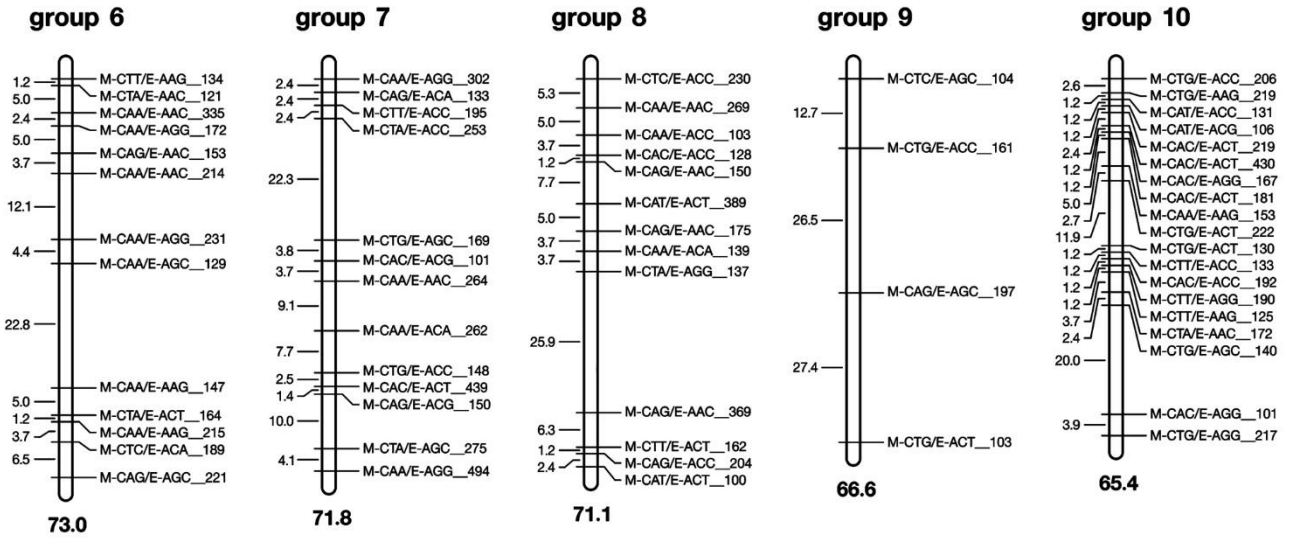

Figure 5-3 continued

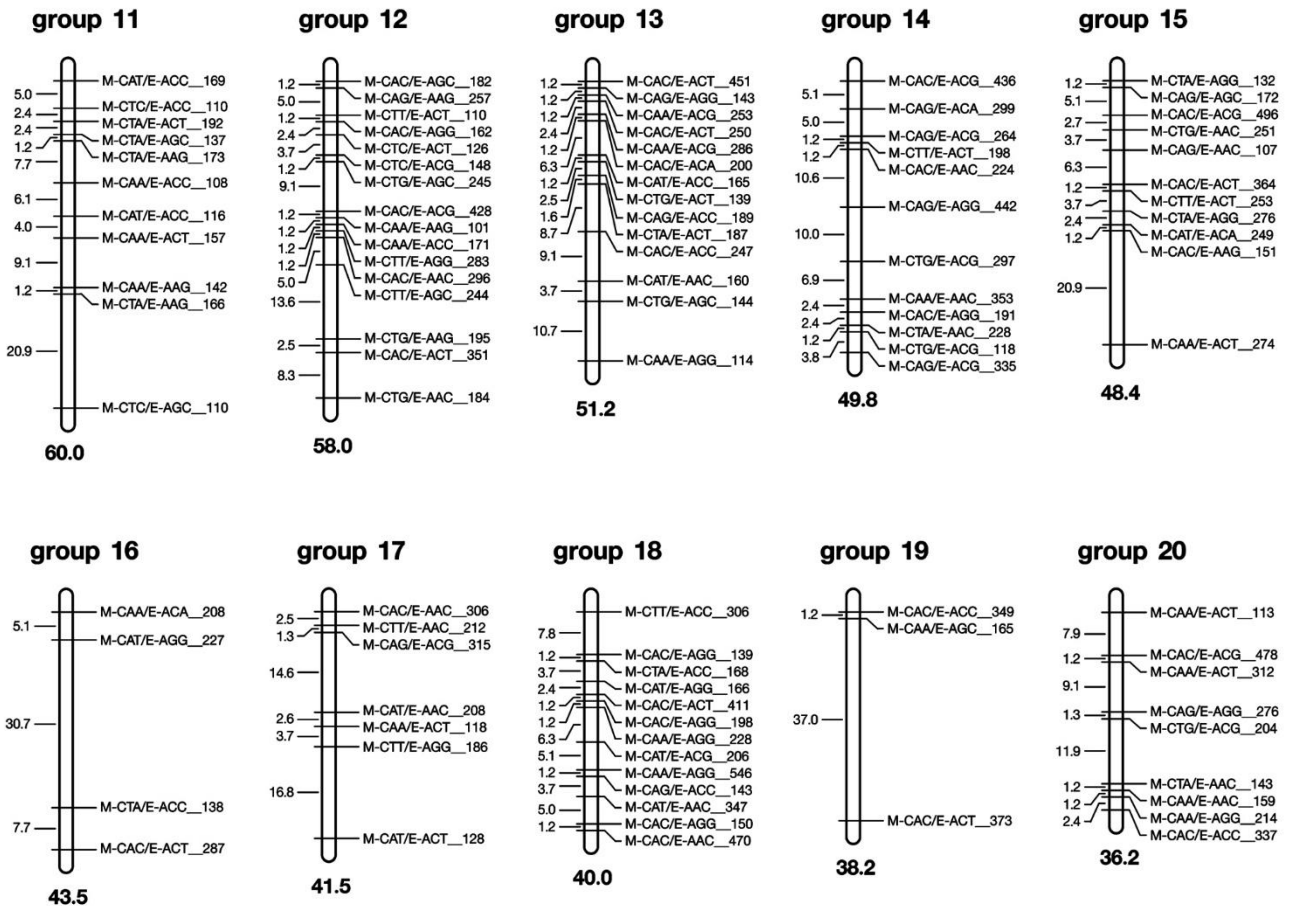

Figure 5-3 continued

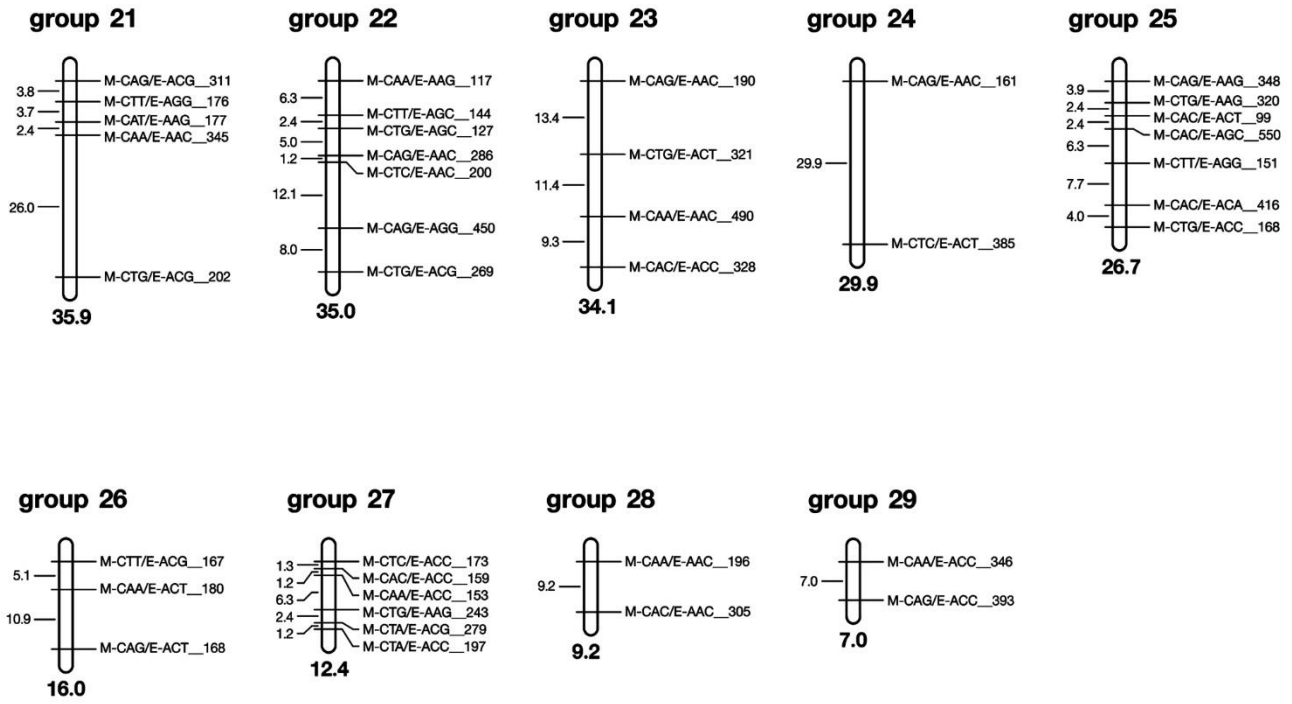

Figure 5-3 continued

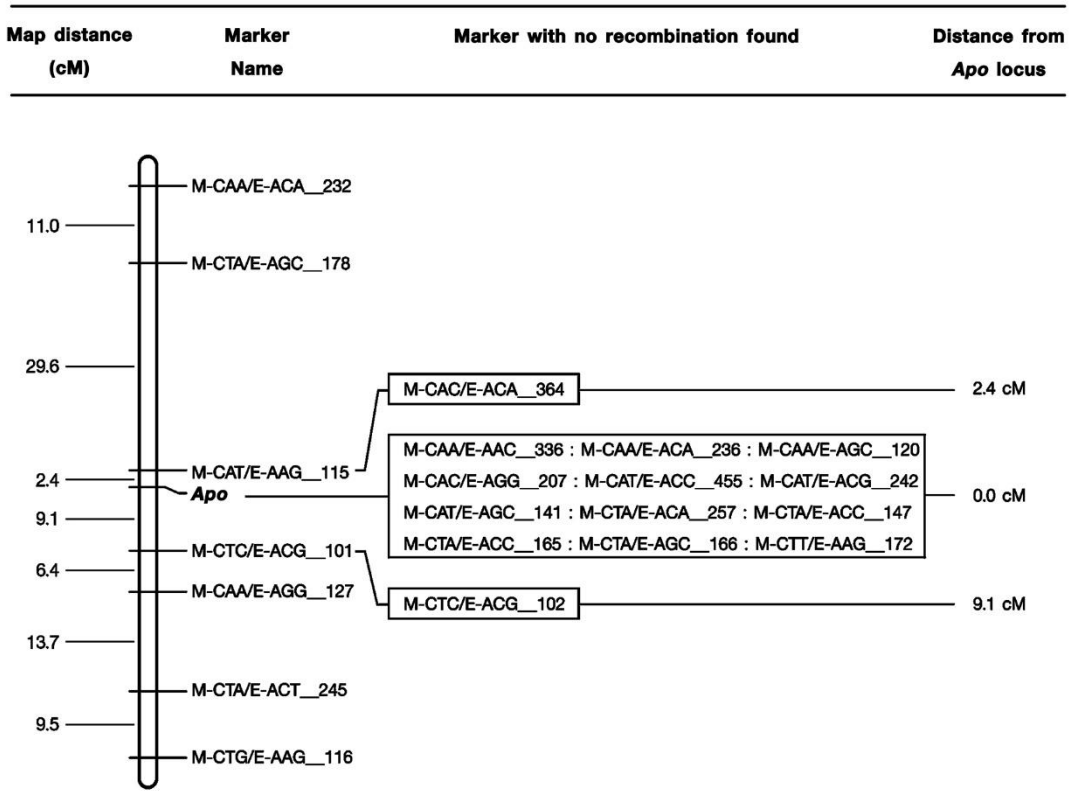

Figure 5-4 An expanded version of the region of linkage group 2 (Fig. 2) that contained the aposporous locus (*Apo*) and 12 tightly linked markers. Map distances are in centimorgans (cM; Kosambi function). Markers that co-segregated with *Apo*, with a zero frequency of recombination, are shown in the boxes at the right of the corresponding marker.

Discussion

This study showed the success of *Brachiaria* spp. AFLP-based linkage map construction using single-dose AFLP fragments. By this method, it was expected to observe 36 (2n) linkage groups based on the 36 chromosomes in 'Mulato' ($2n = 4x = 36$; Miles *et al.* 2004), rather than 18 (n), as any chromosome can contain a simplex allele (Al-Janabi *et al.* 1993). However, only 29 of 36 linkage groups were defined on this map; thus, more markers will be required to create a complete map.

