Chapter 1: General introduction

1.1: Exploiting heterosis in plant breeding

Heterosis—the superiority of a progeny to its parents—underpins many of the yield gains of maize (*Zea mays* L.) as well as other crops (Brummer 1999). The F_1 hybrids exploiting heterosis are outstanding and symbolic examples of agricultural progress in the latter half of the 20th century (Sleper and Poehlman 2006). Maize yield increases have been about 20% per decade during this period (Russell 1984). Although many studies (*e.g.* Stuber *et al*. 1992; Xiao *et al*. 1995; Cockerham and Zeng 1996; Li *et al*. 2001) have been conducted to investigate the genetic bases of heterosis, particularly in maize, no consensus exists about the genetic basis underlying this extremely important phenomenon (Li *et al*. 2008; Zhou *et al*. 2012). Three major genetic hypotheses, dominance, overdominance, and epistasis, have been proposed to explain heterosis. The first hypothesis of dominance attributes heterosis to the canceling of deleterious or inferior recessive alleles contributed by one parent, by beneficial or superior dominant alleles contributed by the other parent in the heterozygous genotypes at different loci (Jones 1917). The second hypothesis of overdominance attributes heterosis to the superior fitness of heterozygous genotypes over homozygous genotypes at a single locus (East 1936). The third hypothesis of epistasis attributes heterosis to positive epistatic interactions between nonallelic genes (Jinks and Jones 1958). Evidence from the results of studies presented above suggests that heterosis might be attributable to dominance (Xiao *et al*. 1995; Cockerham and Zeng 1996), overdominance (Stuber *et al*. 1992; Li *et al*. 2001), or epistasis (Li *et al*. 2001). In any hypothesis, the expression of heterosis requires crossing between genetically divergent parents (Brummer 1999).

According to Russell (1984), maize varieties released in the 1980s have 66% higher grain yield than those released in the 1920s. The long-term rapid yield improvement in maize has been obtained by reciprocal recurrent selection (RRS) procedures with heterotic groups rather than mere hybrid breeding alone. A heterotic group has been defined as a collection of germplasm that tends to exhibit a higher degree of heterosis when crossed with germplasm from an external group than when crossed with a member of its own group (Lee 1995). As related terms, combining ability—the ability to produce superior progeny—is used to identify desirable combinations of genotypes to cross in the breeding of hybrid cultivars or to identify desirable parents to include in a synthetic cultivar of a forage crop (Sleper and Poehlman 2006). General combining ability (GCA) is defined as the average performance of a strain in a series of crosses and is mainly attributable to additive effects. Specific combining ability (SCA) is defined as the performance of specific combinations of genetic strains in crosses in relation to the average performance of all combinations and is mainly attributable to non-additive effects. To exploit heterosis, a breeding approach that capitalizes on SCA using heterotic groups is more beneficial than that based on GCA (Scotti and Brummer 2010).

Most estimates of gains in forage yield in forage grasses or legumes are less than the gains in maize (Casler 1998; Brummer 1999; Wilkins and Humphreys 2003; Brummer and Casler 2009). A significant effect on heterosis or SCA has been found for several forage crops such as alfalfa (*Medicago sativa* L.), tall fescue (*Festuca arundinacea* Schreb.), smooth bromegrass (*Bromus inermis* Leyss.), perennial ryegrass (*Lolium perenne* L.), orchardgrass (*Dactylis glomerata* L.), and timothy (*Phleum pratense* L.) (Gau *et al*. 1989; Brummer 1999; Riday and Brummer 2002; Tamaki *et al*. 2007; Robins *et al*. 2012). Although heterosis breeding has not been used widely as a methodology for forage grasses or legumes because of ineffectiveness in commercial seed production resulted from their small perfect flowers and the lack of stable cytoplasmic male sterility, a potentially effective path to improving forage yield in the future is to capitalize on non-additive gene action by harnessing heterosis or SCA (Li and Brummer 2009), *i.e.* to create and use heterotic groups for yield improvement in forage grasses or legumes.

1.2: Marker-based genetic distances and their relation to yield in plants

Knowledge of germplasm diversity and relations is important for the genetic improvement of plants. Historically, attempts to estimate genetic distance (GD) or genetic diversity based on information of pedigree (Moll *et* *al*. 1965), phenotypes (Prasad and Singh 1986; He 1991), and isozyme polymorphisms (Frei *et al*. 1986; Strauss 1986), and attempts to predict heterosis have been reported for several plants. Molecular marker systems are useful tools for assessing genetic diversity within species (Varshney *et al*. 2005). Because of the initial lack of genetic information, non-species-specific marker systems such as random amplified polymorphic DNA and amplified fragment length polymorphisms have long dominated research on genetic diversity (Kölliker *et al*. 2009). These methods yield multilocus genetic data from single polymerase chain reaction (PCR) assays and therefore enable the investigation of numerous loci at low cost, although these methods must be treated as dominantly inherited (Kölliker *et al*. 2009). Consequently, co-dominant marker systems such as simple sequence repeat (SSR) markers have been developed in several plants. These markers have numerous benefits related to the ability to detect multiple alleles at one specific locus, a high rate of polymorphism, reproducibility and transferability, (Kölliker *et al*. 2009). Additionally, several markers located in coding regions such as expressed sequence tag and single nucleotide polymorphism have been developed, whereas genomic SSR might often be located in non-coding regions (Kölliker *et al*. 2009).

Several studies have described a positive correlation between marker-based GD and yield performance in maize (Marsan *et al*. 1998; Betrán *et al*. 2003; Reif *et al*. 2003; Qi *et al*. 2010), rice (*Oryza sativa* L.; Jaikishan *et al*. 2010), soybean (*Glycine max* [L.] Merr.; Barroso *et al*. 2003) and oilseed rape (*Brassica napus* L.; Riaz *et al*. 2001), although other studies have failed to find such a correlation for maize (Melchinger *et al*. 1992; Benchimol *et al*. 2000), wheat (*Triticum aestivum* L.; Liu *et al*. 1999), soybean (Cerna *et al*. 1997; Manjarrez-Sandoval *et al*. 1997), and potato (*Solanum tuberosum* L.; Bonierbale *et al*. 1993). Heterotic groups have been identified based on molecular marker diversity in maize (Enoki *et al*. 2002; Reif *et al*. 2003), chickpea (*Cicer arietinum* L.; Sant *et al*. 1999), and hop (*Humulus lupulus* L.; Townsend and Henning 2005). Although DNA marker loci assayed in many studies were not substantially associated with genes related to yield components including both additive and non-additive effects, genetic diversity of molecular markers can provide breeders helpful information to support yield improvement by pre-selecting parental combinations and by identifying heterotic groups to exploit heterosis or SCA (Reif *et al*. 2003).

Expression of heterosis in population crosses requires two conditions: directional dominance at loci controlling the trait of interest, and differing allele frequencies at those loci in the populations to be crossed (Brummer 1999). Genetic diversity therefore substantially influences the success of breeding in cross-pollinating forage crop species through various mechanisms such as heterosis, combining ability, and inbreeding depression (Kölliker *et al*. 2005). It is widely accepted that GD itself among parents is correlated with heterosis and synthetic variety performance in forage grasses and legumes (Veronesi *et al*. 2003). Attempts to find relations between marker-based GD and forage yields have been reported in forage grasses and legumes such as alfalfa (Kidwell *et al*. 1994, 1999; Tucak *et al*. 2011), white clover (*Trifolium repens* L.; Joyce *et al*. 1999), perennial ryegrass (Kölliker *et al*. 2005), tall fescue (Amini *et al*. 2011), and orchardgrass (Robins *et al*. 2012). Although failed studies of alfalfa have been reported (Kidwell *et al*. 1999), molecular markers are particularly helpful in identifying genetically similar germplasm when large germplasm collections exist but no heterotic groups are available (Reif *et al*. 2003). For timothy, no report in the relevant literature has characterized the relations between marker-based GD and forage yields. Genetic diversity *per se* is insufficient to show heterosis expression because highly divergent populations or certain cases of multiple allele populations might prevent the expression (Moll *et al*. 1965; Cress 1966; Bonierbale *et al*. 1993). Therefore, various materials on various mating design must be evaluated for relations between marker-based GDs and forage yields.

1.3: Breeding methods in forage grasses and legumes

Posselt (2010) described the structure of breeding methods in outbreeding forage grasses and legumes partitioning into three breeding phases: (i) procuring initial variation, (ii) forming varietal parents, and (iii) testing varieties. In brief, the first phase is the creation of the base population. The second phase is selection of the best individuals as the immediate parents of the first generation used to construct experimental varieties, or to create an improved breeding population. The third phase is construction and evaluation of experimental varieties. In the first phase, forage grass and legume breeders can select for base population from germplasm such as wild relatives, ecotypes, landraces (*e.g.* farm varieties), improved breeding materials (*e.g.* populations, families, clones), and released varieties. Pre-breeding might be necessary before the first phase if non-adopted materials were used. A high degree of genetic diversity within outbreeding forage populations might be desirable because genetic diversity might increase the adaptability and fitness of populations (Reed and Frankham 2003). In the second phase, several population improvement systems were adopted: recurrent restricted phenotypic selection, conventional half-sib progeny test, among- and within-family selection, and recurrent multistep family selection (Vogel and Pedersen 1993). The choice of methods of selection including mating designs in the first phase depends on the breeder, stage of breeding program, stage of germplasm development, and the stage of knowledge of the population and breeding objectives (Posselt 2010). In the third phase, population varieties of two types are distinguishable: open-pollinated varieties, which are the result of population improvement through recurrent selection, and synthetic variety. The development of synthetic varieties, in which a limited number of non-inbred parents possessing superior combining ability were intercrossed (Sleper and Poehlman 2006), was widely adopted in many forage grasses and legumes (Brummer 1999; Tamaki *et al*. 2007).

Historically, forage crop breeders have relied mainly on three approaches to improve forage yields: (i) post-synthesis selection, which involves developing numerous synthetic or open-pollinated varieties from selections made in spaced plantings or sward plots based on various morphological and maturity classes, and increasing seed to an advanced generation; (ii) uni-directional or bi-directional selection for a specific morphological or physiological trait with subsequent determination of its effect on sward-plot forage yield; and (iii) direct selection for forage yield *per se*, mostly on spaced plantings (Casler 1998). Of the three approaches, the post-synthesis selection has been the most widely used in breeding programs. It has achieved steady gains for forage yields (Casler 1998). Recently, genotypic selection systems using progeny test selection such as conventional half-sib progeny test, among- and within-family selection, and recurrent multistep family selection, while using only a fraction of additive genetic variance, have been recommended for producing long-term improvements in forage yield (Vogel and Pedersen 1993). A potentially effective path to improving future forage yields is to capitalize on non-additive gene action by harnessing heterosis or SCA (Chapter 1). Methods of hybrid production with the aim of capturing heterosis or exploiting SCA have been proposed for cross-pollinating species for which inbred development is not feasible because of inbreeding depression. These include the use of partial inbreeding to create free hybrids, synthetic populations, top-cross hybrids, semi-hybrids, modified synthetic variety, clone and strain synthesis (CSS), or the use of male sterility (Brummer 1999; Tamaki *et al*. 2007, 2009; Scotti and Brummer 2010; Robins 2012). Of these methods, a CSS approach, which exploits both GCA and SCA and which minimizes the risk of inbreeding depression, has been used to develop strains producing significantly higher yield performance in timothy (Tamaki *et al*. 2009). The CSS scheme consists of two procedures: (i) crossing two seed parental clones (SPs) with a common pollen parental strain (PP) for seeds harvested from the two SPs as first generation synthetic progenies (Syn1), and (ii) even mixing of the Syn1 seeds to produce subsequent generations, as is done to develop conventional synthetic strains from several parental clones. Therefore, CSS is similar to variety synthesis by combining top-cross progenies.

1.4: Timothy – materials in this thesis

Timothy, an important cool-season perennial and cross-pollinating forage grass species, is used as the material for all experiments in this study. Although wild populations of *Phleum* represent a polyploidy series from diploids to octoploids, the cultivated timothy is a hexaploid ($2n = 6x = 42$) species with a high degree of self-incompatibility. The genomic composition of hexaploid timothy remains unresolved (Cai *et al*. 2003; Tamaki *et al*. 2010), but some evidence suggests that the genome contains two doses (AA) of the diploid *Phleum alpinum* L. and four doses (BBBB) of the diploid *Phleum nodosum* L., *i.e.*, AABBBB (Stewart *et al*. 2009). Timothy is cultivated for hay, silage and pasture across the Northern Hemisphere. In Nordic countries, eastern Canada and Hokkaido, the northernmost Japanese island, timothy is an extremely valuable forage grass species because of its adaption to the cool and humid northern climate, considerable winter hardness, and good quality as fodder (Casler 2001b; Helgadottir and Seveinsson 2006; Tamaki *et al*. 2010). The amount of timothy seed sold in eastern Canada is twice as much as that of all other grasses (Tamaki *et al*. 2010). Timothy occupies approximately 80% of total grassland in Hokkaido (Ashikaga 2012).

Timothy breeding specifically emphasizes dry matter production, forage quality, persistency, and tolerance to biotic and abiotic stresses (Ueda 1990; Larsen and Marum 2006; Tamaki *et al*. 2010). The improvement of forage yield continues to be an important breeding achievement in any site, although the priority differs depending on the breeding site. In Japan, they rely primarily on recurrent selection based on a polycrossing system with spaced-plant and drilled-row evaluations, where synthetic varieties and open-pollinated varieties were developed through among- and within-half-sib family selection, and specifically examine forage yield, disease resistance, forage quality, lodging resistance and competitiveness with legumes (Tamaki *et al*. 2010). A steady yield increases in Japanese breeding programs had been achieved mainly through improvement of regrowth vigor and a higher ratio of internode elongation stems in the second harvests only accounting for approximately one third of annual yields (Tamaki 2005; Tamaki *et al*. 2010). Larsen and Marum (2006) reported that substantial increases in yield through breeding have been difficult to achieve in Europe. In addition, Tamaki *et al*. (2010) described that the yield increase through breeding in Japan was only 3.2% per decade between 1969 and 2004. Unsuccessful selection experiments in Canada have been reported, where no yield increase was observed in either sward or spaced-plant trials by three cycles of recurrent restricted phenotypic selection under spaced-plant plots (Shateryan *et al*. 1995).

With regard to tools for molecular breeding, 822 genomic SSR markers have been developed from hexaploid timothy, with polymorphism in eight hexaploid timothy clones (Cai *et al*. 2003, 2009). Of all 822 SSR markers, 664 markers were also amplified in the diploid *P. nodosum* and the tetraploid *P. alpinum* species. An unsaturated linkage map (total length = 445 cM) was constructed for diploid timothy with 226 SSR markers distributed in seven linkage groups (Cai *et al*. 2009). Several molecular marker tools have been used to assess genetic diversity in timothy, including restriction fragment length polymorphism (Ogawa *et al*. 2001), amplified fragment length polymorphism (Ogawa *et al*. 2001), random amplified polymorphic DNA (Guo *et al*. 2003), universally primed PCR (Guo *et al*. 2003), genomic SSRs (Cai *et al*. 2003), and inter-SSR markers (Jonavičienė *et al*. 2009). Consequently, readily available applications exist of molecular markers for timothy breeding.

1.5: Objectives and composition of this thesis

Grasslands produce vast amounts of forage for ruminants, maintain soil fertility, protect and conserve soil and water resources, harbor a stock of carbon in the terrestrial ecosystem, create a habitat for wildlife, and provide recreational spaces for sport and leisure while contributing to the general benefits simultaneously as maintaining sustainable economic outputs (Yamada 2013). Better production infrastructure for grasslands required for proper material recycling among elements of the soil–grass–cow production change (Ashikaga 2012). However, current dairy production systems being an important industry in Hokkaido, Japan, have depended on imported feed, in particular concentrated feed with high nutritive value. This method can result in the increase of nitrogen from outside regions. The Japanese government produced the Basic Plan for Food, Agriculture and Rural Areas in March 2005, in which increasing the food domestic self-sufficiency rate and shifting to an environmentally friendly agricultural system were proposed (Ashikaga 2012). According to that plan, even greater prevalence of high-yielding superior varieties of forage crops such as maize and forage grasses and legumes was recommended in Hokkaido (Hokkaido Government 2012). Especially, for the food domestic self-sufficiency rate to increase, it is important to raise the cultivated area and unit yield of maize, which can be substituted for concentrated feeds. Besides maize, it is important to raise the unit yield of forage grasses and legumes in limited area of grasslands that can supply protein and mineral components as well as fiber such as cellulose. Improvement of forage yields of timothy, which were cultivated in approximately 80% of all grasslands in Hokkaido, can strongly affect dairy farming in Hokkaido and contribute to the promotion of environmentally friendly agricultural systems based on domestic self-sufficiency feed.

This study was performed to propose timothy breeding schemes based on genetic diversity using DNA marker polymorphism for the long-term improvement of forage yields capturing heterosis or SCA. To realize this breeding strategy, the following knowledge is required: (i) genetic parameters of forage yields such as genetic variation and heritability, (ii) relations between marker-based GDs and forage yields in several mating designs, and (iii) usefulness of genetic diversity based on DNA marker polymorphism and relative contribution of genetic effect in methods of variety development. This dissertation comprises six chapters including the general introduction in this chapter. Chapter 2 presents initial steps of this breeding strategy. Genetic gains and heritability for forage yields were examined and reconfirmed based on datasets of timothy breeding programs at Kitami Agricultural Experiment Station (KAES), Hokkaido Research Organization, Kunneppu, Hokkaido, Japan. Relations between marker-based GDs and forage yields of timothy were examined in a polycross mating design including conventional synthetic varieties and polycross progenies (Chapter 3), top-cross progenies (Chapter 4), and CSS strains (Chapter 5: 5.1). Based on these relations, parental selections in polycross breeding schemes using molecular marker diversity are explained in Chapter 3. The study in Chapter 4 specifically addresses the application of top-cross progenies and potential use of GDs in CSS breeding scheme by synthesizing top-cross progenies. In Chapter 5, some verification on CSS development was conducted for the relative contribution of combining ability (Chapter 5: 5.1), and inbreeding depressions in a CSS strain (Chapter 5: 5.2). In Chapter 6, which presents general discussion, selection simulations were performed based on the results obtained in Chapter 2–5. Finally, a proposed breeding method for yield improvement based on genetic diversity using DNA marker polymorphism is discussed along with its prospects.

Chapter 2: Genetic gains and heritability for forage yields of timothy in Japanese breeding programs

Introduction

Maize yield increases have been approximately 20% per decade since the widespread adoption of single crosses in 1960 (Duvick 1992; Tamaki *et al*. 2007). In cereal crops such as wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), yield increases achieved through breeding have been approximately 10% per decade (Donmez *et al*. 2001; Fischer and Edmeades 2010; Gourdji *et al*. 2013; Khush 2013), although the yield gain has tended to decrease. As one notable example, the rice yield gain has fallen from 23% per decade during 1970–1990 to 15% during 1990s and to less than 10% during the first decade of present century (Khush 2013). By contrast, gains of forage crop yields by breeding are low to nonexistent, despite intensive breeding efforts in numerous forage species (Larsen and Marum 2006; Casler 2008; Casler and Brummer 2008). The most estimates of gains in forage crops yield are less than the gains made for grain yield of cereal crops (Casler 1998; Brummer 1999; Wilkins and Humphreys 2003; Tamaki *et al*. 2007; Brummer and Casler 2009; Tamaki *et al*. 2010). Several reasons for this yield lag in forage crops relative to crop species such as maize are (i) a longer breeding cycle for forage crops, most of which are perennials, (ii) lack of a "harvest index" trait to aid dry-matter partitioning into the economic product, (iii) inability to exploit heterosis in commercial varieties such as severe inbreeding depression on creating inbred lines, self-incompatibility, small perfect flowers and lack of stable cytoplasmic male sterility (Casler 1998, 2001a; Wilkins and Humphreys 2003; Tamaki *et al*. 2007; Casler and Brummer 2008).

Genetic gains (ΔG) are influenced by two main components as $\Delta G = h^2S$, where h^2 is the narrow sense heritability and S denotes the selection differential (Brummer and Casler 2009). Narrow sense heritability, the ratio of additive genetic variance to phenotypic variance, representes the amount of heritable variance that is present in the population. A trait with high narrow sense heritability is expected to respond readily to selection. The additive genetic variance is a property of the population, and the phenotypic variance includes genetic (both additive and non-additive), environmental, and genotype-by-environment components. The latter two components can be decreased by increasing the number of replications and environments in which the breeding material is tested, and by using experimental designs or spatial analyses that reduce error variance. Consequently, narrow sense heritability is not a static feature for a given trait. Rather, it can vary depending on the population being evaluated and the characteristics of the evaluation nursery. The selection differential S is the product of the intensity of selection k and the phenotypic standard deviation (SD) of the population (Brummer and Casler 2009). For the among- and within-half-sib family selection adopted in timothy breeding at KAES, ΔG influences h^2 and S respectively in half-sib family and among individuals within half-sib family.

Until 1965, timothy plants in Hokkaido were represented by only one variety, 'Climax', aside from 'Hokkaido Local', which was originally introduced from the United States in 1874 (Ueda 1990). Since 1964, the national breeding program and network system of timothy started in Hokkaido, where improvement of the forage yield was a continuous major breeding achievement (Ueda 1990). Now, 20 timothy varieties had been released during 1969–2014 through breeding efforts in Hokkaido, accounting for most commercial seeds in Hokkaido. Tamaki *et al*. (2010) calculated the genetic gain of 3.2% per decade during 1969–2004 based on a comparison between 'Senpoku' (Ueda *et al*. 1971) registered in 1969 and 'Horizon' (Tamaki *et al*. 2002) registered in 2004. An initial step to yield improvement of this breeding strategy needs to be the identification of genetic gains or heritability for forage yields in the three phases of timothy breeding in Hokkaido.

This study was conducted to evaluate the following: (i) genetic gains achieved through breeding in Hokkaido based on a dataset of the local-adaptability tests that were done during 1966–2014, (ii) narrow sense heritability among individuals within families for forage yields by a parent–offspring simultaneous test, and (iii) narrow sense heritability among half-sib families for forage yields based on dataset of polycross progeny tests that were done during 1971–2013. In addition, the potential of genetic gains for forage yields was estimated by selection simulation conducted with the genetic parameters listed above for this experiment.

Materials and methods

Timothy local-adaptability tests in Hokkaido since the 1960s

Dataset of timothy local-adaptability tests conducted in Hokkaido during 1966–2014 was used in this experiment (Table 2.1). Details of the outlines of the tests were described earlier in the literature (Ueda *et al*. 1971, 1977; Furuya 1985; Furuya *et al*. 1992a, 1992b; Shimokoji 1994a, 1994b, 1995; Tamaki *et al*. 2002; Fujii *et al*. 2010a, 2010b, 2014; Ashikaga *et al*. 2012, 2014). In these tests, the annual dry matter yields (DMYs) of 16 timothy varieties, which had been had been registered in the forage grasses and legumes variety lists recommended in Hokkaido as nine early maturing hay-types and seven medium maturing hay-types, were examined in each test with a check variety at multiple locations (Table 2.1). The yield performances of the 16 varieties were estimated based on averaged annual DMYs (% of check variety in each local-adaptability test) at multiple locations over experiment-years except for a seedling-year, where the value of ‘Climax’ was equal to 100. Genetic gains of forage yields of early and medium maturing timothy in Japan were estimated based on the yield performance estimates by the slope coefficients of simple linear regression within each maturing group as $PY = \alpha + \Delta G\,Yr$, where PY represents the performance estimate (% of ‘Climax’), α denotes the intercept of the regression, ΔG stands for the slope coefficient defined as the genetic gain (% of ‘Climax’ per year) of forage yield in this experiment, and Yr signifies the year registered in the variety lists recommended in Hokkaido. To compare the slope coefficients of maturing groups, an F-test was performed on variances of the group-by-slope interaction and residual in analysis of covariance, *i.e*., a test of the assumption of homogeneity of regression slopes.

Table 2.1 Variety names, registered year (Reg. yr), yield performance estimates†, maturing type for hay-use of timothy (*Phleum pratense* L.) registered in the forage grass and legume variety lists recommended in Hokkaido, and outlines of their local-adaptability tests at multiple locations of Hokkaido, Japan, since the1960s

Variety name	Reg. yr	Yield perfor-mance esti-mate†	Maturing	Outlines of timothy local-adoptability tests					
				Exp. years‡	N_L§	Check variety¶	Avg. annual DMYs# (Mg ha^{-1})	Avg. annual DMYs# (% of check¶)	References
Hokuo	1969	112	Early	1966–1968	5	Climax	10.9	112	Furuya, 1985
Senpoku	"	106	"	"	"	"	10.3	106	Ueda *et al*., 1971
Hokuren	1971	108	"	"	"	"	10.5	108	Furuya, 1985
Nosappu	1977	114	"	1974–1976	"	Senpoku	9.6	108	Ueda *et al*., 1977
Hokusen	1984	117	Medium	1981–1983	"	Nosappu	10.6	103	Furuya, 1985
Akkeshi	1992	131	"	1988–1990	"	Hokusen	13.1	112	Furuya *et al*., 1992b
Kiritappu	"	127	"	"	"	"	12.7	108	Furuya *et al*., 1992a
Aurora	1994	113	Early	1991–1993	7	Nosappu	12.0	99	Shimokoji, 1994a
Hokusei	"	114	"	"	6	"	12.5	100	Shimokoji, 1994b
Hokuei	1995	128	Medium	1991–1994	"	Hokusen	11.5	109	Shimokoji, 1995
Horizon	2002	120	Early	1999–2001	6	Nosappu	10.0	105	Tamaki *et al*., 2002
Natsuchikara	2010	121	"	2006–2009	"	"	9.1	106	Ashikaga *et al*., 2010
SBT0308	"	143	Medium	2007–2009	"	Kiritappu	12.2	112	Fujii *et al*., 2010a
SBT0310	"	134	"	"	"	"	11.5	105	Fujii *et al*., 2010b
SBT0002	2014	125	Early	2011–2013	5	Horizon	9.5	104	Fujii *et al*., 2014
Kitami30	"	140	Medium	2011–2013	"	Akkeshi	10.5	106	Ashikaga *et al*., 2014

" means ditto.

† Yield performances were estimated based on averaged annual dry matter yields (% of check varieties in each test), where the value of ‘Climax’ was equal to 100.

‡ Experiment years operated in the local-adaptability test including seedling-year.

§ N_L means a number of experimental locations.

¶ Check variety as a control used in each adaptability test.

Averaged annual dry matter yields in multiple locations over experiment years except for a seedling year.

Parent–offspring simultaneous test

This experiment used 15 early maturing parental clones and their polycross half-sib progenies. These materials had been selected for various agronomic traits such as forage yield, lodging resistance, competitiveness with legumes, and seed yields. Two groups derived from different selections constitute the 15 parental clones, which were representatives of current early maturing timothy breeding population. One group included nine parental clones selected mainly for resistance to the purple spot by *Cladosporium phlei* (Gregory) de Vries, and forage yield based on observations at spaced-plant trials in 1987–1988. The other group comprised six parental clones selected mainly for lodging resistance, competitiveness with legumes, and forage yield based on observations at spaced-plant trials in 1999. The parental clones were planted on 31 May 2010 at KAES (43°47′N, 143°42′E) on a high-humic haplic wet andosol with spacing of 0.6 × 0.6 m in a randomized complete block design (RCBD) with two replications. The progenies were sown on 31 May 2010 in single seedling rows 0.85 m long and 0.6 m apart, in an RCBD with three replications in the same field as the parental clones. Seeding-year (planting-year) management consisted of three harvests without data collection to manage annual weeds, with total applications of 120 kg N ha^{-1}, 172 kg P_2O_5 ha^{-1}, and 120 kg K_2O ha^{-1} in spaced-plant plots, and 112 kg N ha^{-1}, 162 kg P_2O_5 ha^{-1}, and 112 kg K_2O ha^{-1} in drilled row plots. Three harvests per year were conducted in 2011 and 2012 in mid-June, early August, and early October, where plants were clipped to a 10 cm stubble height. Spaced-plant plots were fertilized as follows: 75 kg N ha^{-1}, 150 kg P_2O_5 ha^{-1} and 75 kg K_2O ha^{-1} in early spring, 40 kg N ha^{-1}, 46 kg P_2O_5 ha^{-1} and 40 kg K_2O ha^{-1} immediately after the first harvest and 40 kg N ha^{-1}, 46 kg P_2O_5 ha^{-1} and 40 kg K_2O ha^{-1} immediately after the second harvest. Drilled-row plots were fertilized as follows: 78 kg N ha^{-1}, 157 kg P_2O_5 ha^{-1} and 78 kg K_2O ha^{-1} in early spring, 44 kg N ha^{-1}, 50 kg P_2O_5 ha^{-1} and 44 kg K_2O ha^{-1} immediately after the first harvest and 36 kg N ha^{-1}, 41 kg P_2O_5 ha^{-1} and 36 kg K_2O ha^{-1} immediately after the second harvest. Dry matter yields (g $plot^{-1}$) in each plot were measured at each harvest. Annual DMYs of parental clones and their progenies over two years (2011–2012) were subjected to analysis of variance (ANOVA) on an RCBD design with entry and block random effect factors. The narrow-sense heritability (h_N^2) of annual DMY was estimated using the parent–offspring regression method with the following equation (Tamaki 2004): $h_N^2 = 2\, r_{po}\, (\sigma_o\, \mu_p\, /\, \sigma_p\, \mu_o)$. Therein, r_{po} is a correlation coefficient between parents and offspring, σ_p and σ_o respectively denote SD of parents and offspring, for annual DMYs, and μ_p and μ_o respectively represent means of annual DMYs of parents and offspring.

Polycross progeny tests at Kitami Agricultural Experiment Station since the 1970s

Dataset of 100 entries examined in 11 polycross progeny tests at KAES during 1971–2013 were used in this experiment. The 100 entries included five early maturing check varieties, ‘Climax’, ‘Hokuo’ (Furuya 1985), ‘Senpoku’ (Ueda *et al*. 1971), ‘Nosappu’ (Ueda *et al*. 1977), and ‘Aurora’ (Shimokoji 1994a), which were examined over multiple tests. The other 95 entries, some of which were also examined over multiple tests, were early maturing polycross progenies derived from clones selected under spaced-plant evaluations emphasizing several traits in KAES. They were the representatives of the early maturing breeding populations at KAES. Outlines of the 11 polycross progeny tests are shown in Table 2.2. In each polycross progeny tests, the means of annual DMYs (Mg ha^{-1}) over two or three years except for seedling-year were examined. The broad-sense heritability (h_B^2) of the annual DMYs in each polycross progeny test was estimated based on the *F*-value of the entries in ANOVA as follows (Tamaki 2004): $h_B^2 = 1 - 1\, /\, F$-value. The random effects of the entries were estimated using best linear unbiased prediction (BLUP) based on the annual DMYs according to the mixed effect model of $y = X\beta + Z\alpha + \varepsilon$, where y is the vector of annual DMYs, β and α respectively denote vectors of fixed and random effects, X and Z are the associated design matrices, and ε is a random residual vector. The fit of the model by an iterative process gives estimates of the variance components, a prediction of random vector and a solution for the fixed effect vector. The process most used to estimate the components of variance is restricted/residual maximum likelihood (REML), developed by Patterson and Thompson (1971). Heritability (h^2) among half-sib families was estimated according to the expression $h^2 = \sigma^2_G\, /\, (\sigma^2_G + \sigma^2_E)$, where σ^2_G denotes random effect variance and σ^2_E represents residual variance in the REML process.

Table 2.2 Outlines of test designations (Designation), numbers of entries (Entry Num.), experimental design (Exp. design), size of each plot (Plot size), seedling date (Seedling), harvest year (Year), harvests per year (Harvest), fertilization each year (Fertilization), and board-sense heritability (h_B^2) of 11 timothy (*Phleum pratense* L.) polycross progeny tests at Kunneppu, Hokkaido, Japan

Designation	Entry Num.†	Exp. design‡	Plot size§	Seedling	Year	Harvest	Fertilization (kg N ha^{-1})	h_B^2¶
A	80 (31)	RCBD w/ 4 rep.	2 rows, 85, 25	May 20 2011	2010–2013	3	167	0.67
B	44 (43)	"#	1 row, 150, 60	Aug. 31 2000	2001–2003	"	150	0.76
C	40 (37)	"	2 rows, 90, 60	Jun. 29 2001	2002–2003	"	"	0.08
D	54 (7)	"	2 rows, 250, 60	May 28 1997	1998–1999	"	"	0.53
E	35 (12)	"	"	May 29 1995	1996–1997	"	"	0.43
F	39 (10)	"	"	May 21 1990	1991–1993	"	"	0.35
G	38 (10)	"	"	"	"	"	"	0.32
H	51 (8)	"	"	May 11 1989	1990–1992	"	"	0.58
I	42 (10)	"	"	"	"	"	"	unknown
J	40 (6)	"	1 row, 100, 60	Sep. 4 1975	1976–1978	"	"	unknown
K	33 (6)	"	2 rows, 300, 50	May 13 1970	1971–1972	"	"	unknown

† Number in parenthesis is the number of analysis objects within each experiment.
‡ RCBD: randomized complete block design.
§ Plot size shows numbers of drilled-rows, the row length (cm), and intervals (cm) among the rows of each plot, e.g. two drilled-rows 85 cm long 25 cm apart.
¶ The board-sense heritability (h_B^2) was calculated based on ANOVA in each progeny test by the following equation; $h_B^2 = 1 - 1/F$-value.
# " means ditto.

Selection simulation

Based on the grand mean and variance components in REML/BLUP procedure and the narrow sense heritability among individuals within families obtained using Parent–offspring regression method, the potential of genetic gain for the forage yield of timothy in Japan was estimated by selection simulation performed with R statistical software (GNU General Public License). The population size was set as 8000 individuals of 80 half-sib families (100 individuals per half-sib family), which was a conventional population size in timothy breeding at KAES. The selection pressures in among- and within-half-sib family selection were 0.05 (among individuals within half-sib families) and 0.2 (among half-sib families), *i.e.*, 80 superior individuals of 16 superior half-sib families were selected from 8000 individuals of 80 half-sib families. The genetic increases of forage yields through four selection cycles were calculated by generating arbitrary random numbers following the genetic parameters with 100 replications. Predictions of forage yields were calculated as (Posselt 2010) $Y_i = \mu_{i-1} + [2\,(n-1)/n^2]\,\Sigma\mathrm{BLUP}_i$, where Y_i is the prediction of the i th selection cycle, μ_{i-1} is the mean of parental candidates in the i th selection cycle (μ_0 is the grand mean in REML/BLUP procedure), n is the number of selected parents ($n = 80$ in this experiment), and $\Sigma\mathrm{BLUP}_i$ is the sum of BLUP of selected parents in the i th selection cycle.

Results

Genetic gains of forage yields based on timothy adaptability tests in Hokkaido

The yield performance estimates of 16 varieties were 106–143% of ‘Climax’ (Table 2.1). The regression analysis within the group of early maturing varieties indicated –503 as the intercept (t-value = 4.34; df = 7; $p < 0.01$) and 0.31 as the slope coefficient (t-value = 5.33; df = 7; $p < 0.01$), which demonstrated that the genetic gain of forage yields of early maturing timothy in Japan was equal to 3.1% per decade from 1969–2014 (Fig. 2.1). In the cases of the group of medium maturing varieties, the intercept and slope coefficients were –1180 (t-value = 4.17; df = 5; $p < 0.01$) and 0.66 (t-value = 4.64; df = 5; $p < 0.01$), showing that the genetic gain was equal to 6.6% per decade during 1984–2014 (Fig. 2.1). The slope coefficients differed among maturing groups because of significant group-by-slope interaction (means of square = 72.8, 11.8; F-value = 6.19; df = 1, 12; $p < 0.05$).

Heritability of timothy forage yield based on parent–offspring regression method

The annual DMYs of parental clones were 111–556 g $plot^{-1}$ with the mean (μ_p) of 337 g $plot^{-1}$ and the SD (σ_p) of 135 g $plot^{-1}$. Significant (F-value = 8.92; df = 14, 14; $p < 0.001$) difference among the annual DMYs of parental

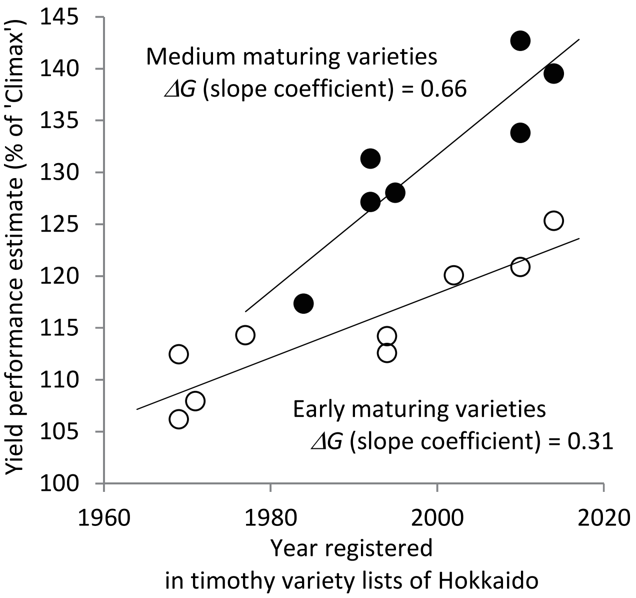

Figure 2.1 Genetic gains (*ΔG*) of forage yields of timothy (*Phleum pratense* L.) in Hokkaido by yield performance estimates and year registered in the variety lists of Hokkaido. The *ΔG* (% of 'Climax' per year) was calculated using the slope coefficient in simple linear regression predicting yield performance estimates (% of 'Climax') based on adaptability tests at multiple locations in Hokkaido since the 1960s. The open circle symbols show nine early maturing varieties ('Hokuo', 'Senpoku', 'Hokuren', 'Nosappu', 'Aurora', 'Hokusei', 'Horizon', 'Natsuchikara', and 'SBT0002'). The closed circle symbols show seven medium maturing varieties ('Hokusen', 'Akkeshi', 'Kiritappu', 'Hokuei', 'SBT0308', 'SBT0310', and 'Kitami30').

clones were found. The annual DMYs of polycross progenies were 726–955 g plot^{-1} with 808 ± 61 g plot^{-1} [the mean ± SD ($\mu_o \pm \sigma_o$)]. Significant difference (F-value = 2.11; df = 14, 42; $p < 0.05$) among polycross progenies was found. The correlation between parents and offspring (r_{po}) for the annual DMYs was 0.07 ($p > 0.05$). The estimate of narrow sense heritability among individuals within family was 0.03 (Fig. 2.2).

Best linear unbiased predictions of forage yields based on timothy polycross progeny tests

The grand mean was 11.1 Mg ha^{-1} in the dataset of the 11 polycross progeny tests. The BLUP values were –0.92 to 0.60 with mean of 0.0 Mg ha^{-1}. Variance components for forage yields of polycross progenies by REML were estimated as 0.115 (σ^2_{G}) and 0.150 (σ^2_{E}). These showed a moderate heritability estimate ($h^2 = 0.43$) among half-sib families.

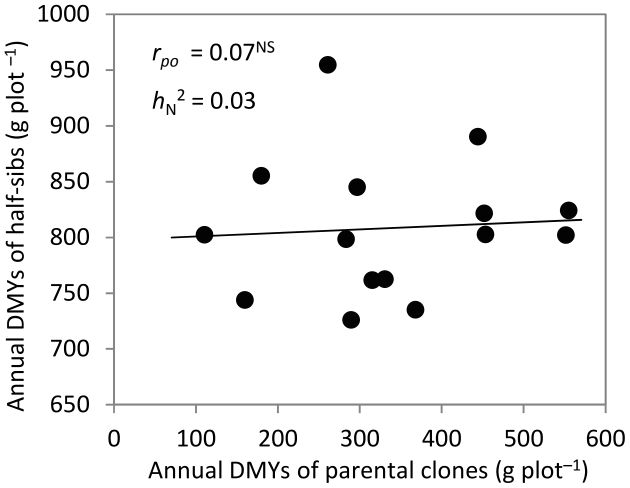

Figure 2.2 Correlation of timothy (*Phleum pratense* L.) parental clones and their polycross half-sib progenies for the annual dry matter yields (DMYs; g plot^{-1}), and narrow-sense heritability (h_{B}^2) estimate of the annual DMY based on the parent–offspring regression method. Annual DMYs were evaluated at Kunneppu, Hokkaido, Japan, in 2011–2012 (three harvests each year).

Selection simulation

The transition of forage yields with selection cycles was shown in Fig. 2.3. The genetic gain by four cycle selection simulation (mean ± SD) was 1.19 ± 0.17 Mg ha^{-1}, corresponding to 10.7 ± 1.5% of an initial selection cycle (Y_0 = 11.1 Mg ha^{-1}). On the assumption that operation of one cycle took the shortest five years (Tamaki 2005), a potential of yield improvement in early maturing population was 5.4% per decade. To realize the genetic gain of 3.1% per decade calculated from the dataset of early maturing timothy local-adaptability tests, time requirements for the among- and within-half-sib family selection were approximately 8–9 years per selection cycle.