It was found that a tight cluster of 12 AFLP markers co-segregated with apospory at a single *Apo* locus in linkage group 2. This result support the association of apomixis loci to large unique chromosomal regions, by showing suppression of recombination and hemizygosity in these regions (Ozias-Akins *et al.* 1998; Akiyama *et al.* 2004, 2011; Ebina *et al.* 2005; Okada T *et al.* 2011; Vasut *et al.* 2014). Furthermore, it was shown that the segregation for the mode of reproduction fit the 1:1 simplex inheritance model (Aaaa). Hence, this results support previous findings for other species, in which apospory is controlled by an apospory-specific genomic region with dominant inheritance (Pessino *et al.* 1997, 1998; Ozias-Akins *et al.* 1998; Barcaccia *et al.* 1998; Dewald and Kindiger 1998; Ebina *et al.* 2005).

The molecular markers tightly linked to apospory from this study will be useful

for screening apomicts in a *Brachiaria* breeding program. These AFLP markers can be used in the F_1 hybrids between *B. ruziziensis* and 'Mulato', and can also be used in crosses between *B. ruziziensis* and 'Marandu' because 'Mulato' is the product of these two parents (Miles *et al*. 2004).

Conclusion

An AFLP-based linkage map of brachiariagrass population of sexual tetraploid 'Miyaokikoku' and apomictic tetraploid 'Mulato' using single-dose AFLP fragments , fragments that segregate in a 1:1 ratio (present : absent) was constructed. Twelve AFLP markers co-segregated with apospory at a single *Apo* locus in linkage group 2. The segregation for the mode of reproduction fit the 1:1 simplex inheritance model (Aaaa).

CHAPTER 6

General Discussion

1. Appropriate materials for *Brachiaria* breeding for increased dry matter digestibility (DMD)

In this study, 17 genotypes of *Brachiaria* spp., including some promising accessions, currently used commercial cultivars, and a newly developed sexual tetraploid cultivar 'Miyaokikoku', were evaluated on DMD values, DMD stability, and their possibly related traits. Most of germplasm were genotypes of *B. brizantha* due to its wide distribution in the nature (Keller-Grein *et al.* 1996). The results showed that variation of all study traits were available. It was found that both ruzigrass, 'Kennedy' and 'Miyaokikoku', showed the highest DMD. It means that the development of new hybrid cultivar with higher DMD than ruzigrass by the conventional hybridization may be impossible. However, in practical, hybridization breeding of brachiariagrass for increased DMD means development of new hybrids with high DMD as ruzigrass combining other important traits, such as increased dry matter yield (DMY), disease resistance and drought tolerance.

The present study focused on breeding for increased DMD combining high

DMD. Although both ruzigrass had high DMD, they had slightly low DMY; therefore, the hybridization of ruzigrass with other potentially high DMY genotypes could provide us the promising candidates for increased DMD with high DMY. However, because of ploidy barrier between sexual diploid and apomictic tetrapolid genotypes, only tetraploid 'Miyaokikoku', not 'diploid Kennedy', can be used as maternal plant as it can be crossed with other apomictic tetraploid *Brachiaria* spp. It was found that some apomictic genotypes, such as CIAT 26318, 'Basilisk', and CIAT 16488, could be the appropriate paternal plant as they had high DMY (Table 2-3, Chapter 2). In Chapter 4, 'Basilisk' was used as the male parent because of the previously successful crossing with sexual plants (Mile *et al.* 2004).

DMD stability was also considered in the present work. 'Miyaokikoku', clearly showed high whole plant DMD with high stability. Therefore, the crossing between 'Miyaokikoku' and 'Basilisk' could provide new promising hybrids having high DMY with high DMD value and stability. Moreover, it was found that some genotypes such as 'Marandu' had higher stem DMD stability than 'Miyaokikoku'; hence, it could be material for improving stem DMD stability of the hybrids of 'Miyaokikoku' and 'Basilisk'.

2. Screening methods for DMD

The concept of the development of screening methods for DMD is to use alternative ways, which are simple, inexpensive, reproducibility, and rapid, for selecting the potentially high DMD progenies from a large breeding population because the standard methods of bioassay are very laborious and expensive. In Chapter 2, the relationship between DMD and other simple traits were clarified among various species of brachiariagras, and it was found that plant water content and some morphological traits had relationship with DMD. However, the appropriate traits for each segregated population of different parent may be not the same due to the different of genetic background. For example, it was found that leaf length had negative relationship with DMD in collected germplasm population (Chapter 2), whereas this relationship was not found in the segregated population of 'Miyaokikoku' x 'Basilisk' (Chapter 4). Therefore, the preliminary analysis of correlation between these promising traits and DMD should be conducted to clarify the appropriate selection criteria for the hybrids of each parent.

This work presented the success of development of selection index for DMD using the combination of some DMD associated traits in 'Miyaokikoku' x 'Basilisk' population. Therefore, this technique could be applied to the other segregated population of other parents.

3. Development of molecular markers linked to apomixis

In Chapter 5, the construction of AFLP linkage map of brachiriagrass in segregated population of 'Miyaokikoku' x 'Mulato' using single-dose DNA fragments was applied, and a tight cluster of 12 AFLP markers linked to apomixis gene were found in linkage group 2. Although, these AFLP markers can only be used for selecting apomict hybrids in segregated population of 'Miyaokikoku' x 'Mulato', and 'Miyaokikoku' x 'Marandu', the success of this technique could be adopted for finding markers linked to apomixes in other populations, including the population of 'Miyaokikoku' x 'Basilisk'.

4. Breeding strategies for increased DMD in *Brachiaria* spp.

As described in Chapter 1 that both open-pollinated and apomictic cultivars can be produced in brachiariagrass; therefore, the breeding strategies for increased DMD could be divided into 2 ways including recurrent selection for the former and hybridization for the latter. In addition, open-pollinated hybrid cultivar could also be produced by using sexual hybrid progenies.

4.1 Open-pollinated cultivar

To date, 'Kennedy' is the only one of open-pollinated cultivar (Cook *et al.* 2005). In this study, it was found that the genotype of 'Kennedy' showed the highest DMD. However, because only one 'Kennedy' genotype was used in this work; therefore, it could

not be concluded whether recurrent selection for increased DMD is possible or not. The future work is needed to identify the variation of DMD within 'Kennedy' population. If DMD variation is available within 'Kennedy' population, recurrent selection for increased DMD is feasible for 'Kennedy'. For each cycle of recurrent selection for increased DMD, selection index for DMD, which is the simple and inexpensive method, is very useful for screening for potentially high DMD genotypes. Furthermore, it was found that leaf DMD of 'Kennedy' was higher than its stem DMD (Chapter 2). This means that recurrent selection for leafiness could indirectly improve DMD of 'Kennedy' population. Moreover, it was also found that leaf DMD stability of 'Kennedy' was higher than its stem DMD stability (Chapter 3), therefore, recurrent selection for leafiness could increase DMD stability as well.

4.2 Apomictic cultivar

As mentioned earlier that this work focused on *Brachiaria* breeding for increased DMD with high DMY. It is clear that only 'Miyaokikoku', which has high DMD, can be maternal plant in the hybridization breeding because it is a sexual tetraploid, and 'Basilisk' could be one of the appropriate paternal plants due to its potentially high DMD. The ideal of superior hybrid should have high DMD as 'Miyaokikoku' with high DMY as 'Basilisk'. However, because, both DMD and DMY traits are controlled by

polygene (Saha *et al.* 2009; Serba *et al.* 2015); therefore, it is very difficult to get the ideal superior hybrid from single cross. Some cycles of backcross, with 'Miyaokikoku' or 'Basilisk' is used as recurrent parent, may be necessary to develop the ideal superior hybrid with high DMD and DMY. For each cross, the simple selection index for DMD is very useful for reducing the labor and cost in breeding program. In addition, the further developed AFLP marker linked to apomixis for 'Miyaokikoku' x 'Basilisk' population, using single-dose AFLP fragment technique, also useful for rapidly selected apomictic hybrids in each cross.

4.3 Open-pollinated hybrid cultivar

In hybridization breeding of 'Miyaokikoku' x 'Basilisk', the excellent apomictic hybrid with high DMD and DMY will be selected as the new cultivar. However, it is possible that some sexual hybrids will also have high DMD and DMY. Therefore, we could use these clones to produced new open-pollinated cultivar having high DMD and DMY, which could be the open-pollinated cultivar of brachiariagrass other than 'Kennedy'.