Discussion

The low genetic gains for forage yields of timothy in Japan (3.1–6.6% per decade) were similar to those of other grasses and legumes (Wilkins and Humphrey 2003). The genetic gain in an early maturing variety (3.1% per decade) showed good agreement with the value reported by Tamaki *et al.* (2010). The reasons for this yield lag of timothy to grain crops such as maize include the three aspects already described in the Introduction: a longer breeding cycle, lack of a "harvest index" and inability to

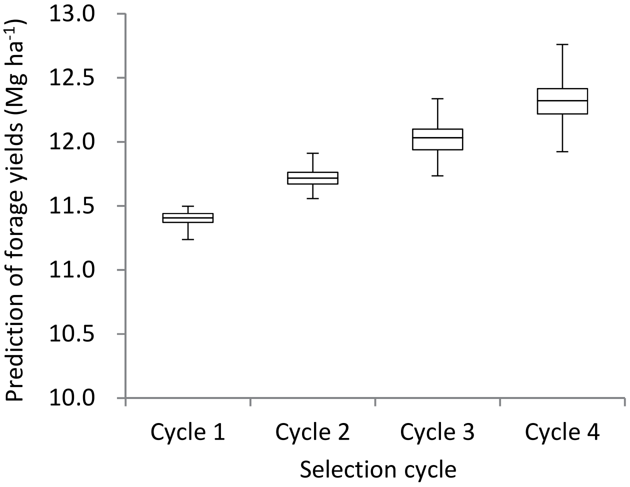

Figure 2.3 Transition of forage yields with selection cycles of among and within half-sib selection in timothy (*Phleum pratense* L.) by computer simulation based on genetic parameters calculated from dataset of the timothy breeding program in Hokkaido, Japan. The forage yield of the initial population in simulation was settled 11.1 Mg ha^{-1}.

exploit heterosis. In addition, difficulties for yield improvement in timothy lie on polyploidy, low heritability and selection intensity, interactions under spaced-plant plot and sward-plot trials, and a wide array of other target traits. Polyploidy in many forage grasses and legumes species complicates inheritance of the traits and results in low selection efficiency (Yamada 2013), that applied to hexaploid nature of cultivated timothy. The low narrow-sense heritability estimate among individuals within half-sib family (h_N^2 = 0.03) is attributable to an intrinsic feature itself for forage yields and interactions under spaced-plant plot and drilled-row plot trials. Low correlations, inconsistent rankings, and genotype by spacing interactions have been reported for forage yields between spaced plants and sward plots in several forage grasses including timothy (Helgadóttir and Björnsson 1994; Waldron *et al.* 2008). In addition, visual vigor ratings are often used as a proxy for yield in case of individual selections, although the genetic correlation between forage yield and vigor rating might not be strong (Wilkins and Humphreys 2003). The moderate heritability (0.43) among half-sib families indicated promising genetic gains for forage yields by polycross progeny tests evaluating additive effect. However, these progeny tests were typically based on fewer families and lower selection intensities than those among individuals. The lack of yield improvement can be ascribed in many cases simply to the practice of not selecting for yield *per se* (Casler and Brummer 2008). Many other traits including nutritive value and disease resistance must also be improved. Yield improvement is often not the emphasis of the program except for post-synthesis selection because yield is not easily measured. In the cases of timothy breeding in Japan, breeding efforts have also been devoted to resistance to the purple spot disease caused by *Cladosporium phlei* (Gregory) de Vries, winter hardness, competitive ability toward legumes (or weeds), seed yields, logging tolerance in the first harvest, nutritive values including dry matter digestibility, and water-soluble carbohydrate concentration and assortment of maturing type (Tamaki 2005; Tamaki *et al.* 2010; Ashikaga 2012). Most of the traits have no or negative relation to forage yields, although the improvement of disease resistance, winter hardness, and competitive ability indirectly cause forage yield gains. In terms of the assortment of maturing type, extensive breeding efforts have been undertaken because different base populations have been established for each of the maturity groups.

Differences of genetic gains among early and medium maturing types (3.1 vs. 6.6% per decade) were found from this study. One reason might be a stage of the breeding program. Whereas the breeding for early maturing type started since the 1960s based mainly on ‘Hokkaido Local’ originally introduced from the United States by the “Hokkaido Kaitakushi (Commission of Hokkaido Colonization)” in 1874 (Ueda 1990; Tamaki *et al.* 2010), that for medium maturing-type were started from the 1970s using materials derived from ‘Hokkaido Local’ and introduced materials having several foreign origins (Furuya *et al.* 1992a, 1992b). These might result in different amounts of variance of additive effects for forage yields. Greater variation on GCA values evaluated by polycross progeny tests of medium maturing population in the breeding program at KAES was found than that in early maturing population (data not shown). Another reason might be a difference of growth period, which strongly influences light scattering characteristics such as the leaf area index contributing to additive effect for forage yields (Kawata and Matsunaka 1998, 1999).

Results in these experiments have indicated that some measures should be taken for more effective yield improvement, especially in early maturing populations. Selection of breeding aims and concentration of breeder efforts can shorten the selection time, which would be approximately 8–9 years per selection cycle in the conventional breeding scheme because the potential for yield improvement in early maturing populations is 5.4% per decade. However, the selection and the concentration have limitations for forage breeders having many requirements from farmers, companies, researchers, and governments. Furthermore, the potential of gains *per se* was not so high. These indicated limitations of the conventional timothy breeding scheme at KAES only for exploiting an additive effect. In the following chapters (Chapters 3–6), the author aims at proposing a breeding scheme that exploits non-additive effects for forage yield improvement.

Chapter 3: Marker-based genetic distances among timothy genotypes in polycross breeding and its relation to forage yield

Introduction

The improvement of timothy forage yield is a major breeding achievement in Japan (Ueda 1990; Tamaki *et al.* 2010). In fact, timothy yield increase by breeding in Japan has been about 3.1–6.6% per decade since the 1960s (Chapter 2), which is similar to gains achieved in other forage grasses or legumes (Casler and Brummer 2008). Improvement of self-incompatible forage crops, including timothy, often relies on the production of synthetic varieties (Tamaki *et al.* 2007). A variety developed by synthetic population development originated from intercrossing among a limited number of non-inbred parents possessing high GCA for specific traits (Sleper and Poehlman 2006). Theoretically, the phenotypic variance among half-sib families is equivalent to the GCA of the polycross parents from which they were derived (Nguyen and Sleper 1983). It is regarded as a quarter of the inherent additive genetic variance (Casler and Brummer 2008). General combining ability values are themselves useful in the prediction of trait performance in synthetics (Posselt 2010).

Contrary to the theoretical expectations based on GCA values for forage yield from polycross progeny tests (Posselt 2010), several synthetic timothy populations originating from KAES since the 1960s have failed to produce high yields (Tamaki *et al.* 2007). Reports that attempt to explain this lack of performance suggest that SCA or inbreeding depression influenced selection success, as shown for other perennial and self-incompatible forage crop species (Michaelson-Yeates *et al.* 1997; Riday and Brummer 2002). Alternatively, the GCA value itself might include both additive and non-additive effects (Hayward 1979) and/or precise GCA estimation might be masked by genotype-by-environment interactions (GEI) (Wilkins and Humphreys 2003; Casler and Brummer 2008; Amini *et al.* 2011). Molecular markers are regarded as powerful tools in forage grasses for the analysis of genetic diversity and the identification of parents for variety development (Kölliker *et al.* 2005; Amini *et al.* 2011). The relations between marker-based GD and forage yield have been characterized in forage grasses and legumes (Joyce *et al.* 1999; Kidwell *et al.* 1999; Kölliker *et al.* 2005; Amini *et al.* 2011; Tucak *et al.* 2011; Robins *et al.* 2012). Investigations in maize and oilseed rape have shown that GDs among parents as estimated by molecular marker analysis correlate (r = 0.58–0.93) with hybrid performance and that molecular markers might have value as predictors of yield heterosis (Smith *et al.* 1990; Raiz *et al.* 2001; Betrán *et al.* 2003).

The emphasis of most marker-based genetic relation studies related to forage yield improvement has been on selecting (or preselecting) parental combinations (Kidwell *et al.* 1999; Kölliker *et al.* 2005; Amini *et al.* 2011; Tucak *et al.* 2011) and not on estimating the proportion of yield-associated additive and non-additive effects in synthetic varieties. Some studies have produced results suggesting that the partitioning of GD into general genetic distance (GGD) and specific genetic distance (SGD) components, which provides some indications of genotype-specific allelic constitutions, might furnish information related to parental genetic diversity that has some value for breeding (Melchinger *et al.* 1990b; Betrán *et al.* 2003). A large positive or negative GGD indicates that a parent might possess alleles that are occurring respectively at a low or high frequency, when compared to other parents (Melchinger *et al.* 1990b). A large positive or negative SGD between parents indicates that cross-progeny are more or less heterozygous than might be expected based on the GGD of their parents (Melchinger *et al.* 1990b). Because GGD values are indicative of allelic complementation, such analyses of polycross progeny might be used to estimate non-additive effects from GCA values. Nevertheless, no reports of studies of forage grasses have described the relation between GGD and GCA as obtained from polycross progeny test analyses. Therefore, a study was designed to evaluate (i) the potential utility of GD estimation to predict forage yield among timothy synthetic strains and polycross progeny and (ii) the utility of molecular marker-based GD values for forage yield improvement during polycross breeding. The dissection of yield performance in polycross progeny into additive and non-additive component effects is used herein to elucidate the potential role of marker-based GD estimations.

Materials and methods

Field evaluations in Experiments 1 (Exp. 1) and 2 (Exp. 2) were conducted at KAES, Hokkaido Research Organization, Kunneppu, Hokkaido, Japan (43°47′N, 143°42′E) on a high-humic haplic wet andosol.

Experiment 1: Synthetic strains

Two timothy synthetic strains, ‘Kitakei 98301’ and ‘Kitakei 98303’, and their parental clones were used in Exp. 1. ‘Kitakei 98301’ and ‘Kitakei 98303’ were synthesized respectively through polycross breeding among five and six early maturing clones, which were derived from Japanese local materials, introduced materials, and advanced breeding materials in 1998. Selection for high forage yields in five and six parental clones from 35 and 34 parental candidates, respectively, occurred between 1991 and 1998 based on polycross progeny tests (Tables 3.1 and 3.2).

Seeds of the two synthetic strains and two check varieties (‘Nosappu’ and ‘Aurora’) were planted in an RCBD having four replications in May 1999 at a seeding rate of 20 kg ha^{-1}. Each plot was drilled in two seeding rows 2.5 m long and 0.6 m apart. Seeding-year management consisted of two harvests without data collection to manage annual weeds, and one application of 70 kg N ha^{-1}, 80 kg P_2O_5 ha^{-1}, and 70 kg K_2O ha^{-1}. Three harvests per year were conducted in 2000 and 2001, where plants were clipped to a 10 cm stubble height. Plots were fertilized as follows: 75 kg N ha^{-1}, 150 kg P_2O_5 ha^{-1} and 75 kg K_2O ha^{-1} in early spring, 45 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 45 kg K_2O ha^{-1} immediately after the first harvest and 30 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 30 kg K_2O ha^{-1} immediately after the second harvest. Annual DMYs in 2000 and 2001, and cumulative annual DMYs for each plot were subjected to ANOVA based on an RCBD linear model with entry and block random effect factors.

Table 3.1 General combining ability (GCA) for forage yields of five parental timothy (*Phleum pratense* L.) clones of the synthetic polycross strain, ‘Kitakei 98301’, based on cumulative dry matter yields (DMYs) in a polycross progenies test over three years (1991–1993) at Kunneppu, Hokkaido, Japan

Parental clones names	Cumulative annual DMY in a polycross progeny and GCA†		
	($Mg\ ha^{-1}$)	(% of ‘Nosappu’‡)	(GCA§)
17thPC-06	18.74	101	4.4
17thPC-20	19.14	104	6.6
17thPC-24	19.02	103	5.9
17thPC-32	18.69	101	4.1
17thPC-35	19.30	104	7.5
Mean of selections¶	18.98	103	5.7
Mean of entries#	17.98	97	
Nossapu	18.48	100	

† Experimental plots were harvested three times per year during 1991–1993.

‡ The check variety was used as the comparative control.

§ GCA calculated using the following equation: $GCA_i = [(n-1) / (n-2)] (X_i - \mu)$, where n is the number of parents in a polycross, X_i is DMY (percentage of ‘Nosappu’) of a polycross progeny i, and μ stands for the means of DMY (percentage of ‘Nosappu’) of all polycross progenies (Griffing 1956).

¶ Mean of polycross progenies derived from five selected parental clones.

Mean of all 35 polycross progenies evaluated in the test.

Experiment 2: Polycross progenies

Forty-one early maturing timothy polycross progenies resulting from three cycles of phenotypic recurrent

Table 3.2 General combining ability (GCA) for forage yields of six parental timothy (*Phleum pratense* L.) clones of the synthetic polycross strain, ‘Kitakei 98303’, based on cumulative dry matter yields (DMYs) in a polycross progeny evaluated over two years (1996–1997) at Kunneppu, Hokkaido, Japan

Parental clone names	Cumulative annual DMY in polycross progeny and GCA†		
	($Mg\ ha^{-1}$)	(% of ‘Nosappu’‡)	(GCA§)
23thPC-01	18.27	104	5.9
23thPC-15	18.30	104	6.1
23thPC-27	17.56	99	1.8
23thPC-30	17.71	100	2.7
23thPC-31	17.58	100	1.9
23thPC-34	18.34	104	6.4
Mean of selections¶	17.96	102	4.2
Mean of entries#	17.24	98	
Nossapu	17.66	100	

† Experimental plots were harvested three times per year during 1996–1997.

‡ The check variety was used as the comparative control.

§ GCA calculated using the following equation: $GCAi = [(n-1) / (n-2)] (Xi - \mu)$, where n is the number of parents in a polycross, Xi is DMY (percentage of ‘Nosappu’) of a polycross progeny i, and μ stands for the means of DMY (percentage of ‘Nosappu’) of all polycross progenies (Griffing 1956).

¶ Mean of polycross progenies derived from five selected parental clones.

#Mean of all 34 polycross progenies evaluated in a progenies test.

selection and their parental clones were used in Exp. 2. Of those 41 parental clones examined, one clone (‘27thPC-05’) was not used in genetic diversity and yield performance comparisons because it died during experimentation.

In Exp. 2, cumulative annual DMYs for the 41 polycross progenies together with three check varieties (‘Nosappu’, ‘Aurora’, and ‘Hokusei’) were evaluated over two years. These seeds for this experiment were produced from a polycross among 41 parental clones described above between 1999 and 2000. The seeds of polycross progenies and the check varieties were planted in an RCBD having four replications in August 2000. Each plot was drilled in one seeding row 1.5 m long and 0.6 m apart. Seeding-year management consisted of an application of 40 kg N ha^{-1}, 80 kg P_2O_5 ha^{-1} and 40 kg K_2O ha^{-1} without harvesting. Experimental plots were harvested three times per year during 2001–2003. Fertilizer application and seeding rates were the same as those in Exp. 1. Dry matter yields for each plot were summed only over all six harvests in 2002 and 2003 (*i.e.*, three harvests per year for two years) because missing values in 2001 data precluded its inclusion for analysis. Cumulative annual DMYs were analyzed using the same procedure as that described in Exp. 1. General combining ability values for cumulative annual DMYs of polycross progenies in Exp. 2 were calculated using the following equation: $GCA_i = [(n-1)/(n-2)](X_i - \mu)$, where GCA_i is the GCA value of the parental clone i, X_i is the value of the polycross progeny i, μ is the mean of all polycross progenies, and n is the parental number of the polycross (Griffing 1956).

Genotyping protocol

Young leaves were collected from a single plant clone of each genotype that had been maintained in a greenhouse. Individual tissue samples were freeze-dried and stored in a –80°C freezer. Each sample was placed in a 2-mL microcentrifuge tube with a 3-mm bead. The tubes were placed on a horizontal shaker (BioMedical Science Co., Ltd., Tokyo, Japan) until the leaf tissues were ground to a fine powder. The DNA was extracted using the DNeasy™ Plant Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer’s instructions. The PCR was performed in an Applied Biosystems Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) under the following amplification conditions: a ‘touchdown’ PCR consisting of 95°C for 10 min; 2 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min; 10 cycles of 94°C for 1 min, 65°C to 55°C for 1 min decreasing by 1°C/cycle and 72°C for 1.5 min; and 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by 72°C for 7 min and 4°C as the holding step. The 20 µl reaction mix consisted of 0.1 µM of each primer, 2.5 U of Taq polymerase (AmpliTaq Gold DNA polymerase; Applied Biosystems), 0.16 mM of each dNTP, 1 x GeneAmp PCR Gold Buffer (150 mM Tris–HCl, pH 8.0 500 mM KCl), 2.5 mM $MgCl_2$ and 1 µl template DNA. The PCR products were fractionated on 6% (w/v) polyacrylamide gels with 0.5 × TBE buffer. Electrophoresis was performed at 100 mA, for approximately 2 h on a 38.0 × 32.5 cm gel apparatus (Nihon Eido Corp., Tokyo, Japan) and was revealed using the ethidium bromide staining.

Screening of simple sequence repeat markers

Based on information from an unsaturated linkage map of diploid timothy (*P. nodosum*) (Cai *et al*. 2009), we chose 55 SSR primer pairs that were able to amplify in all [hexaploid, tetraploid (*P. alpinum*) and diploid] species of *Phleum* from 226 markers that belong to the map. The SSR primer pairs showing no amplification, no polymorphism and unclear bands were excluded based on primary experiment data using 24 genotypes of timothy. Among a final set of 28 SSR primer pairs, at least three SSR loci were located on each linkage group on the diploid timothy map (Table 3.3), where the mean marker interval was approximately 13.9 cM (Fig. 3.1). The 28 SSR markers produced 373 and 437 polymorphic bands among 11 (Exp. 1) and 40 (Exp. 2) parental clones, respectively, in which allelic dosages were indistinguishable. Therefore, bands produced from DNA amplification of individual genotypes were scored as presence or absence.

Genetic distance estimation and statistical analyses

Genetic distance estimates were calculated from SSR data for all possible pairs of parental clone comparisons using the following equation: $GD_{i,j} = 1 - [2N_{i,j} / (N_i + N_j)]$, where $GD_{i,j}$ represents the GD estimate between clone i and j, $N_{i,j}$ denotes the total number of bands common to clone i and j, and N_i is the total number of bands present in i (Dice 1945; Nei and Li 1979). Principal coordinate analysis (PCOA) was performed using GD estimates in Exp. 1 employing the function ‘pcoa’ in the R statistical package ‘ape’ (GNU

Table 3.3 Timothy simple sequence repeat (SSR) marker primer pairs used for this study

Marker name	LG†	Repeat motif	Forward primer sequence (5’–3’)	Reverse primer sequence (5’–3’)
B10-G03#	1	(TC)$_{17}$	CAGCTATGGTTACCTACACATG	ATGGCGTCTTTATTAATCACA
D07-H01#	1	(GA)$_{30}$	ATCTCGTTCGGTTAGAAACA	AGGGTGGAGAAACTTTGG
B08-G12#	1	(TC)$_{25}$	CAGCTATGGTTACCTACACATG	ATGGCGTCTTTATTAATCACA
D01-E04††	1	(CAA)$_{8}$(TAA)$_{10}$	TCTGTTGCCTATTCTGCTG	GCATTTCACTAAGACTGTGACA
A08-C05††	2	(TG)$_{7}$…(TG)$_{13}$	ATCCTTATCGGGACGATC	GTAATATCACTCCCGATCCA
A01-F03††	2	(TG)$_{21}$	GCATCCGTGCATTAACTC	TACACCTTTCACAAGGAATACA
B03-D03††	2	(TC)$_{17}$	GTCTACAGATCTTTTGCATGG	TTTTCAGGAACTCGTCAAGT
D13-G03#	2	(TAA)$_{12}$ Imperfect	AACACCTCATGCAGAAGTAGA	GGTCAATTGCACTTAGTTTTG
B08-C01#	3	(TC)$_{23}$	AATCCACATCCAATGCAC	ATATGGAAAGAAGAGGACGTC
D03-G10#	3	(GA)$_{25}$	CAACGTTTAGAACATCATATGC	AAGAGCTCTTTAGAAGTGATGC
B03-C05††	3	(TC)$_{15}$	ACGATCTGACCATATGATCTG	CAAAGGTTATGTACTCGATGC
A03-A04††	3	(TG)$_{10}$	TAGTAGCATTTAGCGATGCA	ACGCATGTCATCAGTAGTGA
B06-E07#	3	(TC)$_{13}$	AAGTAAGAGCAGAGGCGAG	CAATTCCTCTGTGATGCAC
B03-A09††	4	(GA)$_{19}$	AACTAGGTGAACCGTTTGG	CCCGTTATGTCCTTGTATGT
D05-H01#	4	(GA)$_{18}$	ATCGACAGATGGTTGCAC	AATAGAGATTTGGAGGAGCC
B08-E02#	4	(GA)$_{14}$	AGTAAACTGTCAGCCTGATGA	GACGTACAAGTCGAGAAACC
A15-A11††	4	(TG)$_{22}$	TCTCTGCTCCTGGTTCTTT	TTCACTGGCGATAGAGATTT
D03-F04#	5	(GA)$_{25}$	ATGTACGTGGGAGTGAGAAC	AACTGAGGATTCGTCACATC
D09-B01#	5	(TC)$_{20}$	TGATAATGATTAACCAGCAGC	CAGCATTGTGTGCATGATAC
D05-D06#	5	(GA)$_{27}$	ATTCTCCTCCTCGGATATATG	ATCACGACATACAACTATGGG
A16-G10††	6	(CA)$_{35}$	GACTTGTAATTTGAGACCCG	CTTTATTATGTGATAGCAGGCA
B07-G02#	6	(GA)$_{19}$	AGGGTCACATTCAAAGAGG	GCACTCTAATCAAGTATTGCTC
C02-C08††	6	(AAG)$_{13}$	TACAAACTCTGCCCACGT	ACTCAGAACAGGTATGGGC
B08-E07#	6	(GA)$_{16}$	CTAGGTAATATCCGCATGTTG	CAGTTGTGGTTCAAATTGC
A10-A03††	7	(CA)$_{21}$(CGCA)$_{9}$(CA)$_{17}$	AGGAGACATGCATCCATG	CCTAGTGTAGCTTCGCAGAT
B03-E10††	7	(GA)$_{19}$	TAAGGGTTGAATTGACTTTGA	TTGGGGTAGCCATCTCTAC
B-T21††	7	(TC)$_{11}$(TACA)$_{11}$	AACAATTACCTAGCGCAATC	TCACATGCTCCTTTATTTTTT
C01-E10††	7	(TTC)$_{13}$	CGATGTGATGTTTGAGCTC	ACGTTCCAAAGCGGAC

† Linkage groups in unsaturated diploid timothy (*Phleum nodosum* L.) map (Cai *et al.*, 2009).
‡ Total number of alleles in all 67 genotypes.
§ Range of number of alleles per genotype at each locus.
¶ Mean number of alleles per genotype at each locus.
# Unpublished.
†† Repeat motif and primer sequence were referred from Cai *et al.* (2003).

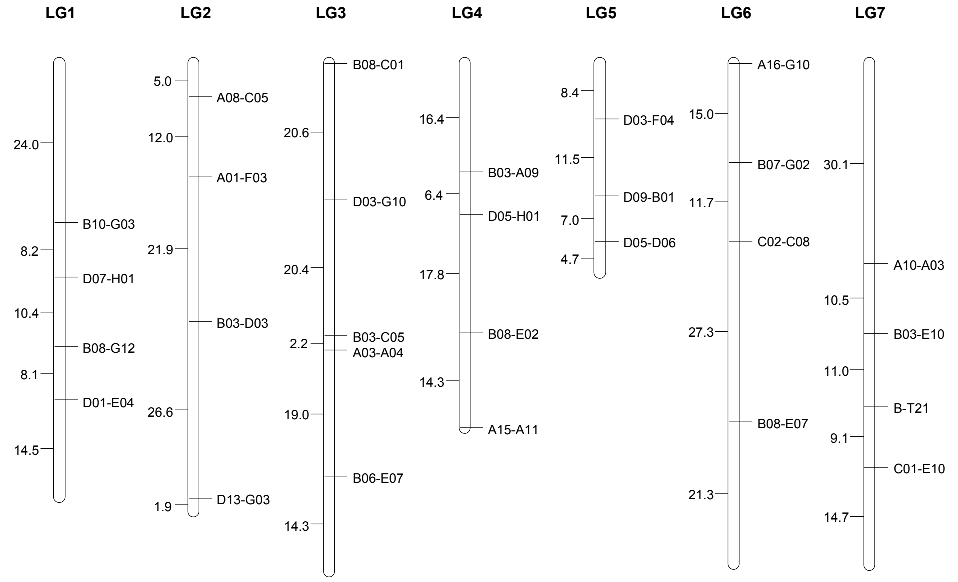

Figure 3.1 Simple sequence repeat (SSR) markers used for this study of unsaturated diploid timothy (*Phleum nodosum* L.) map (Cai *et al.*, 2009).

General Public License) according to Paradis *et al.* (2004). An average GD of the Synthetic strains in Exp. 1 based on the pairwise GD estimates within each strain, ‘Kitakei 98301’ and ‘Kitakei 98303’, and the significance of the averaged GD estimates was determined using *t*-test comparison (Enoki *et al.* 2005). General genetic distance estimates of 40 parental clones of the polycross in Exp. 2 were calculated from GD estimates using the following equation based on a method analogous to

Griffing's Model I of Method 4 (Griffing 1956; Melchinger *et al.* 1990b): $GGD_i = [(n - 1) / (n - 2)]\,(GD_{i,\cdot} - \overline{GD})$, where GGD_i denotes the GGD estimate of clone i within the polycross, $GD_{i,\cdot}$ signifies the average of GD estimates between clone i and the other parental clones of the polycross, $\overline{GD}$ stands for the average of GD estimates among all possible pairs of parental clones of the polycross, and n is the parental number of the polycross. The significance of GGD estimates was assessed using F-test evaluation after ANOVA according to Griffing's Model I of Method 4 (Griffing 1956). The correlation between the GGD estimates and GCA values for cumulative annual DMY was estimated using the Pearson product-moment correlation coefficient. The dataset including the GGD and DMY of 40 polycross progenies in Exp. 2 was analyzed to evaluate the contributions of genetic effects associated with and without allelic complementation on DMYs. The dataset was calculated according to linear regression as $y_{i,j} = \mu + B_j + \beta GGD_i + R_i + \varepsilon_{i,j}$, where $y_{i,j}$ is in cumulative annual DMY in a plot of entry i and block j, μ signifies a mean of cumulative annual DMYs, B_j represents the effect for being in block j, βGGD_i denotes estimates by a regression with GGD of entry i, R_i is residuals of a regression of entry i, and $\varepsilon_{i,j}$ stands for errors.

Results

Experiment 1: Synthetic strains

Significant differences ($p < 0.05$) among all entries examined were detected for DMY in 2000 and cumulative annual DMY over two years (Table 3.4). The DMY of 'Kitakei 98301' was 18, 5, and 12% greater, respectively, than the check variety 'Nosappu' in 2000, 2001, and in cumulative annual DMY (Table 3.4). In contrast, the DMY of 'Kitakei 98303' was comparable to 'Nosappu' and lower than 'Kitakei 98301' in 2000, 2001, and the cumulative annual DMY (Table 3.4).

The GD for all pairwise combinations among the 11 parental clones examined were 0.61–0.78, where the average GD for all pairs was 0.70. The GD estimates among parental clones for each synthetic were 0.68–0.78 for 'Kitakei 98301', where the average GD was 0.74, and were 0.61–0.75 for 'Kitakei 98303', where the average GD was 0.68. The difference in the average GD between the two synthetics examined was significant (t-value = 4.34, $p < 0.001$). The SSR-based PCOA [principal components (PC) = PC1 and PC2 explained 16.1% and 14.9% of the variation, respectively], which isn't a goodness-of-fit test, did not provide for distinctions among the 11 parental clones examined (data not shown).

Table 3.4 Annual dry matter yields (DMYs) of two timothy (*Phleum pratense* L.) synthetic populations in a yield trial over two years (2000–2001) at Kunneppu, Hokkaido, Japan

Entry	Annual DMY (Mg ha^{-1})†		
	2000	2001	Cumulative
Synthetic strains			
Kitakei 98301	6.73	4.66	11.39
Kitakei 98303	5.77	4.30	10.07
Check varieties			
Nossapu	5.69	4.46	10.15
Aurora	5.69	4.29	9.98
LSD$_{0.05}$‡	0.59	NS§	1.06
CV (%)¶	8.5	3.9	6.4

† Experimental plots were harvested three times per year during 2000–2001.
‡ Least significant difference at the 0.05 probability level.
§ NS, not significant.
¶ Coefficient of genetic variation.

Experiment 2: Polycross progenies

The 41 polycross progenies showed considerable variation for all traits, except for the heading date (data not shown). The DMYs of 41 progenies were 93–115% of 'Nosappu' (Table 3.5). Moreover, the DMYs of all 44 entries examined were significantly different over years [F-value of 2.95 ($p < 0.001$)]. General combining ability values based on cumulative annual DMY among progenies were significantly different ($p < 0.001$) and were –10.0 to 11.4% of the values of the control 'Nosappu' (Table 3.5). Pairwise GD estimates among the 40 parental clones of the polycross (excluding '27thPC-05') were 0.59–0.91 (data not shown). The GGD estimates of the parental clones were –0.0244 to 0.0394 (Table 3.5), and the F-value of GGD estimate was 3.73 ($p < 0.001$). The correlation coefficient (r) between GCA values for cumulative annual DMY and GGD estimates was 0.45 ($p < 0.01$) (Fig. 3.2). Regression analysis of GGD on cumulative annual DMYs among the 40 polycross progenies (excluding '27thPC-05'; Table 3.6) showed that the mean square values among polycross progenies (F-value = 2.40), regression with GGD (F-value = 9.67) and the residual of the regression (F-value = 1.96) were significant ($p < 0.01$).

Table 3.5 General combining ability (GCA) for cumulative annual dry matter yields (DMYs) over two years (2002–2003) in timothy (*Phleum pratense* L.) polycross progeny test and general genetic distances (GGD) within the parental clones of a polycross evaluated at Kunneppu, Hokkaido, Japan

Parental clone names	Cumulative annual DMY of polycross progeny†			GGD¶
	Mg ha^{-1}	% of ‘Nosappu’‡	GCA§	
27thPC-01	24.76	101	–2.2	0.0209
27thPC-02	25.76	105	1.8	0.0113
27thPC-03	23.93	98	–5.5	–0.0215
27thPC-04	25.78	105	1.9	–0.0047
27thPC-05	26.35	108	4.1	–#
27thPC-06	25.37	104	0.2	–0.0061
27thPC-07	26.57	109	5.0	0.0205
27thPC-08	25.39	104	0.3	0.0329
27thPC-09	24.61	101	–2.8	0.0072
27thPC-10	26.39	108	4.3	–0.0051
27thPC-11	25.40	104	0.3	–0.0031
27thPC-12	25.24	103	–0.3	–0.0105
27thPC-13	25.96	106	2.6	–0.0057
27thPC-14	25.73	105	1.7	–0.0047
27thPC-15	25.66	105	1.4	–0.0145
27thPC-16	25.62	105	1.2	–0.0047
27thPC-17	25.60	105	1.1	0.0141
27thPC-18	25.20	103	–0.5	–0.0061
27thPC-19	23.83	97	–5.9	–0.0074
27thPC-20	24.44	100	–3.5	0.0032
27thPC-21	24.41	100	–3.6	–0.0116
27thPC-22	26.41	108	4.4	0.0394
27thPC-23	28.17	115	11.4	0.0107
27thPC-24	24.88	102	–1.7	–0.0227
27thPC-25	25.93	106	2.5	–0.0131
27thPC-26	24.34	99	–3.9	–0.0115
27thPC-27	23.35	95	–7.8	–0.0244
27thPC-28	26.53	108	4.9	–0.0040
27thPC-29	25.32	103	0.0	–0.0011
27thPC-30	24.01	98	–5.2	–0.0074
27thPC-31	24.84	102	–1.9	–0.0201
27thPC-32	24.81	101	–2.0	0.0001
27thPC-33	26.02	106	2.8	0.0150
27thPC-34	24.80	101	–2.0	0.0218
27thPC-35	23.84	97	–5.9	–0.0042
27thPC-36	25.91	106	2.4	0.0112
27thPC-37	24.24	99	–4.3	–0.0084
27thPC-38	22.81	93	–10.0	–0.0014
27thPC-39	27.95	114	10.5	0.0147
27thPC-40	24.41	100	–3.6	–0.0111
27thPC-41	27.28	111	7.8	0.0121
Mean of all 41 polycross progeny	25.31	103		
Check varieties Nossapu	24.47	100		
Aurora	21.36	87		
Hokusei	23.92	98		
LSD(0.05) ††	2.08	8	8.4	
CV (%)‡‡	5.1			

† Experimental plots were harvested three times per year during 2002–2003.

‡ The check variety was used as the comparative control.

§ GCA calculated using the following equation: $GCA_i = [(n-1) / (n-2)] (X_i - \mu)$, where n is the number of parents in a polycross, X_i is DMY (percentage of ‘Nosappu’) of a polycross progeny i, and μ stands for the means of DMY (percentage of ‘Nosappu’) of all polycross progenies.

¶ GGD calculated from genetic distances based on marker-based diversity by methodology analogous to Griffing’s Model I of Method 4 (Griffing, 1956; Melchinger *et al*., 1990b).

The clone was excluded from the SSR analysis because of its non-existence.

†† Least significant difference at the 0.05 probability level.

‡‡ Coefficient of genetic variation.

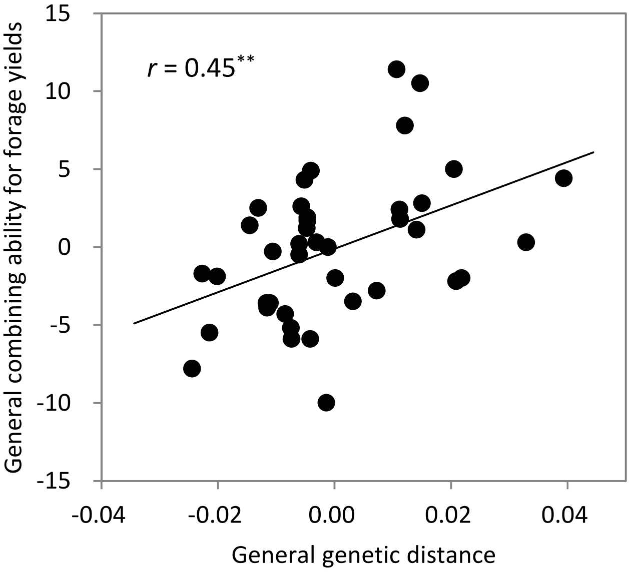

Figure 3.2 Relation between general combining ability (GCA) values for forage yields based on cumulative annual dry matter yields (DMYs) of timothy (*Phleum pratense* L.) polycross progenies and general genetic distance (GGD) estimates within the polycross parents. The polycross progenies derived from crossing among 41 parental clones. Each experimental plot was harvested three times per year during 2002–2003. Pearson product-moment correlation coefficient is denoted by "*r*". ** denotes a significance *r*-value ($p < 0.01$). $GCA_i = [(n–1) / (n–2)] (X_i – \mu)$, where *n* is the number of parents in a polycross, X_i is DMY (percentage of 'Nosappu') of a polycross progeny *i*, and μ is means of DMY (percentage of 'Nosappu') of all polycross progenies, respectively. GGD were calculated from genetic distances based on marker-based genetic diversity by methodology analogous to Griffing's Model I of Method 4 (Griffing, 1956; Melchinger *et al.*, 1990b).

Table 3.6 Regression analysis of variance of marker-based general genetic distances (GGD) on cumulative annual dry matter yields (DMYs) of 40 timothy (*Phleum pratense* L.) polycross progeny over two years (2002–2003) at Kunneppu, Hokkaido, Japan

Source of variance	df	MS	*F*-value	*P*-value
Block	3	7216.8	32.91	<0.001
Among PXPs†	39	525.9	2.40	<0.001
R. with GGD‡	1	4159.5	9.67	0.004
Residual	38	430.3	1.96	0.003
Error	117	219.3		
Total	159	426.6		

† Among polycross progenies.
‡ Regression based on 28 SSR markers diversity genotyping parental clones used for creating polycross progeny.

Discussion

The synthetic strains 'Kitakei 98301' and 'Kitakei 98303', both derived from parental clones with high GCA (Tables 3.1 and 3.2), exhibited contrasting forage yield levels under the conditions in which they were evaluated (Table 3.4). The significant difference in average GD between two synthetic strains (0.74 vs. 0.68) might be a reason for these contrasting yields, which might be associated with non-additive gene effects and/or inbreeding depression. These observations agree with those of a previous study in perennial ryegrass, where high molecular marker diversity was associated with increased DMY among synthetic varieties (Kölliker *et al.* 2005). Furthermore, these data support the hypothesis that selection of polycross parents with contrasting genetic diversity exploits heterosis and/or avoids inbreeding depression. However, populations derived from genetically divergent parents are not always high yielding (Boller *et al.* 2007). For instance, lack of gain from selection for forage yield based on marker diversity analysis has been reported for alfalfa (Kidwell *et al.* 1999). Additive and dominant gene effects and complementary gene interactions are, in fact, important for improving forage yield in forage grasses or legumes (Casler and Brummer 2008). Consequently, an understanding of both additive and non-additive effects is likely to be important when marker-based GD analysis was used as a tool for improving the forage yield in timothy.

Although estimates of GCA can be useful for the prediction of cross-progeny performance in synthetics (Posselt 2010), unsuccessful GCA-based selection experiments in the timothy synthetic strain 'Kitakei 98303' have been documented as shown herein (Tables 3.2 and 3.4) and in the first generation of synthetics tall fescue progenies (HGCA in Amini *et al.* 2011). A polycross can provide satisfactory discrimination between the breeding values of genotypes when both additive and dominance effects are present under a situation of equal gene frequencies (Hayward 1979). However, as Hayward (1979) reported, unequal gene frequencies are likely to arise as a consequence of selection. Discrimination among genotypes would, in such cases, be more difficult than under equal gene frequencies.

This study demonstrated that differences in GGD among the parental clones of a polycross correlated with their respective GCA values for DMY based on polycross progeny tests (Fig. 3.2), and demonstrated that the regression mean square of GGD for DMY is significant (Table 3.6). These results indicate that these GCA estimates included a genetic effect associated with allelic complementation (*i.e.* non-additive effects) in addition to additive effects. Low levels of additive genetic variation in the GCA values might be attributable to the materials used for the present study. The low levels are consistent with the predictions of Hayward (1979) associated with non-additive gene action. Although genetic variation among individuals within timothy populations and their interrelations have traditionally been assessed using morphological and agronomic trait analyses, these might not be sufficient in themselves to produce precise estimates of genetic relatedness. Marker analysis is a useful tool for assessing genetic relatedness during selection within genetically broad-based populations (Guo *et al*. 2003; results present herein). The results of regression analysis (*i.e.* residual and GGD regression) conducted herein were significant (Table 3.6), indicating that the residual mean square likely includes considerable genetic effects that are unrelated to allelic complementation (*i.e.* additive effects). Approaches that compare marker-based GGD within polycross parents and forage yield in polycross progeny tests such as those defined herein can be used to elucidate the genetic effects on yield by allowing for the division of inherent GCA values into additive and non-additive effects. Such approaches might engender more efficient improvement of forage yield during timothy polycross breeding by exploiting heterosis and/or reducing inbreeding depression.

Chapter 4: DNA profiling of seed parents and a top-cross tester and its application for yield improvement in timothy

Introduction

Molecular markers provide effective methods for assessing the genetic diversity among parental genotypes within heterogeneous populations (Barker and Warnke 2001). Studies of genetic diversity in relation to heterosis have been undertaken for several forage grasses and legumes (Kidwell *et al*. 1994, 1999; Joyce *et al*. 1999; Kölliker *et al*. 2005; Amini *et al*. 2011; Tucak *et al*. 2011; Robins *et al*. 2012). Kölliker *et al*. (2005) studied an effect of increased amplified fragment length polymorphism diversity among parental plants on polycross progenies in perennial ryegrass. First and second generations of synthetic progenies derived from wider polycrosses showed consistently higher yields when compared to progenies derived from narrower ones. The observations presented in Chapter 3 agreed with those from a study by Kölliker *et al*. (2005). They support the hypothesis that selection of polycross parents with contrasting genetic diversity exploits heterosis and/or avoids inbreeding depression. In forage breeding programs, screening combinations of parental clones for high yield performance is a costly and time-consuming process. With DNA profiling, the possibility exists of selecting parents for which combinations show a high yield performance more effectively. Most studies of marker diversity in relation to yield have been undertaken using polycrosses or single crosses in forage grasses and legumes (Kidwell *et al*. 1994; Joyce *et al*. 1999; Kölliker *et al*. 2005; Amini *et al*. 2011; Tucak *et al*. 2011). However, no report in the literature describes a study of the assessment of relations between forage yield and marker diversity using top-cross progenies derived from crosses between individuals and a population or variety.

The yield increase is only 3.1–6.6% per decade in timothy, which has lagged behind the approximate 20% per decade in maize (Chapter 2). To improve the yield in timothy more efficiently, Tamaki *et al*. (2009) proposed CSS, a breeding method for perennial self-incompatible forage grasses such as timothy in which a high-yield variety can be synthesized by combining two top-cross progeny lines, *i.e*., two seed parental clones (SPs) and a pollen parental strain (PP) having a mutually high GCA as well as SCA. Moreover, Tamaki *et al*. (2009) explained that high yield performance was exhibited in strains that were developed by CSS using top-cross progenies showing high performance on yields. Top-cross progeny tests are also adopted in a typical RRS scheme (Posselt 2010). Therefore, top-cross progenies are expected to be applicable for yield improvement in timothy.