Summary

One of the most important traits for forage quality is dry matter digestibility (DMD). Genetic improvement in DMD of pasture generally resulted in improved an animal production. This study aimed to evaluate DMD and related traits of *Brachiaria* spp., which is one of the most important tropical pastures, for clarifying the most appropriate materials in the breeding for increased DMD. Moreover, the study aimed to develop the screening methods in the hybridization breeding for increased DMD. In addition, the objective of the study was also to develop molecular markers linked to apomixes gene as the quicker and reliable method to assess the reproductive mode in a segregating breeding population.

Evaluation on digestibility and the relation to morphology and water content of *Brachiaria* spp. germplasm and their heritability

The objectives of this experiment were to evaluate DMD of brachiariagrass germplasm as material in breeding program, and also to determine the appropriate selection criteria for high digestibility. *In vitro* digestibility (IVDMD) of 17 genotypes of *Brachiaria* germplasm, including 12 genotypes of *B. brizantha*, 1 genotype of *B.*

decumbens, 2 genotypes of *B. ruziziensis* (ruzigrass), and 2 genotypes of *B.* hybrid, ranged from 48.9 to 59.0 % in winter, and 42.9 to 54.4 % in early summer. It was found that both ruzigrass, diploid 'Kennedy' and tetraploid 'Miyaokikoku', tended to have highest IVDMD. For the relationship between traits, leaf-stem index (ratio of leaf width/stem diameter) showed the highest correlation with whole plant IVDMD (r = 0.74) followed by leaf shape ratio (ratio of leaf width/leaf length)(r = 0.72). Leaf water content (LWC) also highly correlated with whole plant IVDMD (r = 0.79). The broad sense heritability of IVDMD and the related traits of two harvest combinations were high with the range of 0.75 to 0.93. The results demonstrate that 'Miyaokikoku' could be the most appropriate material in the breeding for increased DMD due to the ploidy level and IVDMD value, and leaf-stem index, leaf shape ratio, and plant water content could be the useful selection criteria for *Brachiaria* breeding for increased DMD.

Evaluation on dry matter digestibility stability of *Brachiaria* spp.

The objectives of this experiment were to estimate the DMD stability, which shows an ability of plants to hold the decrease degree of DMD with advanced age, of *Brachiaria* germplasm by regression method, and to identify the plant traits that affect DMD stability. This experiment continued from the first experiment, with the total of 5

harvests. ‘Miyaokikoku’, showed the high leaf IVDMD stability (*b* = 0.67) with high IVDMD (58.7%). *B. brizantha* CIAT 16306 showed the highest stem IVDMD stability (*b* = 0.57) with high IVDMD (55.0%). ‘Miyaokikoku’ showed the highest whole plant IVDMD stability (*b* = 0.74) with high IVDMD (58.6%), whereas ‘Kennedy’ showed very low whole plant IVDMD stability (*b* = 1.27) with high IVDMD (59.0%). Leaf width positively correlated with stem and whole plant IVDMD stability. Stem diameter positively correlated with stem IVDMD stability. Leaf/stem ratio positively correlated with whole plant IVDMD stability. This work demonstrated that variation of DMD stability in both leaves and stems is available within *Brachiaria* germplasm; therefore, the development of new superior *Brachiaria* hybrids for increased DMD stability is possible.

Development of selection indexes as the screening method in *Brachiaria* breeding for increased DMD

This experiment aimed to construct selection indexes for DMD in ‘Miyaokikoku’ x ‘Basilisk’ population using leaf water content and leaf morphological traits combination. Forty-nine individual hybrids were examined for IVDMD, LWC, leaf width (LW), leaf length (LL) and leaf shape ratio (LR; LW/LL) of 5 first fully expanded leaves. For simple

standardize, values of LWC and leaf morphological traits were adjusted to be percentage of maximum value of each trait. Selection indexes were constructed by combining adjusted values of each trait together, with different weight of trait according to partial regression coefficient. LWC, LW and LR were positively associated with leaf IVDMD.