The objective of this study was to assess the usefulness of GD estimates based on SSR markers for yield improvement in timothy breeding. The specific objectives were (i) to evaluate genetic polymorphism among clones of early maturing breeding populations and the variety ‘Aurora’ which was used as top-cross tester, (ii) to investigate the mean of GD estimates between the clones and ‘Aurora’, and (iii) to assess its relations to yields of top-cross progenies.

Materials and methods

Plant materials

Fifty-one out of 152 clones derived from breeding populations. They were crossed as seed parents with ‘Aurora’ as the pollen parent top-cross tester. Then 16 randomly chosen individuals from ‘Aurora’ were used to assess genetic diversity based on DNA markers. The 51 clones, chosen from 152 clones for covering the whole variations on their origins and yield levels of their progenies to the greatest extent possible, were selected from Japanese local materials or foreign cultivars including some synthetic parental clones and varieties bred at KAES, Hokkaido. Therefore, they adapt well to the local environment (Table 4.1). ‘Aurora’ is an early maturing variety bred using mass selection method from 91 parents by the Hokuren Federation of Agricultural Cooperatives (HFAC), Japan (Shimokoji 1994a).

Field trials

All field experiments were conducted at KAES (43°47′N, 143°42′E) on a high-humic haplic wet andosol. Cumulative DMYs of 152 top-cross progenies bred from crosses between each clone and ‘Aurora’ were evaluated over two years (2003–2004). A top-crossing of clones by ‘Aurora’ was conducted at KAES. Seeds of ‘Aurora’ were

Table 4.1 Derivation of 51 timothy (*Phleum pratense* L.) clones used in SSR analyses, marker-based genetic distances (GD$_T$) toward a top-cross tester ‘Aurora’, and the cumulative annual dry matter yields (DMYs) during two years (2003–2004) of evaluations in field trials of their top-cross progenies at Kunneppu, Hokkaido, Japan

Clone designations†	Derivation			DMY (Mg ha^{-1})	GD$_T$§
	Parent material	Origin	Group‡		
b-04	Clair	USA	A	23.79	0.72
b-08	Clair	USA	A	22.76	0.69
b-12	Climax	Canada	A	20.94	0.69
b-15	Champ	Canada	A	24.83	0.74
b-31	Champ	Canada	A	22.86	0.76
b-40	Canadian	Canada	A	21.17	0.71
c-09	Climax	Canada	A	20.62	0.69
c-33	Labelle	Canada	A	20.79	0.69
d-09	Champ	Canada	A	21.50	0.71
d-39	O-296	Canada	A	22.02	0.73
a-05	Vanadis	Sweden	E	19.78	0.66
a-06	Barenza-Hay	Netherland	E	18.22	0.65
a-13	Eskimo	Netherland	E	21.93	0.69
a-22	Oberhaunstader	Germany	E	22.53	0.71
a-25	No.90	UK	E	22.78	0.73
b-24	PI-345663	Russia	E	23.02	0.72
b-29	Czech local material	Czech	E	23.47	0.71
c-08	Hokuo	Ukraine	E	24.19	0.73
c-13	Polish local material	Poland	E	20.56	0.71
c-24	Lischower	Germany	E	22.49	0.71
c-34	A-4	Norway	E	20.62	0.71
d-17	Toro L84	Italia	E	22.27	0.73
d-28	Landsberger	Germany	E	20.75	0.72
a-07	Senpoku	Japan	J	22.60	0.72
a-10	Horonobe (Japanese local material)	Japan	J	22.94	0.72
a-38	Shichinohe (Japanese local material)	Japan	J	20.54	0.69
a-40	Yamanashi (Japanese local material)	Japan	J	19.83	0.68
b-09	Hidaka (Japanese local material)	Japan	J	22.29	0.68
b-19	Hokunoushi (Japanese local material)	Japan	J	21.98	0.72
b-26	Kitakei 77304	Japan	J	21.75	0.69
b-32	Aurora	Japan	J	21.07	0.69
b-37	84HR-9	Japan	J	19.15	0.68
c-04	Hokuren	Japan	J	21.94	0.72
c-17	Kitami (Japanese local material)	Japan	J	23.40	0.74
c-23	Bekkai (Japanese local material)	Japan	J	24.59	0.72
c-36	Hidaka (Japanese local material)	Japan	J	21.97	0.74
c-39	Kitakei 5313	Japan	J	22.96	0.74
d-03	Kitami (Japanese local material)	Japan	J	19.50	0.66
d-07	Yamanashi (Japanese local material)	Japan	J	22.79	0.72
d-10	Bekkai (Japanese local material)	Japan	J	23.36	0.70
d-23	Bekkai (Japanese local material)	Japan	J	24.50	0.74
d-24	Shiranuka (Japanese local material)	Japan	J	21.73	0.72
d-33	Hidaka (Japanese local material)	Japan	J	21.38	0.73
a-01	9thCE-287	miscellaneous	M	20.60	0.69
a-11	HXIV-32	miscellaneous	M	21.72	0.72
a-23	HXV-12	miscellaneous	M	22,04	0.75
a-37	Nosappu	miscellaneous	M	22.28	0.69
b-11	HXIV-32	miscellaneous	M	21.58	0.72
b-22	Kitakei 0301	miscellaneous	M	23.36	0.72
c-01	11thIS-064	miscellaneous	M	23.50	0.70
c-02	HXV-14	miscellaneous	M	23.40	0.74

† a-, b-, c-, and d- show that their progenies were investigated in each subset of top-progenies test which were divided into four groups for the convenience of field management.

‡ A, E, J, and M refer respectively to America or Canada, Europe or Russia, Japan, and miscellaneous.

§ Genetic distances were estimated using Dice coefficients framed by 28 simple sequence repeat markers.

drilled in a grid pattern as the pollen parent top-cross tester. Each clone was transplanted in August 2001 at a sufficient distance from others (at least 2.4 m distance) and was surrounded with 'Aurora' plants. Seeds of top-cross progenies were harvested from each clone in August 2002. These entries and two checks including 'Aurora' were seeded in August 2002. Plots held four replicates of each entry in two rows, 83 cm long and 60 cm apart, and arranged in an RCBD. The respective amounts of fertilizer applied in the spring and after the first and second cuts were 83, 50, and 33 kg N ha^{-1}. In all, six harvests were collected three times a year.

Genetic distance estimation and statistical analyses

The genotyping protocol used for this study was described in Chapter 3. Briefly, the 28 SSR primer pairs used herein were developed from hexaploid timothy and showed polymorphic in diploid (*Phleum nodosum* L.) and hexaploid timothy (Cai *et al.* 2009). The 28 SSR markers produced 408 polymorphic bands among 67 genotypes, in which the allelic dosage was indistinguishable. Bands produced from the amplification of individual genotypes were scored for presence or absence. Genetic distance estimates were calculated from SSR data for all possible pairs of genotypes using the following equation of one minus Dice coefficients (Dice 1945; Nei and Li 1979): $GD_{i,j} = 1 - [2N_{i,j} / (N_i + N_j)]$, where $GD_{i,j}$ is the GD estimate between clone i and j, $N_{i,j}$ is the total number of bands common to clone i and j, and N_i represents the total number of bands present in i. The mean of GD estimates between each clone and a tester 'Aurora' (GD_T) was calculated from GD estimates between each clone and 16 individuals of Aurora. The unweighted pair group mean average (UPGMA) cluster analysis and PCOA were performed with the matrix of Dice coefficients using appropriate procedures of the program NTSYSpc (Rohlf 2009) and GenAlEx (Peakall and Smouse 2006). Analysis of molecular variance (AMOVA) based on a Euclidean distances matrix was performed using software (Arlequin ver 3.0; Excoffier *et al.* 2005) to test the significance of the differentiation among geographical groups within the 51 clones of breeding population and between 'Aurora' and the 51 clones.

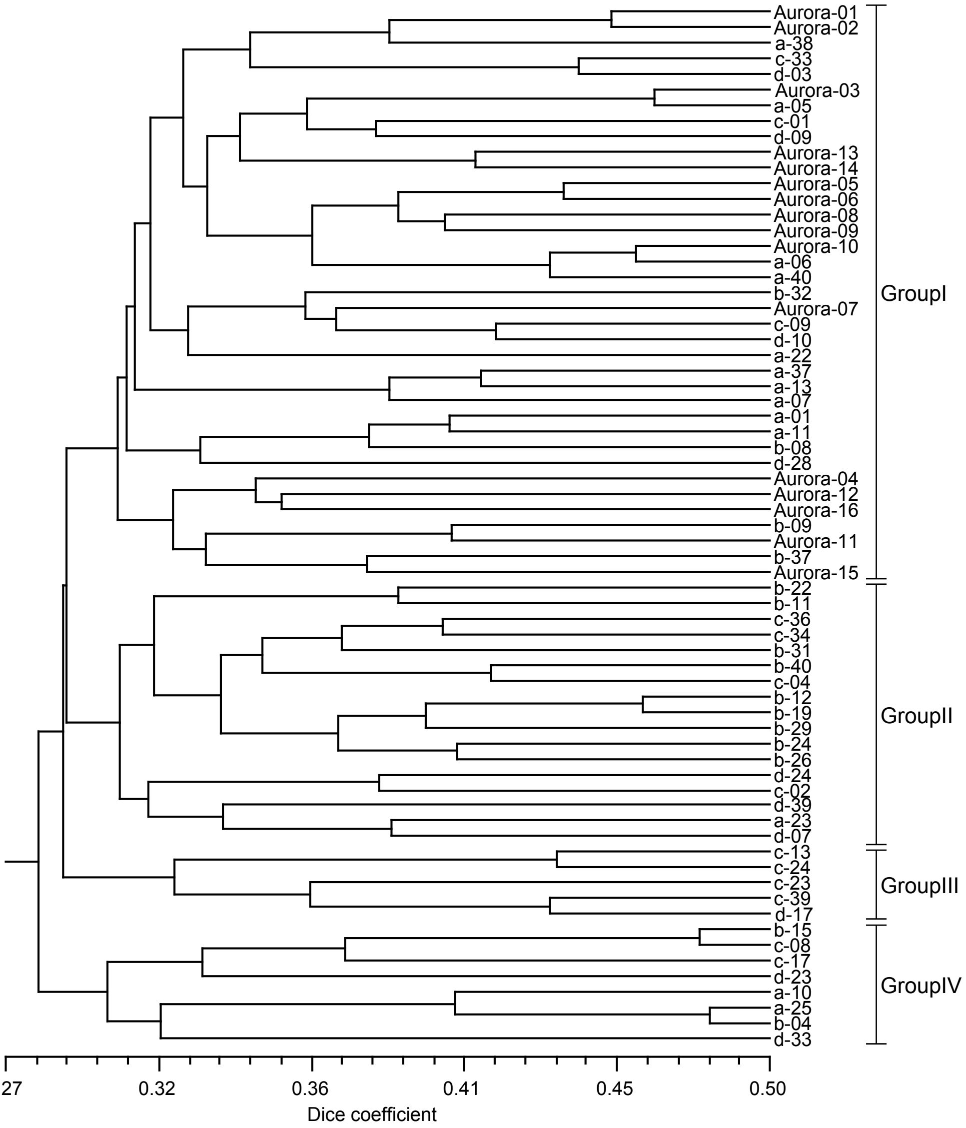

Figure 4.1 Unweighted pair group method using arithmetic average (UPGMA) clustering of 67 timothy (*Phleum pratense* L.) genotypes using a matrix of Dice coefficients. The genotypes included 16 individuals of 'Aurora' and 51 clones from timothy breeding populations in Kitami Agricultural Experiment Station.

Results

Simple sequence repeat marker evaluation and genetic distance estimation

Genetic distance estimates for all pairs among the 67 genotypes had a mean of 0.70 and were 0.52–0.86. For the GD_T estimates of the 51 clones showing relations between each clone and ‘Aurora’, the pollen parent for top-cross testers were 0.65–0.76 with the mean of 0.71. The dendrogram from UPGMA cluster analysis of Dice coefficients based on SSR markers is presented in Fig. 4.1. Clustering produced four major groups designated as group I to IV. Clone ‘b–32’ derived from ‘Aurora’ and 16 individuals of ‘Aurora’ belong to group I. Other seed parents of top-cross progenies were distributed into all four groups. The seed parents of top-cross progenies could not be classified clearly according to their geographical origins. In the PCOA based on the Dice coefficients, the first and second principal coordinates, termed PC1 and PC2, respectively explained 24.3% and 12.8% of total variation in the SSR data (Fig. 4.2). The 16 individuals of ‘Aurora’ were likely to be separated from the 51 clones with respect to PC1. The distribution of the 51 clones was more widely dispersed than ‘Aurora’ in a scatter plot of PCOA. Six clones (‘b-15’, ‘c-01’, ‘c-02’, ‘c-08’, ‘c-17’ and ‘c-23’) for which top-cross progenies showed high performance on yields and had been selected for demonstrating the potential of CSS (Tamaki *et al*. 2009) are presented in Fig. 4.2. Five of these clones, ‘b-15’, ‘c-02’, ‘c-08’, ‘c-17’ and ‘c-23’, were located far from the 16 individuals of ‘Aurora’ in the scatter plot by PCOA. The remaining clone, ‘c-01’, was closer to the 16 individuals of ‘Aurora’. The AMOVA demonstrated that the difference between ‘Aurora’ and the 51 clones was significant. However, greater variation was found among genotypes within populations (Table 4.2). The variance within populations accounted for 96.3% of the total variance, although variance between ‘Aurora’ and the 51 clones of breeding population contributed only 3.7% (Table 4.2). The differences among geographical groups within the 51 clones were unclear from AMOVA, as was the case for UPGMA cluster analysis and PCOA (data not shown).

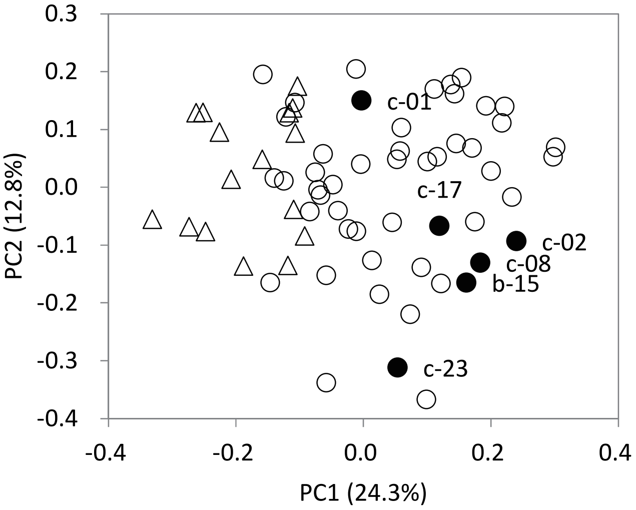

Figure 4.2 Scatter plot of the first two principal coordinate scales for 67 timothy (*Phleum pratense* L.) genotypes including 16 individuals of ‘Aurora’ and 51 clones from breeding population in principle coordinate analysis (PCOA) performed with the matrix of genetic distance (GD) estimates. PC1 and PC2 respectvely represent first and second principal coordinates. Symbols ●, ○ and △ denotes clones (‘b-15’, ‘c-01’, ‘c-02’, ‘c-08’, ‘c-17’, and ‘c-23’) used also in a report by Tamaki *et al*. (2009), the other 45 clones, and 16 individuals of ‘Aurora’.

Table 4.2 Analysis of molecular variance (AMOVA) for 16 genotypes of timothy (*Phleum pratense* L.) cultivar ‘Aurora’ and 51 genotypes from timothy breeding populations (pop.) in the Kitami Agricultural Experiment Station based on 28 SSR markers

Source of variation	df	SS†	VC‡	%§	*P*-value¶
‘Aurora’ vs. breeding population					
Among pop.	1	138.1	2.75	3.7	<0.00001
Within pop.	65	4629.0	71.22	96.3	
Total	66	4767.1	73.96		

† Sum of squares.
‡ Variance components.
§ Percentage of variation.
¶ Based on 1023 permutations.

Relations between means of genetic distance estimates and yields of top-cross progenies

Significant differences among yields of all 152 top-cross progenies were detected in top-cross progeny yield trials. The mean of cumulative DMY over two years of all top-cross progenies was 21.9 Mg ha^{-1}, SD was 1.1 Mg ha^{-1}. Cumulative DMY over two years of the progenies developed from the 51 clones used in the SSR evaluation are shown in Table 4.1. The mean of cumulative DMY of the 51 progenies was 22.0 Mg ha^{-1}, SD was 1.4 Mg ha^{-1}, and the values (mean and SD) were similar to that for all 152 progenies. The relation between GD and yield was evaluated by calculating the Pearson product-moment correlation coefficient between GD_T estimates and yield

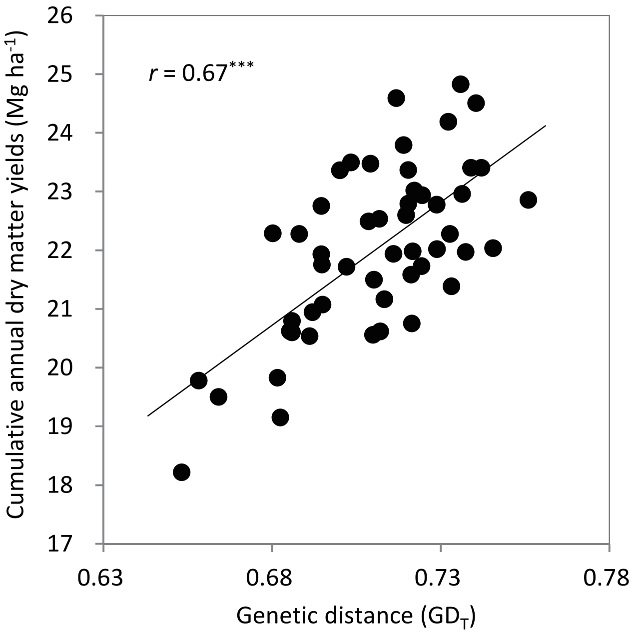

Figure 4.3 Relation between cumulative annual dry matter yields (Mg ha^{-1}) of top-cross progenies and genetic distance (GD$_T$) among their parents. The top-cross progenies derived from crossing between each timothy (*Phleum pratense* L.) clone and timothy variety 'Aurora'. Each experimental plot was harvested three times per year during 2003–2004. Pearson product-moment correlation coefficient is signified by "*r*". *** denotes significance *r*-value ($p < 0.001$).

data obtained from top-cross progenies (Fig. 4.3). The correlation coefficient between GD$_T$ estimates and yields was computed as 0.67 ($p < 0.001$).

Discussion

The GD$_T$ estimates were 0.65–0.76. The values resembled those in maize reported by Enoki *et al.* (2005), in which GD estimates between each miscellaneous maize inbred line and 35 dent inbred lines using 60 SSR markers carrying 463 alleles among 88 genotypes were 0.65–0.80. In general, GD calculated using many DNA markers reflects genetic diversity across the board genome. Therefore, GD is used for predicting heterozygosity on the assumption that overall genetic diversity based on the GD estimate is relevant to probability of heterozygosity at the locus associated with heterosis in crossing. As presented in Fig. 4.3, GD$_T$ estimates are closely related with the yields of their progenies developed from the top-cross with 'Aurora'. DNA marker loci assayed in many studies were not substantially associated with genes related to components of yields including both additive and non-additive effects. This lack of association produced poor correlation between heterosis and genetic diversity in some studies (Melchinger *et al.* 1990a; Bernardo 1992). Positive correlation in our study, however, resulted from (i) significant difference between 'Aurora' and clones based on the AMOVA, (ii) reasonable variation among GD$_T$ estimates of each clone, and (iii) adaptation to the local environment that all clones had acquired. In a cross between adapted potato breeding lines, maximum heterozygosity was positively correlated with the proportion of tuber yield in the large-size fraction (Bonierbale *et al.* 1993). Considering this, these results confirmed SSR marker diversity as a useful tool for screening genotype combinations inducing high yield performance within the limits of a breeding population with better adaptability.

Positive correlation between GD$_T$ estimates and yields of top-cross progenies strongly suggests that DNA profiling will provide reliable information related to the yield of the assumed top-cross progeny lines and that it can be expected to help breeders to accelerate yield improvement by CSS in timothy or other perennial self-incompatible forage grasses. In addition, Figs. 4.1 and 4.2 clearly present that five out of the six clones ('b-15', 'c-02', 'c-08', 'c-17' and 'c-23'), of which the top-cross progenies showed high yield performance and which had been selected for demonstrating the potential of CSS (Tamaki *et al.* 2009) were distantly located from 'Aurora'. Distant locations of these clones identified by SSR profiling also supported the potential for DNA profiling. As presented from several studies of maize (Betrán *et al.* 2003; Enoki *et al.* 2005), pairs with large GD led to their progenies' high yield through their high SCA resulting from high heterozygosity. Moreover, the relation between GD$_T$ estimates and the yield of the top-cross progenies found in this study also seems to have derived from the SCA between their seed and pollen parents. Clone 'c-01', which differs from the other five, is much closer to 'Aurora' (Figs. 4.1 and 4.2), although the yield of its top-cross progeny was as high as those of the others. Taking the following two facts into consideration, the high yield in the top-cross progeny of clone 'c-01' is likely to be attributable to GCA rather than SCA. First, an earlier study by KAES (Tamaki *et al.* 2009) demonstrated that the yield in all CSS strains derived from crosses with 'c-01' as SP, without exception, were remarkably higher than those of other strains with no involvement of ‘c-01’. Secondly, ‘c-01’ showed the third highest combining ability in another polycross progenies trial having 41 entries at KAES (cumulative DMY for two years from 2001 was 123.0% of ‘Aurora’ and 106.2% of the average of all progenies). The difference between ‘c-01’ and the other five clones implies that it is important to improve both GCA and SCA of parent candidates for effective yield improvement in CSS. Consequently, efforts must be made for effective GCA improvement by developing techniques similar to those used for this study.

Ecotypes or landrace collections have been established in many regions by contemporary large-scale breeding programs. Remarkable differences have been observed among them in various traits (Larsen and Marum 2006, Tamaki *et al*. 2010). In spite of such differences, no clear distinction was observed in this study using SSR markers in terms of the geographical origin of clones (Figs. 4.1 and 4.2). In field trials for the 152 top-cross progenies for which outlines are described above, the geographical or varietal origins of each SP did not account well for the difference between the yield levels of their progenies (H. Tamaki, unpublished). Therefore, it is possible that breeders adopting CSS need not be troubled about the details of the origins of SP candidates as long as they have sufficient adaptability to the breeding sites. These results seem to accord with those of Casler *et al*. (2007), who conducted amplified fragment length polymorphism marker analyses in switchgrass (*Panicum virgatum* L.) and found that variation among the wild populations collected from the east coast of United States to the coast of Lake Superior was much smaller than the variation within them. Similar results have been reported also for perennial ryegrass, meadow fescue (*Festuca pratensis* Huds.), orchardgrass, rhodesgrass (*Chloris gayana* Kunth), and timothy (Huff 1997; Kölliker *et al*. 1999; Ubi *et al*. 2003; Tanhuanpää and Manninen 2012). However, the difference between the 16 genotypes representing ‘Aurora’ and the others was significant in this study. ‘Aurora’ had been developed for a specific target trait: the lodging resistance. It is possible that ‘Aurora’ has a narrow genetic background, but it is difficult to accurately explain the reasons for smaller within-population variation in ‘Aurora’ because little information has been announced in relation to its breeding history.

As an alternative to applying DNA profiling to CSS, the clear relation between the GD_T estimates and the yield of the top-cross progenies found in this study also suggests the potential for applying DNA profiling techniques to practical breeding methodologies of perennial self-incompatible forage grasses such as timothy. In general, top-cross progenies were handled similarly, with open-cross or polycross progenies in half-sib selection. A new cultivar can be developed efficiently using half-sibs derived from a cross between a leading variety and superior clones with expected high yield performance inferred from DNA profiling. In fact, DNA profiling is expected to be more efficient for a breeding program targeting heterosis and SCA (Liu *et al*. 2002). In addition, top-cross progenies between a leading variety and superior clones derived from polycrosses through recurrent selections using genetically broad-based populations can be expected to show high yield performance including both GCA and SCA. The method requires no expensive markers such as gene function markers. Moreover, it is reliably applicable for a practical timothy breeding program. Integration of DNA profiling with field trials can be expected to help develop efficient breeding programs, and can therefore expedite a new cultivar development process for important forage grass species such as timothy.

Chapter 5: Development of timothy “clone and strain synthesis” strains by combining top-cross progenies

5.1: Relative contributions of general and specific combining abilities in timothy “clone and strain synthesis” breeding scheme

Introduction

A potentially effective way to improve forage yield in the future is to capitalize on non-additive gene action by harnessing heterosis or SCA (Li and Brummer 2009) as in the F_1 hybrids produced with RRS in maize, which is an outstanding and symbolic example of agricultural progress during the latter half of the 20th century (Sleper and Poehlman 2006). Because long-term rapid yield improvement in maize has been achieved using RRS procedures rather than merely by hybrid breeding itself, the most critical point is not hybrid development *per se* but the introduction of RRS to create and use heterotic groups for yield improvement in forage grasses or legumes.

Estimates of GCA and SCA help breeders devise breeding and selection strategies. Several breeding methods have been proposed for effective yield improvement in forage grasses or legumes with the aim of capturing heterosis or exploiting SCA (Burton 1948; Rotilli *et al.* 1996; Brummer 1999; Tamaki *et al.* 2007, 2009). One method is a CSS approach that exploits both GCA and SCA and minimizes the risk of inbreeding depression. This method has been used to develop strains producing significantly higher yield performance in timothy (Tamaki *et al.* 2009). The CSS scheme consists of two procedures: (i) crossing two SPs with a common PP for the seeds harvested from the two SPs as first generation synthetic progenies (Syn1), *i.e.*, developing two top-cross progenies; and (ii) even mixing of the Syn1 seeds (two top-cross progenies) to produce subsequent generations, as is done to develop conventional synthetic strains from several parental clones. Clone and strain synthesis strains therefore include 1/2 derived from crossing among two top-cross progenies and 1/2 within each top-cross progenies. The allele frequency of a CSS strain is 1/4 derived from one SP being one genotype, 1/4 from another SP being one genotype, and 1/2 from PP being numerous genotypes (Tamaki *et al.* 2009). Various genetic effects, such as GCA of parental materials and SCA among parental materials, are expected to influence forage yields of Syn1 and the second synthetic generation (Syn2) of CSS, respectively. Knowledge of quantitative genetic parameters is therefore crucial for allocating resources for parental selection in the CSS breeding scheme.

Information on genetic relations within species is used to facilitate selection of breeding materials in some crop-breeding programs (Lee 1995; Kiula *et al.* 2008). In Chapter 4, a positive correlation ($r = 0.68$, $p < 0.001$) was found between marker-based GD estimates toward a tester and forage yields of top-cross progenies corresponding to Syn1 of the CSS breeding scheme. However, reports of the literature describe few attempts to establish a relation between GD estimates among parents and observed yields of Syn2 of well-developed CSS strains in timothy breeding programs. The aims of this study were to examine (i) the association of the GD estimates among the SPs of each CSS strain with its forage yield in Syn2 generation and (ii) the relative contribution of GCA and SCA to forage yield in CSS breeding scheme by predicting the yields of Syn1 and Syn2 with molecular marker and field evaluation data.

Materials and methods

First synthetic generation of clone and strain synthesis strains

Timothy 51 top-cross progenies, which correspond to the Syn1 generation of CSS, and their SPs were used in this experiment. The cumulative DMYs over two years (2003–2004) of the progenies and marker-based GDs of SPs toward a PP (GD_T) are presented in Chapter 4 (Table 4.1). Field trails and the genotyping protocol are described in Chapter 4. In addition, GCA values of the 51 SPs are examined using BLUP in Chapter 2 (Table 5.1), except for ‘a-13’, which had not been examined in polycross progeny tests. Effective progeny numbers of SPs (n_E in Table 5.1) indicating the connectedness of data in BLUP procedure were calculated as follows (Robertson and Rendel 1954): $n_{Ei} = \Sigma\{n_{i,j}\ (1 - n_{i,j}\ /\ n_j)\}$, where $n_{i,j}$ is a number of i th entries in j th progeny test ($n_{i,j} = 1$ in all cases of this experiment), n_j is the number of targeted entries in the j th

Table 5.1 Design matrix† in 11 timothy (*Phleum pratense* L.) polycross progeny tests (A–K), the effective progeny number (n_E)‡, and general combining ability for annual dry matter yields (GCA)§ of 50 parental clones

Parental clone	Entry of polycross progeny tests												
designations	A	B	C	D	E	F	G	H	I	J	K	n_E	GCA
b-04	Y								Y			1.87	0.162
b-08	Y						Y					1.87	0.157
b-12										Y		0.83	0.103
b-15	Y											0.97	0.378
b-31	Y											0.97	0.367
b-40						Y						0.90	0.039
c-09						Y						0.90	–0.027
c-33									Y			0.90	–0.075
d-09						Y						0.90	0.025
d-39							Y					0.90	–0.016
a-05									Y			0.90	–0.074
a-06									Y			0.90	–0.029
a-22							Y					0.90	–0.010
a-25										Y		0.83	–0.081
b-24	Y				Y							1.88	–0.132
b-29	Y											0.97	0.036
c-08	Y						Y					1.87	0.269
c-13	Y											0.97	–0.281
c-24	Y										Y	1.80	0.124
c-34							Y					0.90	–0.087
d-17				Y								0.86	–0.187
d-28											Y	0.83	–0.153
a-07								Y				0.88	0.081
a-10				Y								0.86	0.076
a-38											Y	0.83	0.514
a-40						Y						0.90	–0.069
b-09						Y						0.90	0.064
b-19								Y				0.88	–0.017
b-26	Y											0.97	0.250
b-32		Y	Y									1.95	0.409
b-37					Y							0.92	–0.063
c-04									Y			0.90	–0.013
c-17				Y								0.86	–0.109
c-23	Y				Y							1.88	0.330
c-36							Y					0.90	0.004
c-39	Y						Y					1.87	–0.049
d-03									Y			0.90	–0.039
d-07								Y				0.88	0.055
d-10	Y				Y							1.88	0.028
d-23	Y				Y							1.88	0.336
d-24											Y	0.83	0.085
d-33								Y				0.88	0.011
a-01		Y										0.98	0.226
a-11					Y							0.92	–0.059
a-23	Y				Y							1.88	–0.181
a-37	Y				Y							1.88	0.168
b-11					Y							0.92	–0.030
b-22	Y					Y						0.97	–0.035
c-01	Y	Y										1.94	0.595
c-02	Y			Y								1.82	–0.175

† Y means entry to each polycross progeny tests: A–K.

‡ The effective progeny number (n_E) is calculated according to Robertson and Rendel (1954).

§ GCA is estimated by fitting to a mixed additive model based on annual dry matter yields in 11 polycross progeny tests with 180 data and 100 entries.

progeny test. To investigate the relative contributions of GCA and SCA to yields of Syn1 generation of CSS strains (*i.e.* top-cross progenies), the dataset including the cumulative DMYs over two years of top-cross progenies, $\mathrm{GD_T}$ and GCA, was analyzed according to the following multiple linear regression: $y = \beta_0 + \beta_1\, GD_{\mathrm{T}} + \beta_2\, GCA$, where y is the cumulative DMYs of top-cross progenies, β_0, β_1 and β_2 are partial regression coefficients (PRCs), GD_{T} represents marker-based *GD*s toward a PP, and *GCA* is a BLUP value based on polycross progeny tests. In addition, standardized PRCs and the Pearson product-moment correlation coefficients among the three data (DMY of top-cross progenies, $\mathrm{GD_T}$, and GCA) were calculated.

Second synthetic generation of clone and strain synthesis strains

Second synthetic generation of 15 CSS strains reported by Tamaki *et al.* (2009) and their parental materials were used in this experiment. Seed parent clones of the 15 (= ${}_6C_2$) CSS strains were 'b-15', 'c-01', 'c-02', 'c-08', 'c-17', and 'c-23' (Table 5.2), which were selected for high yields out of 152 entries in top-cross progeny tests described in Chapter 4. The common PP of the CSS strains was 'Aurora' (Shimokoji 1994a).

The cumulative DMYs of 15 CSS strains, which had been developed from all possible pairs of six top-cross progenies derived from crosses between the SPs and 'Aurora', were evaluated at KAES, Hokkaido Research Organization, Kunneppu, Hokkaido, Japan, (43°47′N, 143°42′E) on a high-humic haplic wet andosol over two years (2005–2006). The CSS strains and five check varieties including 'Aurora' were planted in an RCBD with four replicates in August 2004. The plots were 2.0 by 1.0 m with four drilled rows. The amounts of fertilizer used on the plots were the following: 90 kg N ha^{-1}, 180 kg P_2O_5 ha^{-1} and 90 kg K_2O ha^{-1} in early spring, 50 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 50 kg K_2O ha^{-1} after the first harvest and 40 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 40 kg K_2O ha^{-1} after the second harvest. Cumulative DMY of each plot over the two years (six harvests – three per year) were processed with ANOVA to detect the significance among the entries.

The six SPs and 16 individuals of 'Aurora' as a PP were used in SSR evaluation. The outlines of the genotyping protocol used for this study were described in Chapters 3 and 4. Genetic distance estimates were calculated from SSR data for all possible pairs of genotypes using the following equation: $GD_{i,j} = 1 - [2N_{i,j} / (N_i + N_j)]$, where $GD_{i,j}$ is the GD estimate between clone i and j, $N_{i,j}$ represents the total number of bands common to clone i and j, and N_i denotes the total number of bands present in i (Dice 1945; Nei and Li 1979). Principal coordinate analyses were performed on the matrix of the GD estimates with the GenAlEx program (Peakall and Smouse 2006).

To investigate the relative contribution of GCA and SCA to yields of Syn2 generation of CSS strains, a dataset including the cumulative DMYs of Syn2 of CSS strains, the cumulative DMYs of Syn1, sum of GCA (ΣGCA) of the combinations in the six SPs examined using BLUP (Chapter 2), marker-based GD estimates among SPs, and marker-based GD between SPs and a PP ($\mathrm{GD_T}$) was analyzed according to multiple linear regression analysis with selection of variables based on Akaike's information criterion (AIC) employing the function 'lm' in the R

Table 5.2 Derivation of seed parental clones of 'clone and strain synthesis (CSS)' timothy (*Phleum pratense* L.) strains, genetic distance (GD) estimates among seed parental clones using 28 simple sequence repeat markers and cumulative annual dry matter yields (DMYs) over two years (2005–2006) of CSS strains† at Kunnepu, Hokkaido, Japan

Seed parental clones		GD estimates‡						DMY§					
Designation	Derivation¶	b-15	c-01	c-02	c-08	c-17	c-23	b-15	c-01	c-02	c-08	c-17	c-23
b-15	Champ		0.76	0.68	0.52	0.66	0.74		31.78	30.47	26.96	29.14	30.73
c-01	11thIS-064			0.75	0.75	0.67	0.72	109.0		30.26	31.19	31.57	31.31
c-02	HXV-14				0.72	0.70	0.70	104.5	103.8		28.22	28.08	28.63
c-08	Hokuo					0.59	0.67	92.5	107.0	96.8		27.96	31.12
c-17	Kitami						0.71	100.0	108.3	96.3	95.9		28.09
c-23	Bekkai							105.4	107.4	98.2	103.3	96.4	

† Fifteen CSS strains developed from all possible pairs of seed parental clones and a common pollen parent, 'Aurora'.

‡ GD estimates were calculated for all possible pairs of individuals according to Dice (1945) and Nei and Li (1979).

§ Mg ha^{-1} (above diagonal) and percentage of a check variety 'Aurora' (below diagonal). Experimental plots were harvested three times per year during 2005–2006.

¶ 'Champ' and 'Hokuou' were registered timothy varieties in OECD lists, '11thIS-064' and 'HXV-14' were half-sib materials in Kitami Agricultural Experiment Station and 'Kitami' and 'Bekkai' were local Japanese materials.

statistical software package (GNU General Public License).

Results

Predicting yields of top-cross progenies

The correlations (r) among DMYs of 50 top-cross progenies, GD_T and GCA of their SPs were –0.02 ($p > 0.05$, GD_T vs. GCA), 0.68 ($p < 0.001$, DMY vs. GD_T), 0.33 ($p < 0.05$, DMY vs. GCA), respectively. The multiple linear regression analysis predicting DMYs of top-cross progenies with GD_T and GCA produced significant (F-value = 32.6; df = 2, 47; $p < 0.001$) regression models (Table 5.3). The respective partial regression coefficients (β_0, β_1 and β_2) in the regression analysis were –8.57 (β_0; intercept), 42.8 (β_1; the PRC of GD_T), and 2.69 (β_2; the PRC of GCA). All PRCs were significant (Table 5.4). The standardized PRCs of GD_T (0.68) was larger than that of GCA (0.35) (Table 5.4). The multiple correlation coefficient was 0.76. The squared multiple correlation coefficient adjusted for the degrees of freedom was 0.56.

Table 5.3 Regression analysis of variance of marker-based genetic distances (GD_T) toward a tester and general combining ability (GCA) estimates of seed parents on cumulative annual dry matter yields (DMYs)† of 50 timothy (*Phleum pratense* L.) top-cross progenies over two years (2003–2004) at Kunneppu, Hokkaido, Japan

Source of variance	df	Mean of squares	F-value	P-value
Regression	2	29.8	32.6	< 0.001
GD_T‡	1	48.0	52.6	< 0.001
GCA§	1	11.5	12.6	< 0.001
Residual	47	0.914		
Total	49			

† Experimental plots were harvested three times per year during 2003–2004.
‡ GD_T was calculated from the average of genetic distances (GDs) between each seed parent and 16 individuals of tester ‘Aurora’ based on 28 simple sequence repeat marker diversity.
§ Best linear unbiased prediction values of SPs in mixed effect models based on 11 polycross progeny tests with 180 data and 100 entries, where polycross progenies of the SPs were examined.

Yields of the second synthetic generation of clone and strain synthesis strains

Significant differences were detected among the yield of 15 CSS strains in field trials. The mean of DMY over two years of 15 CSS strains was 29.63 Mg ha^{-1} and DMY were 26.96–31.78 Mg ha^{-1} (Table 5.2). Four strains out of the 15 showed significantly higher yield levels than ‘Aurora’ (29.15 Mg ha^{-1}), their PP, although the mean DMY of 15 strains (29.63 Mg ha^{-1}) was similar to that of ‘Aurora’.

Table 5.4 Partial regression coefficients (PRCs) of a linear model with marker-based genetic distances (GD_T) toward a tester and general combining ability (GCA) estimates of seed parents on cumulative annual dry matter yields (Mg ha^{-1})† of 50 timothy (*Phleum pratense* L.) top-cross progenies over two years (2003–2004) at Kunneppu, Hokkaido, Japan

Factors	PRC	SE‡	t-value	P-value	Standardized PRC
GD_T§	42.8	5.9	7.25	< 0.001	0.68
GCA¶	2.70	0.73	3.69	< 0.001	0.35
Intercept	–8.57	4.20	2.04	0.047	

† Experimental plots were harvested three times per year during 2003–2004.
‡ Standardized error.
§ GD_T was calculated from average of genetic distances (GDs) between each seed parent and 16 individuals of tester ‘Aurora’ based on 28 simple sequence repeat marker diversity.
¶ Best linear unbiased prediction values of SPs in mixed effect models based on 11 polycross progeny tests with 180 data and 100 entries, where polycross progenies of the SPs were examined.

Simple sequence repeat marker evaluation and genetic distance among parents

The marker-based GD estimates among the six SPs were 0.52 (between ‘b-15’ and ‘c-08’) to 0.76 (between ‘b-15’ and ‘c-01’) (Table 5.2). The marker-based GD estimates for all possible pairs among the 22 individuals including the 16 individuals of ‘Aurora’ were 0.52–0.83. In the PCOA based on the GD estimates, the first and second principal coordinates, termed PC1 and PC2, explained 8.7% and 6.8% respectively of the total variation in the SSR data (Fig. 5.1). The SPs, portrayed in Fig. 5.1, were separated from the 16 individuals of ‘Aurora’ with respect to PC1. Five out of the six SPs, ‘b-15’, ‘c-02’, ‘c-08’, ‘c-17’, and ‘c-23’, were located distally from the 16 individuals of ‘Aurora’, although ‘c-01’ was closer to ‘Aurora’.

Predicting yields of clone and strain synthesis strains

As results of the selection of the explanatory variables based on AIC in the multiple linear regression analysis, two factors which were marker-based GD among SPs and ΣGCA of the two SPs examined using BLUP were selected for prediction of the yield of Syn2 of CSS strains (Tables

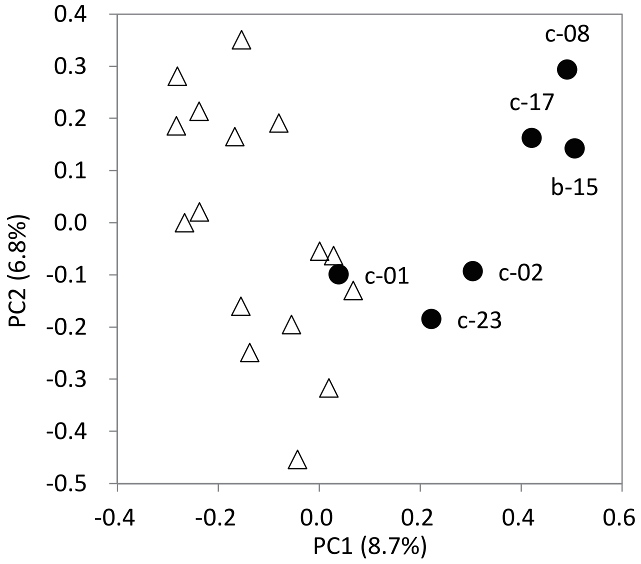

Figure 5.1 Scatter plot of the first two principal coordinate scales for 22 timothy (*Phleum pratense* L.) genotypes including 16 individuals of 'Aurora' and six parental clones constituting 15 (= $_6C_2$) 'clone and strain synthesis' strains in principal coordinate analysis (PCOA) performed with matrix of genetic similarity coefficients. PC1 and PC2 respectively denote the first and second principal coordinates respectively. Symbols ● and △ respectively denote six clones ('b-15', 'c-01', 'c-02', 'c-08', 'c-17' and 'c-23') and 16 individuals of 'Aurora'.