Three selection indexes were constructed as follows:

1) LWC-LW index (index value = 0.31 LWC + 0.10 LW), with R^2 of 0.35

2) LWC-LR index (index value = 0.30 LWC + 0.06 LR), with R^2 of 0.35

3) LWC-LW-LR index (index value = 0.30 LWC + 0.07 LW + 0.04 LR), with R^2 of 0.39. While R^2 of LWC, LW and LR were 0.27, 0.14 and 0.14, respectively. Other 45 plants in the same ‘Miyaokikoku’ x ‘Basilisk’ population were sampled for validating selection index candidates. The validity of selection index candidates was as follows: LWC-LW index (R^2 = 0.35); LWC-LR index (R^2 = 0.20); and LWC-LW-LR index (R^2 = 0.31). The results showed the potentiality of using plant water content and morphological combination as the index for screening high IVDMD *Brachiaria* hybrids.

Tightly clustered markers linked to an apospory-related gene region in *Brachiaria* hybrids

This experiment aimed to construct an AFLP-based linkage map and identify

markers linked to an apospory (a type of apomixis) gene in brachiariagrass using single-dose fragment, a fragment that segregates in a 1:1 ratio (present : absent). The obligate sexual autotetraploid *B. ruziziensis* cv. 'Miyaokikoku' was crossed with the apomictic hybrid cultivar 'Mulato' to generate a mapping population with 84 progeny. Sixty-nine plants out of 84 plants were able to define the mode of reproduction. The segregation for the mode of reproduction in this population was 37aposporous plants versus 32 sexual plants, which fits the model of a single dominant gene with tetrasomic monogenic inheritance ($\chi 2$ =0.547, P>0.05). The map contained 29 linkage groups with 272 markers. The linkage map of aposporous 'Mulato' had a total length of 1423.2 cM, with 272 loci and an average interval of 5.23 cM between markers. The apospory apomixis locus co-segregated with 12 tightly clustered AFLP markers, and mapped near the center of the linkage group 2. The segregation for the mode of reproduction fit the 1:1 simplex inheritance model (Aaaa). These markers will be valuable tools for marker-assisted selection in brachiariagrass improvement programs.

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要　約

牧草の品質に関わる形質のなかで最も重要な形質の 1 つとして，消化性を挙げることができる。乾物消化率 (Digestibility of dry matter，DMD) を遺伝的に改良した牧草種の草地へ導入は，一般的に家畜，主として反芻家畜の生産性の向上につながる。本研究は，主要な暖地型イネ科牧草であるブラキアリア属草種 (*Brachiaria* spp.) の DMD とその関連形質を評価し，育種技術で本草種の乾物消化率の向上を図るための育種母材選抜および交雑後代の選抜手法を開発しようとするものである。同時に，本草種の特徴的な主要形質であるアポミクシス形質の分子マーカーに着目し，育種集団における迅速な選抜を可能とするアポミクシスマーカーの探索を行った。

ブラキアリア属 (*Brachiaria* spp.) 遺伝資源における消化性と形態形質や水分含量との相関関係とそれらの遺伝率の評価

ブラキアリア属草種の遺伝資源の母材としての能力を評価するため，乾物消化率(DMD)に注目し，その母系の適切な選抜手法を *in vitro* DMD (IVDMD)を指標として開発した。供試したブラキアリア属草種の遺伝資源 17 系統の遺伝子型は，12 系統の *B. brizantha*，1 系統の *B. decumbens*，2 系統の *B. ruziziensis* (ruzigrass) および 2 系統の *B.* hybrid である。これらについて IVDMD を評価したところ，冬季では 48.9-59.0%，夏季は 42.9-54.4%でそれぞれ推移した。形態形質のうち，

葉幅/茎径長比 leaf-stem index (葉幅/茎径比)は，IVDMD と最も高い相関関係(r = 0.74)を示し，次いで，葉幅/葉長比 leaf-shape ratio が高い相関関係(r = 0.72)を示した。葉部水分含量 Leaf water content も同様に IVDMD と高い相関関係(r = 0.79)を示した。IVDMD およびこれらの相関する形質について，刈取り毎に広義の遺伝率を検討したところ，0.75-0.93 の範囲内ではあるものの，比較的高い値となった。

これらのことから，葉幅/茎径長比，葉幅/葉長比および葉部水分含量は消化率との間に遺伝率の高さが示され，高い消化性のブラキアリア属草種の育種選抜のための優れた指標となることを示している。