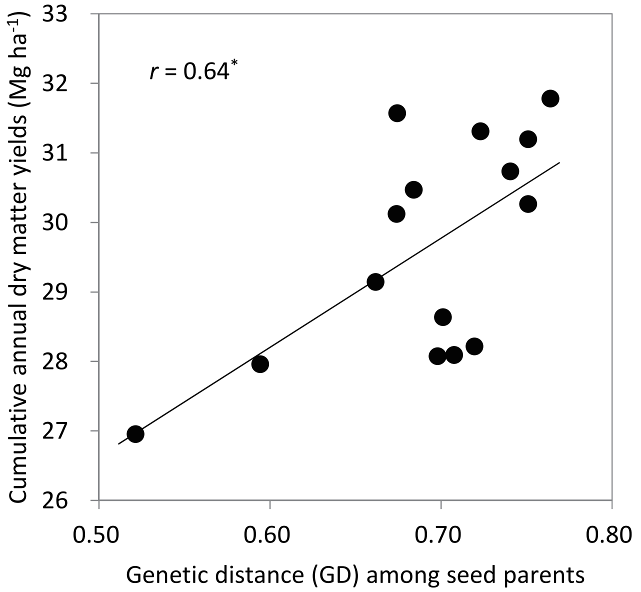

Figure 5.2 Relation between cumulative annual dry matter yields of 15 'clone and strain synthesis (CSS)' timothy (*Phleum pratense* L.) strains and genetic distance estimates among pairs of two seed parents constituting each CSS strains. *r* denotes Pearson's correlation coefficient. * represents significance *r*-value ($p < 0.05$). The cumulative annual dry matter yields were examined over two years (2005–2006) at Kunneppu, Hokkaido, Japan.

5.5 and 5.6). The correlations between response and explanatory variables were, respectively, 0.66 ($p < 0.01$, DMY vs. ΣGCA) and 0.64 ($p < 0.05$, DMY vs. GD; Fig. 5.2). Analysis of variance of the regression (Table 5.5) found significance of regression (*F*-value = 13.8; df = 2, 12; $p < 0.001$). The prediction model, in which all PRCs were significant, was the following equation: $y_{i,j} = 19.7 + 12.9\ GD_{i,j} + 2.40\ (GCA_i + GCA_j)$, where $y_{i,j}$ is DMY of Syn2 of CSS strain derived from SP *i* and *j*, $GD_{i,j}$ represents marker-based GD estimates between SP *i* and *j*, and GCA_i stands for the GCA estimate of SP *i* (Table 5.6). The standardized PRCs of GD (0.53) were similar to that of ΣGCA (0.55) (Table 5.6). The multiple correlation coefficient was 0.83. The squared multiple correlation coefficient adjusted for the degrees of freedom was 0.65 (Fig. 5.3).

Table 5.5 Regression analysis of variance of marker-based genetic distances (GDs) among timothy (*Phleum pratense* L.) seed parents (SPs) of 'clone and strain synthesis (CSS)' strains and sum of general combining ability (GCA) estimates of SPs on cumulative annual dry matter yields (Mg ha^{-1})† of 15 CSS strains over two years (2005–2006) at Kunneppu, Hokkaido, Japan

Source of variance	df	Mean of squares	*F*-value	*P*-value
Regression	2	11.9	13.8	0.0007805
GD‡	1	9.12	10.6	0.006955
GCA§	1	14.7	17.0	0.001418
Residual	12	0.863		
Total	14			

† Experimental plots were harvested three times per year during 2005–2006.
‡ GD were calculated based on 28 simple sequence repeat markers diversity according to Dice (1945) and Nei and Li (1979).
§ Best linear unbiased prediction values of SPs in mixed effect models based on 11 polycross progeny tests with 180 data and 100 entries, where polycross progenies of the SPs were examined.

Discussion

It was found in maize that marker-based GD among parents is associated with yields of their progenies because of a SCA effect (Qi *et al.* 2010). The GD_T and BLUP of SPs can be regarded respectively as SCA and GCA. Positive correlation between GD_T estimates and yields of top-cross progenies (Chapter 4 and results present herein) suggests that yields of top-cross progenies had come from the SCA between their SP and PP. The multiple linear

Table 5.6 Partial regression coefficients (PRCs) of a linear model with marker-based genetic distances (GD) among timothy (*Phleum pratense* L.) seed parents (SPs) of 'clone and strain synthesis (CSS)' strains and sum of general combining ability (ΣGCA) estimates of SPs on cumulative annual dry matter yields (Mg ha^{-1})† of 15 CSS strains over two years (2005–2006) at Kunneppu, Hokkaido, Japan

Factors	PRC	SE‡	*t*-value	*P*-value	Standardized PRC
GD§	12.9	4.0	3.25	0.007	0.53
ΣGCA¶	2.40	0.71	3.36	0.006	0.55
Intercept	19.7	2.7	7.24	< 0.001	

† Experimental plots were harvested three times per year during 2005–2006.

‡ Standardized error.

§ GD were calculated based on 28 simple sequence repeat markers diversity according to Dice (1945) and Nei and Li (1979).

¶ Sum of best linear unbiased prediction values of SPs in mixed effect models based on 11 polycross progeny tests with 180 data and 100 entries, where polycross progenies of the SPs were examined.

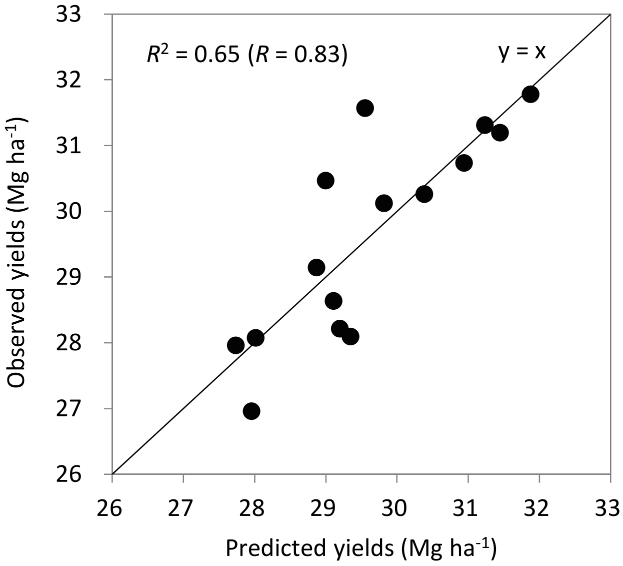

Figure 5.3 Observed and predicted cumulative annual dry matter yields of 15 'clone and strain synthesis (CSS)' timothy (*Phleum pratense* L.) strains. Prediction were performed multiple regression based on sum of GCA of two parental clones (PCs) and genetic distances (GDs) among two SPs as follows: $y = 19.7 + 2.40\ \Sigma\text{GCA} + 12.9\ \text{GD}$ (see Table 5.6).

regression analysis predicting yields of top-cross progenies with GD_T and GCA in this study, where the PRCs of GD_T and GCA were significantly ($p < 0.01$) larger than zero (Table 5.4), showed that both GCA and SCA contributed to yields of top-cross progenies. Regarding relative contribution GCA and SCA to yields of top-cross progenies, SCA (standardized PRC of $\text{GD}_\text{T} = 0.68$) were larger than GCA (standardized PRC of BLUP = 0.35) (Table 5.4). The lack of significant correlation of GD_T and GCA of SPs ($r = -0.02$, $p > 0.05$) suggests that GCA and SCA were mutually independent. As one might expect, simultaneously maximizing both SCA toward a tester and GCA of SPs would be difficult in parental selections for SPs candidates in top-cross progeny tests.

The significant positive correlation ($r = 0.64$, $p < 0.05$) between forage yields of 15 CSS strains and marker-based GD estimates among SPs (Fig. 5.2) suggests that considerable SCA effects among SPs contributed to the yield performances of the CSS strains, as proposed by Tamaki *et al.* (2009), and suggests that the use of elite clones as SPs of CSS strain, selected from putative heterotic group formed based on molecular marker, leads to efficient development of high-yielding strains with exploitation of SCA effect among SPs. Various genetic effects, which were GCA of a PP, GCA of SPs, SCA among SPs, and SCA between PP and SPs, can be expected to contribute to yields of Syn2 of CSS strains. In multiple linear regression analysis predicting yields of Syn2 of CSS strains, GCA of SPs and GD among SPs were selected as the predictor variables (Tables 5.5 and 5.6, Fig. 5.3), although GCA of a PP was excluded from the analysis because of 15 CSS strains derived from the common PP 'Aurora'. These results indicate that selection based on GCA of SPs and GD among SPs is promising for a CSS breeding program.

Creation and utilization of heterotic groups are expected to play key roles for CSS breeding programs in timothy. A significant positive correlation between GD estimates and yields of top-cross progenies in previous study in timothy (Chapter 4) suggests that parents separated based on molecular marker diversity might be in different heterotic groups. In crops such as maize, where an F_1 hybrid advantage is established and for which heterotic groups have been identified clearly, molecular marker approaches have been used to show relations between GD and yield performance in single-cross hybrids (Betrán *et al.* 2003; Reif *et al.* 2003; Qi *et al.* 2010) and to identify different heterotic groups (Enoki *et al.* 2002; Reif *et al.* 2003). Distinct groups have been identified based on molecular markers also in some forage grasses including perennial ryegrass (Bolaric *et al.* 2005; Posselt 2010) and timothy (Guo *et al.* 2003). As improvement for yield with our emphasis on a wide array of important traits, more effort should be devoted to using putative heterotic groups based on molecular marker diversity within a well-adopted current breeding population, which was often established as one population by breeders, rather than a search for distinct populations within non-adopted germplasm. In an earlier study (Tamaki *et al*. 2009), the selection of SPs in top-cross progeny tests derived from crossing SPs and a PP 'Aurora' caused separation of SPs and a PP (Fig. 5.1) and high-yielding because of SCA between SPs and a PP (Tables 5.3 and 5.4) except for 'c-01'. However, the results of the present study (Tables 5.5 and 5.6) suggest that utilization of putative heterotic groups as the first step in a RRS approach should be selection based on GCA of SPs and GD among SPs. Replacing the parents as described in Tamaki *et al*. (2009) should be selecting SPs from each marker-based putative heterotic group.

A CSS strain is not the same as a hybrid cultivar, it is a kind of synthetic variety because a number of parents (or genotypes) are included in the strain. Comparing with conventional variety synthesis shows that CSS presents some benefits for breeding for high yield as well as high adaptability. In general, the development of conventional synthetic varieties by intercrossing a limited number of clones (generally 5–12) having superior combining ability just partially exploits heterosis during a limited number of generations of seed increase. In perennial ryegrass, first and second generations of synthetic cultivars derived from genetically wider polycrosses showed higher yields than those derived from genetically narrower ones based on amplified fragment length polymorphism diversity (Kölliker *et al*. 2005). However, it is not feasible to create 5–12 subgroups that tend to exhibit high performance when crossed mutually and to intercross individuals selected from each subgroup in conventional variety synthesis. A three-parent approach using a PP and two SPs in CSS is expected to be more suitable than conventional synthetic cultivars for RRS with two or three heterotic groups. Another benefit of CSS that merits further discussion is the adoption of current leading varieties as PPs in CSS. The use of leading cultivars as PPs in CSS development is expected to engender the development of new upgraded strains with strong selling points and without fatal shortcomings as well as originally competent leading varieties. Other noteworthy points are related to the role of PPs in CSS. Except for the PP, only two genotypes constitute CSS strains. As few as two clones have been used in conventional synthetic varieties of alfalfa and tall fescue (Sleper and Poehlman 2006). The synthetic varieties based on two clones of alfalfa were unsuccessful as a result of inbreeding depression because pollinators tended to favor a specific clone, leading to seed produced by self-pollination or sib-pollination rather than by cross-pollination. However, Tamaki *et al*. (2009) estimated that the inbreeding coefficient of a CSS strain is similar to that of a conventional synthetic cultivar bred from six parental clones. From this viewpoint, PPs help CSS strains to be dealt with in the same way as conventional synthetic varieties for commercial distribution.

5.2: Evaluation of forage yield performance in advanced generations of timothy "clone and strain synthesis" strain

Introduction

Timothy national breeding program and network system in Japan started since 1964 (Ueda 1990). Raising the forage yield remains a high-priority target of timothy breeding programs in Japan (Ueda 1990; Tamaki *et al*. 2010). Tamaki *et al*. (2009) proposed CSS, which is similar to variety synthesis, to boost improvement of forage yield by exploiting both GCA and SCA in perennial self-incompatible forage grasses. As a result of selection for forage yield in the CSS scheme, a high-yield variety can be synthesized by combining two SPs and a PP having high GCA as well as SCA toward each other.

Breeding of most forage species including timothy is based largely on the development of synthetic varieties in which a limited number of non-inbred parents possessing superior combining ability were intercrossed (Brummer 1999; Tamaki *et al*. 2007). The development of synthetic varieties just partially exploits heterosis during a limited number of generations of seed increase, although inbreeding depressions might occur in advanced generations (Sleper and Poehlman 2006). Therefore the success of synthetic varieties depends on a balance between these two effects of heterosis (and combining ability) and inbreeding depression among their parents. Regarding CSS development, although high yield performance was found in Syn2 of timothy CSS strains (Tamaki *et al*. 2009), no report describes yield performances in their subsequent generations.

Inbreeding allogamous forage crops severely affects fitness, forage yield, plant height, vigor, and seed production (Suginobu *et al*. 1983; Gallais 1984; Jones and Bingham 1995). The inbreeding coefficient is a useful parameter for predicting the degree of inbreeding depression because many reports have described results showing that the effect of inbreeding is approximately linear over widely varied values of the coefficient (*e.g*. Mayo 1987). Under the assumption of diploidy and no relation among parents, the inbreeding coefficient of a CSS strain is theoretically equivalent to that of its PP if the PP has been synthesized from six clones (Tamaki *et al*. 2009). However, it is necessary to examine the performance in advanced generations of CSS, especially in the case of the hexaploid nature of timothy with a controversial genome structure and little information related to their genetic relatedness. This study was undertaken to investigate the scale of inbreeding depression in advanced generations of a CSS strain by comparing forage yield, plan height, winter survival, and regrowth vigor among its second to fourth synthetic generations (Syn2–4).

Materials and methods

All experiments were conducted at KAES, Hokkaido Research Organization, Kunneppu, Hokkaido, Japan (43°47′N, 143°42′E) on a high-humic haplic wet andosol.

Seed production of a clone and strain synthesis strain in advanced generations

Seed production in each generation of the CSS strain 'Kitakei 04306', designated as No. 6 in our previous studies (Tamaki *et al*. 2009; Chapter 5), was conducted during 2004–2008. Two SPs ('c-01' and 'c-02') with two clonal replicates and seeds of the PP ('Aurora') were planted in September 2004. These parental materials ('c-01'–'Aurora' and 'c-02'–'Aurora') were transferred separately into isolation greenhouses in May 2005, and came into flower in late June 2005. Syn1 seeds were harvested in July 2005. Equal quantities of seed from each of the two SPs were mixed together to form the Syn1 balanced bulk. In all, 640 seedlings of Syn1 were transplanted to isolated fields in October 2005 for Syn2 seed production. The Syn2 seeds were harvested in August 2006. The third synthetic generation (Syn3) seed production with 950 Syn2 seedlings was conducted similarly to Syn2 seed production during 2006–2007. Two hundred seedlings of Syn3 were grown in incubators for vernalization during 4 months (December 2007 – March 2008) and were transplanted into isolation greenhouses in April 2008. The fourth synthetic generation (Syn4) seeds were harvested in August 2008. The respective quantity of Syn2, Syn3, and Syn4 seed harvested was 378, 783, and 40.9 g.

Experiment 1: Drilled-row plots

This experiment evaluated nine early maturing entries: the three subsequent synthetic generations (Syn2–4) of ‘Kitakei 04306’, four check varieties including ‘Aurora’ and ‘Natsuchikara’, and two strains. Seeds of the nine entries were planted in an RCBD with four replicates in May 2009 at a planting rate of 20 kg ha^{-1}. Each plot consisted of four 2.0-m locations drilled 0.25 m apart. Two herbage harvests were conducted in the first year: mid-July 2009 and early September 2009. Fertilizer applications of 95 kg N ha^{-1}, 80 kg P_2O_5 ha^{-1} and 95 kg K_2O ha^{-1} were conducted in the first year. Three herbage harvests per year were made in 2010 and 2011: mid-June, early August, and early October, where plants were clipped to a 10-cm stubble height. Plots were fertilized as follows: 90 kg N ha^{-1}, 180 kg P_2O_5 ha^{-1} and 90 kg K_2O ha^{-1} in early spring, 55 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 55 kg K_2O ha^{-1} immediately after the first harvest and 37 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 37 kg K_2O ha^{-1} immediately after the second harvest. Dry matter yield (Mg ha^{-1}) and plant height (cm) in each plot were measured at each harvest. Date of head emergence in each plot was recorded as the day after 31 May when the sixth panicle had emerged from the boot leaf, *i.e.* three heading panicles per square meter. Winter survival, based on the observation of crown health and amount of regrowth, was scored on a 1 = poor to 9 = good scale on 30 April 2010 and 25 April 2011. Regrowth vigor, based on the observation of plant density and amount of vegetative regrowth, was scored on a 1 = poor to 9 = good scale approximately 2 weeks after first harvests in 2010 and 2011. Statistical analyses were performed using two-way ANOVA with the entries and block as fixed effects, followed by Tukey’s honestly significant difference (HSD) test (Yandell 1997) for post hoc comparisons. Regarding the annual DMY, data were analyzed using ANOVA with the entries, year, and year by entry interaction effects to investigate the year by entry interaction.

Experiment 2: Spaced plant plots

The entries in this experiment consisted of the Syn2–4 of ‘Kitakei 04306’ and variety ‘Aurora’. Seeds were germinated in March 2009 in the greenhouse. Two-month-old seedlings of the four entries were transplanted into the field to establish spaced plant plots according to an RCBD with six replicates in May 2009. Each plot was a 6-m row containing 10 individuals planted 60 cm apart. The rows were spaced 75 cm apart. Three herbage harvests per year were made in 2010 and 2011 – early July, late August and mid-October, where plants were clipped to a 10-cm stubble height. Plots were fertilized as follows: 60 kg N ha^{-1}, 69 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1} in early spring, 60 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1} immediately after the first harvest and 60 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1} immediately after the second harvest. Four traits as plant height, date of head emergence, winter survival, and regrowth vigor after the first harvests were measured in 2010 and 2011. Statistical analyses were performed using one-way ANOVA with the entries as fixed effects, followed by Tukey’s HSD test for post hoc comparisons.

Results

Significant differences ($p < 0.05$) were found among the nine entries for DMYs of the first and second harvests in

Table 5.7 Dry matter yields (Mg ha^{-1}) of advanced generations of ‘clone and strain synthesis (CSS)’ timothy (*Phleum pratense* L.) strain, ‘Kitakei 04306’, in drilled–row plots (Experiment 1) over three years (2009–2011) at Kunneppu, Hokkaido, Japan

Entry	Generation†	2009			2010				2011				Cumulative (2009–11)
		1st	2nd	Annual	1st	2nd	3rd	Annual	1st	2nd	3rd	Annual	
Kitakei 04306	syn2	1.45^{b}‡	5.14^{ab}	6.59	7.21^{a}	3.30^{ab}	1.84	12.35^{a}	9.19	4.02	2.48	15.69	34.62
	syn3	1.40^{b}	5.14^{ab}	6.55	7.18^{a}	3.48^{ab}	1.82	12.48^{a}	8.97	3.81	2.47	15.25	34.27
	syn4	1.32^{b}	5.29^{a}	6.60	6.96^{ab}	3.42^{ab}	1.91	12.28^{a}	8.65	4.04	2.43	15.11	34.00
Aurora§	CS	1.90^{a}	4.36^{c}	6.26	6.12^{b}	2.86^{c}	1.60	10.58^{b}	8.04	3.87	2.47	14.37	31.20
Natsuchikara	BS	1.89^{a}	4.58^{bc}	6.47	6.70^{ab}	3.58^{a}	1.70	11.97^{ab}	8.78	4.15	2.51	15.43	33.88
F-value¶		15.0^{**}	12.0^{**}	2.8^{*}	3.6^{**}	8.2^{**}	1.1^{NS}	4.4^{**}	0.4^{NS}	1.7^{NS}	0.7^{NS}	0.6^{NS}	2.1^{NS}

† CS and BS mean commercial seeds and breeder seeds, respectively.