ブラキアリア属（*Brachiaria* spp.）遺伝資源における消化率の安定性評価

ブラキアリア属草種の遺伝資源において，生育に伴う消化率低下を抑える能力を指す，乾物消化率の安定性(DMD stability)につき，回帰分析法を用いて検討すると共に，DMD stability に影響する形質を明らかにすることを目的とした。

ブラキアリア属 17 系統の遺伝子型を用い，それぞれ計 5 回の刈取り調査を行った。DMD stability は *in vitro* DMD を測定し，IVDMD stability として表した。その結果，4 倍体ルジグラス (*B. ruziziensis*) ‘Miyaokikoku’ は最も高い葉部 IVDMD stability (b = 0.67) を示し，高い IVDMD (58.7%) であった。また，*B. brizantha* CIAT 16306 は最も高い茎部 IVDMD stability (b = 0.57) を示し，IVDMD (55.0%)も比較的高かった。 ‘Miyaokikoku’ はまた，最も高い全体の

IVDMD stability (b = 0.74) を示し， IVDMD (58.6%)も高かった。一方，2 倍体ルジグラス ‘Kennedy’ では，非常に低い全体 IVDMD stability (b = 1.27) を示したが，IVDMD は 59.0%と最も高かった。葉幅は全体 IVDMD stability と高い正の相関を示した。 茎径は茎の IVDMD stability と高い正の相関を示した。Leaf/stem ratio は全体の IVDMD stability と高い正の相関を示した。

***Brachiaria* 属牧草の乾物消化性の育種的向上のための選抜指標の開発**

ブラキアリア属の有性系統とアポミクシス系統との交配系統個体群において，乾物消化性に優れる系統個体を選抜するため，葉の水分含量（LWC）と葉の形態形質から選抜指標を構築し，高い IVDMD を示す選抜個体を評価することを目的としている．最初に，49 交配系統のそれぞれ 5 個体の展開葉から第 5 葉までの分析試料につき，IVDMD，LWC，葉幅（LW），葉身長（LL）及び葉形態比（LR; LW/LL） の測定値を基に，IVDMD を目的変数とし，1 形質あるいは複合形質の説明変数からそれぞれ回帰式を求め，選抜指標とした。その結果，LWC-LW index（0.31 LWC + 0.10 LW），LWC-LR index（0.30 LWC + 0.06 LR），及び LWC-LW-LR index（0.30 LWC + 0.07 LW + 0.04 LR）の決定係数 R^2 はそれぞれ 0.35，0.35 及び 0.39 となり，LWC，LW 及び LR 単独については 0.27，0.14 及び 0.14 となった。次に，これらの選抜指標の妥当性を評価するため，別の 45 交配系統において，上述のそれぞれの形質の測定を行い，LWC-LW index， LWC-LR index 及び LWC-LW-LR index により，ブラキアリア属の交

配系統のうちから，IVDMD の高い系統個体を選抜することが可能であることが示された。

ブラキアリア交雑種における近傍でクラスター化し連鎖しているアポスポリー連鎖領域

ブラキアリア属交雑種の品種育成をするうえで有利な形質であるアポスポリー（アポミクシスの一種）によるブラキアリア牧草の育種選抜を可能とするため，AFLP による連鎖地図構築を行い，育種選抜に有効なアポミクシスマーカーを探索した。AFLP 連鎖地図作成は，ブラキアリアが高次倍数体であるため，単式遺伝子型様式を 1:1 の遺伝分離で検定して行った。4 倍体有性生殖系統である *B. ruziziensis* cv. ‘Miyaokikoku’ とハイブリッドアポミクシス品種 ‘Mulato’ の交雑後代 84 個体を連鎖解析集団として解析した。その結果，アポスポリーは 37 個体（アポスポリー）と 32 個体（有性生殖）に遺伝分離し，同質 4 倍体での 1 優性遺伝子による遺伝支配モデルに一致 (χ^2 =0.547, P>0.05) した。272AFLP マーカーによる 29 連鎖群からなるアポスポリー花粉親 ‘Mulato’ の 1423.2cM に渡る連鎖地図 (マーカーの平均間隔 5.23cM) を構築することができた。アポスポリー連鎖領域は，連鎖群 2 の中央付近に位置し，12 の近傍でクラスター化した AFLP マーカーとともに特定することができた。これらのマーカーは，マーカー利用育種技術に応用することができると考えられた。