‡ Values with different letters within a column are significantly different by Tukey’s honestly significant difference (HSD) test at the 0.05 level.

§ Pollen parental variety of the CSS strain, ‘Kitakei 04306’.

¶ *F*-value in analysis of variance with the entry as fixed effects. ** and * show significant difference among the entries at the 0.01 and 0.05 level, respectively. ^{NS} shows no significant difference among the entries at the 0.05 level.

2009, and for the first, second, and annual harvests in 2010 (Table 5.7). In ANOVA for the annual DMYs (Table 5.8), the year and entry effects were significant, although the year by entry interaction effect was not significant. The cumulative annual DMYs of each generation of ‘Kitakei 04306’ were 9.0–10.9% higher than that of the PP ‘Aurora’, and were similar to that of ‘Natsuchikara’, which was the highest yielding variety among check varieties in this experiment. Although no significant differences ($p > 0.05$) for DMYs was found among Syn2–4 generations of ‘Kitakei 04306’, it was observed that the first harvests in both 2010 and 2011, and the cumulative annual DMYs tended to decline from Syn2 to Syn4 (Table 5.7), where the rates on the cumulative annual DMYs were –1.0% (from Syn2 to Syn3) and –1.7% (from Syn2 to Syn4). Moreover, the trend for DMYs decrease among advanced generations seemed to worsen with the lapse of the harvests during 2009–2011 (Table 5.7).

Table 5.8 Analysis of variance (ANOVA) on the annual dry matter yields (Mg ha^{-1})† of 9 timothy (*Phleum pratense* L.) early maturing entries in drilled-row plots (Experiment 1) over three years (2009–2011) at Kunneppu, Hokkaido, Japan

Source of variance	df	SS	MS	*F*-value	*P*-value
Year	2	1368	684	963	< 0.001
Entry	8	16.95	2.12	2.98	< 0.01
GEI‡	16	4.97	0.31	0.438	0.967
Residuals	81	57.52	0.71		

† Experimental plots were harvested twice in 2009, and three times per year during 2010–2011.
‡ Genotype (entry) by environment (year) interaction.

Significant differences ($p < 0.05$) were found among entries for plant height (cm) in the first and second harvests of Experiment (Exp.) 1 (Table 5.9). Although no significant differences ($p > 0.05$) were found among advanced generations of the CSS strain, the plant heights of the first harvests in Exp. 2 tended to decline with the advance of generation (Table 5.9).

Regarding other traits, the date of head emergence, winter survival, and regrowth vigor after the first harvest, significant differences ($p < 0.05$) were found among the entries only in Exp. 1. The differences among advanced generations of the CSS were not significant ($p > 0.05$) in Exp. 1 or 2 (Table 5.10).

Table 5.9 Means of plant height (cm) of advanced generations of ‘clone and strain synthesis (CSS)’ timothy (*Phleum pratense* L.) strain, ‘Kitakei 04306’, in drilled-row plots (Experiment 1) and spaced plant plots (Experiment 2) over two years (2010–2011) at Kunneppu, Hokkaido, Japan

Entry	Generation†	Experiment 1			Experiment 2		
		1st	2nd	3rd	1st	2nd	3rd
Kitakei 04306	Syn2	100	83	44	132	94	46
	Syn3	99	84	43	130	89	47
	Syn4	100	85	46	128	93	47
Aurora‡	CS	96	81	46	132	91	49
Natsuchikara	BS	101	87	45			
F-value§		8.1^{**}	3.3^{*}	0.8^{NS}	1.0^{NS}	1.1^{NS}	1.7^{NS}

† CS and BS mean commercial seeds and breeder seeds, respectively.
‡ Pollen parental variety of the CSS strain, ‘Kitakei 04306’.
§ *F*–value in analysis of variance with the entry as fixed effects. ** and * respectively show significant difference among the entries at the 0.01 and 0.05 level. ^{NS} shows no significant difference among the entries at the 0.05 level.

Table 5.10 Date of head emergence, winter survival, and regrowth vigor of advanced generations of ‘clone and strain synthesis (CSS)’ timothy (*Phleum pratense* L.) strain, ‘Kitakei 04306’, in drilled-row plots (Exp. 1) and spaced plant plots (Exp. 2) over two years (2010–2011) at Kunneppu, Hokkaido, Japan

Entry	Generation	Date of head emergence†		Winter survival‡		Regrowth vigor after first harvest‡	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Kitakei 04306	Syn2	12.6	17.5	5.1	4.6	5.5a§	4.6
	Syn3	12.6	17.5	5.1	4.5	5.5a	4.4
	Syn4	12.6	18.0	5.1	4.6	5.6a	4.6
Aurora¶	Commercial seeds	12.6	17.7	4.8	4.4	4.4b	4.2
Natsuchikara	Breeder seeds	13.1		4.8		5.9a	
F-value#		10.0^{**}	0.7^{NS}	4.6^{**}	0.9^{NS}	8.7^{**}	1.7^{NS}

† Day after 31 May.
‡ Index is scored on a 1 (poor) to 9 (good) scale based on visual observation.
§ Values with different letters within a column are significantly different by Tukey’s honestly significant difference (HSD) test at the 0.05% level.
¶ Pollen parental variety of the CSS strain, ‘Kitakei 04306’.
# *F*-value in analysis of variance with the entry as fixed effects. ** and * show significant difference among the entries at the 0.01 and 0.05 level, respectively. ^{NS} shows no significant difference among the entries at the 0.05 level.

Discussion

The controversy related to the timothy genome structure remains unresolved: auto-, allo- or autoallo-hexaploid (Tamaki *et al*. 2010). A yield decrease is theoretically expected also in advanced generations unless it is an allohexaploid. Moreover, the three parental materials of ‘Kitakei 04306’ had genetic relatedness because the parents had several common alleles based on simple sequence repeat markers (Chapter 5). Therefore, it is necessary to accumulate additional empirical results related to the performance of advanced generations for demonstrations of yield superiority in CSS. The results obtained in this study were not significance ($p > 0.05$). They showed a slight decrease with the advance of generation for forage yield and plant height (Tables 5.7 and 5.9). These results resemble those of reports describing that inbreeding depression was not observed between Syn1 and Syn2 of conventional synthetic varieties with four parents of timothy (Piano *et al*. 2007; Tamaki *et al*. 2007). The risk of severe inbreeding depression in CSS was not revealed by our limited experiments, which were conducted for only one CSS strain in one location.

In the breeding strategy currently assumed for a CSS variety, Syn4 corresponds to the generation of its commercial seeds. The cumulative annual DMY of Syn4 remained 9.0% higher than that of ‘Aurora’. One of the two seed parental materials of the CSS strain ‘c-01’ was an elite clone having high GCA (Chapter 5) and was one of the parents of ‘Natsuchikara’. The other material ‘c-02’ was an undesirable clone, which could not be selected as parents of current well-developed strains (data not shown). Nevertheless, ‘Kitakei 04306’ derived from ‘Aurora’ and ‘c-02’, which were inferior to ‘Natsuchikara’ in terms of forage yield, the cumulative annual DMY of Syn4 was similar to that of ‘Natsuchikara’. These results strongly suggest the yield superiority of ‘Kitakei 04306’ and the important benefits of yield improvement of CSS in timothy breeding.

The theory of prediction of yield of conventional synthetic variety has provided perennial and self-incompatible plant breeders with various options such as the optimum number of parents based only on GCA estimates, or the concept of general synthesizing ability, which takes account of GCA and performance of selfed progenies (Posselt 2010). In addition, the theory has been extended to autopolyploids (Posselt 2010). Regarding CSS development, Tamaki *et al*. (2009) described differences between Syn1 and Syn2 for yield levels by genotype frequencies derived from each parental material. Future studies elucidating the theoretical performance of CSS strains can suggest alternative selection schemes. Our results will provide helpful information for the formulation of CSS prediction.

Chapter 6: General discussion

Genetic gains for forage yield of timothy in the Japanese breeding program (3.1–6.6% per decade) were low, as were those of other forage grasses and legumes breeding programs in Europe and North America (Chapter 2). The results in early maturing population at KAES, Hokkaido Research Organization, Kunneppu, Hokkaido, which showed a lower genetic gain of 3.1% per decade, the low narrow-sense heritability among individuals within half-sib family (h^2 = 0.03), and the medium narrow-sense heritability among half-sib family (h^2 = 0.43), indicated that accumulating only an additive effect was difficult for a breakthrough in the stagnation of yield improvement (Chapter 2).

Marker-based GDs are clearly related to forage yields in several mating designs such as synthetic varieties (Exp. 1 in Chapter 3), polycross progenies (Exp. 2 in Chapter 3), top-cross progenies (Chapter 4), and CSS strains (Chapter 5). These results provide evidence that marker-based GDs were useful tools for parental selection exhibiting genetic effects related to genetic diversity, *i.e.* non-additive effects. Many cases of using DNA markers can be (i) pre-selecting parental combinations in a conventional synthetic development (Chapter 3) and combinations of SPs in a CSS development (Chapter 5), (ii) partitioning combining ability into GCA and SCA in yields of polycross progenies (Chapter 3), top-cross progenies (Chapter 4) and CSS strains (Chapter 5), (iii) creating putative heterotic groups (Chapter 4 and Chapter 5), and (iv) introducing new germplasm into breeding population by top-cross (Chapter 4). The methods require no expensive marker such as gene function markers. They are reliably applicable for a practical timothy breeding program.

Because long-term rapid yield improvement in maize has been obtained using RRS procedures rather than just hybrid breeding itself, the most critical point is not only hybrid development *per se* but also the introduction of RRS, *i.e.* to create and use heterotic groups for yield improvement in forage grasses or legumes. The CSS has the capacity to include RRS because one of its main aims is to exploit combining ability including SCA among a small number of parents (Chapter 5). In addition, SCA effect among SPs partly contributed in the high-yielding performance of Syn2 of CSS strains as well as GCA effect of SPs (Chapter 5). Moreover, its high-yielding performance was confirmed in its subsequent generations (third and fourth synthetic generation) (Chapter 5). These results indicate that a potentially effective way to improve forage yield in the future is RRS in CSS development with an application of genetic diversity based on DNA marker polymorphism. Difficulties associated with the creation of heterotic groups starting RRS in timothy are that breeders have often established only one breeding population and that defining and dividing heterotic groups based only on field trials are labor-intensive and time-consuming. The author discusses the usefulness of genetic diversity based on DNA marker polymorphism to propose an effective RRS scheme for an acceleratable yield improvement of timothy judged from the results of selection simulations in this chapter (Chapter 6). In addition, the author suggests the application of SSR marker diversity and development of CSS in actual timothy breeding program, and discusses their prospects for the development of high yield varieties (Chapter 6).

6.1: Proposal for reciprocal recurrent selections in timothy breeding using molecular marker diversity

Posselt (2010) described a generalized RSS scheme for improving two parent populations in hybrid breeding. Feasible RRS approaches were selections of GCA of SPs and GD among SPs because these genetic effects contributed to forage yield in the CSS strains (Chapter 5). Taking these two perspectives into consideration, the breeding scheme in Fig. 6.1 was proposed, which consists of three breeding phases. In brief, current breeding populations were divided into two groups based on molecular marker diversity in the first phase of creation of the base population (upper part of Fig. 6.1). In the second phase of parental selections, SP candidates were selected based on individual selections within each of the two pre-divided groups and top-cross progeny tests with a tester derived from another group (middle part of Fig. 6.1). In the third phase of constructions and evaluation of experimental varieties, CSS strains were developed by combinations of SP candidates and leading varieties or well-developed strains as a PP (lower part of Fig. 6.1).

In the first breeding phase, the maintenance of a series of elites and genetically divergent populations will

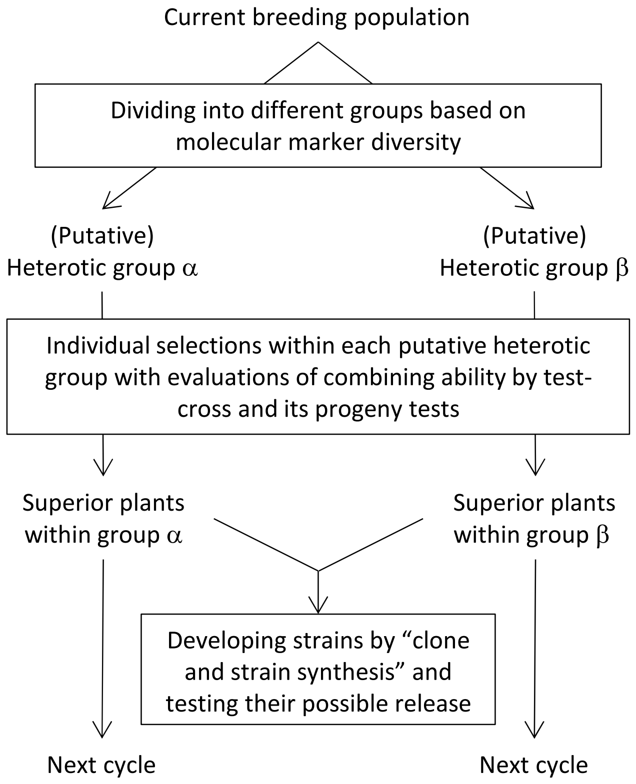

Figure 6.1 General reciprocal recurrent selection (RRS) scheme with “clone and strain synthesis (CSS)” development based on molecular marker diversity and test-cross field data.

enhance breeders’ ability to develop variety partial exploiting heterosis recurrently (Scotti and Brummer 2010). Posselt (2010) described that it is suggested to preselect parents for GD based on molecular or geographic data, and to produce and test diallel crosses among them as for heterotic groups and patterns. In addition, some evidence suggests that adapted populations isolated by time and space are the most promising candidates for heterotic patterns (Posselt 2010). In perennial ryegrass, pre-grouping of genetic materials by geographic distances engenders a rather high association (r = 0.64) between marker-based GDs and performances of the diallel crosses (Posselt 2010). However, numerous studies have found the largest molecular variance within populations rather than among populations in forage grasses (Huff 1997; Kölliker 1999; Bolaric *et al.* 2005; Casler *et al.* 2007; Chapter 4). In current timothy breeding programs, advanced breeding materials and varieties well adapted to the climate are the main genetic resources (Tamaki *et al.* 2010). Breeders often have established only one breeding population for labor saving. Therefore most advanced breeding materials are miscellaneous because they had already attempted introgressions by intercrossing among various resources in recurrent selections. As improvement for yield with our emphasis on a wide array of important traits, more effort should be devoted to exploiting heterotic effects for yield within well-adapted current breeding populations rather than using distinct populations that are inferior to current breeding population at the beginning of a creation of heterotic groups. According to Humphreys (2001), crossing early and late heading accessions produced valuable gene pools which have been used to improve consistency of seasonal yield and nitrogen use efficiency in perennial ryegrass. Populations derived from crossing with other maturing materials of advanced breeding populations, which had been identified as distinct groups based on molecular markers diversity, might be genetically divergent population as well-adapted heterotic group candidates. No evidence that three putative heterotic groups exist within breeding populations were obtained in this study, although the three-parents approach in CSS development require three divergent populations for two SP candidates and a PP. Therefore, two divergent populations shown in Fig. 6.1, where current breeding population were divided into the putative heterotic groups based on DNA marker diversity, is expected to be the most feasible approach at the initial step to RRS.

In terms of the second breeding phase, the selection of SPs in RRS and improvement of PP candidates are key points to improve the process of CSS construction. Difficulties associated with the selection of SPs are that breeding efforts were distributed for SCA among SPs and GCA of SPs, *i.e.* either top-cross or polycross progeny tests were established. Top-cross progeny tests were more feasible procedures in selection for SCA than polycross progeny tests (Chapter 4) were, whereas GCA defined as performances in polycross progeny tests contributed to forage yields in CSS strains as well as SCA among SPs (Chapter 5). Successful SPs selection is expected to depend on how genetic gains for forage yield can be anticipated within a limited of current breeding population possessing additive variation evaluated in Chapter 2 and genetic diversity on SSR markers evaluated in Chapter 4. In any case, DNA profiling provides applicable tools to distinguish the relative contributions of GCA and SCA effects, as explained in Chapter 3 and Chapter 4. Development of PP candidates can be operated using the following three approaches: (i) a new leading cultivar as

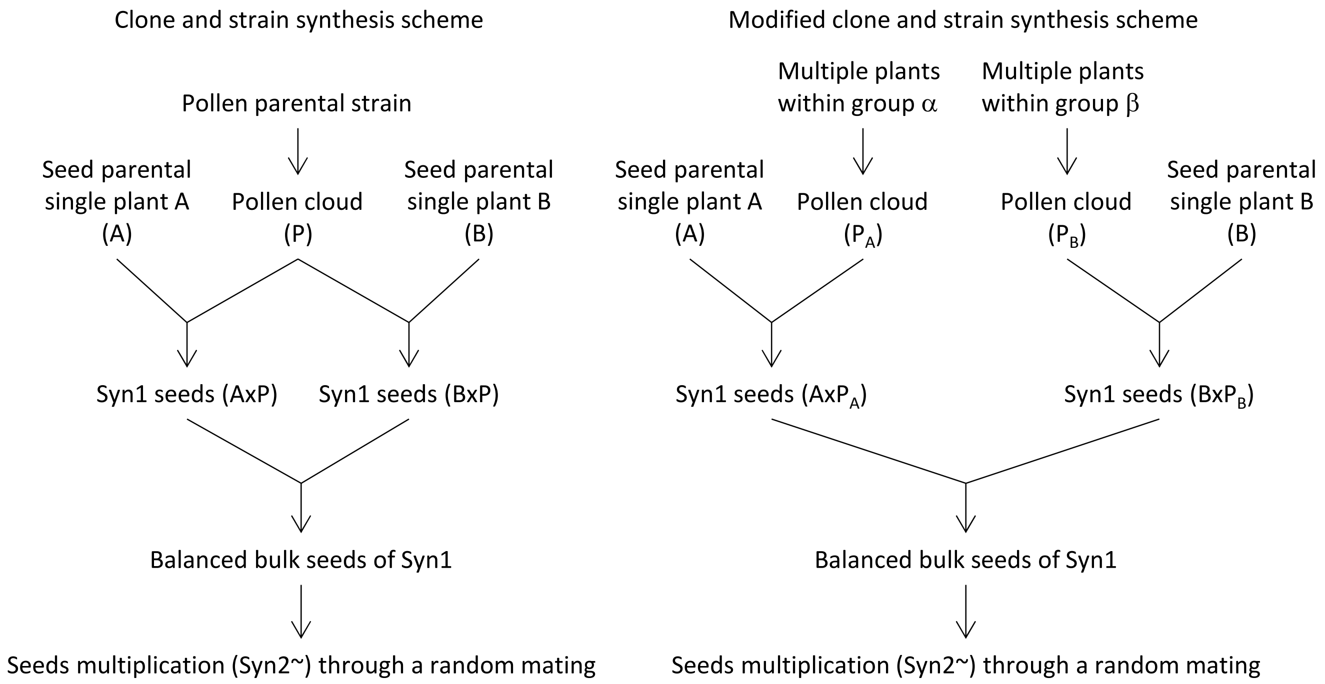

Figure 6.2 Typical scheme of “clone and strain synthesis (CSS)” (Left) and modified CSS scheme (Right).

PP of CSS strains, (ii) a conventional synthetic strain derived from the third genetically divergent group from SPs, and (iii) a conventional synthetic strain derived from two putative heterotic groups of SPs. At a breeding stage of transition from a conventional recurrent selection to a RRS, a leading cultivar would be suited to the first choice, as discussed in Chapter 5. Establishment of PP in a succeeding CSS development requires more empirical results at an advanced breeding stage for long-term yield increases.

Post-synthesis selections, which are historically the most proven approach to improving forage yields, still have a key role in the third breeding phase. The CSS development might be more beneficial for the post-synthesis selection than conventional synthetic variety. Synthesizing of the only six SPs and a PP in Chapter 5 found 15 CSS strains having large variations for forage yields, whereas a few experimental strains could be developed through one selection cycle in conventional synthetic development. An experimental strain might include failure parents masked by GEI in parental selections (Amini *et al.* 2011). Breeders must also assume the cases of a conventional synthetic development in RRS scheme and reverting to conventional recurrent selection such as among- and within-half-sib family selection in a breeding stage of the transition. These facts imply the necessary of avoiding the loss of accumulating additive effects within breeding populations through RRS. In a breeding stage attaining several cycles in RRS, the CSS scheme was modified to fit two heterotic groups shown in Fig. 6.2, although no evidence exists for this. General aspects of the scheme has been described by Tamaki *et al.* (2007) as proposing “modified synthetic variety,” where strains were synthesized by two polycross progenies with exploitation of SCA among SPs of the two progenies and minimizing a risk of inbreeding depression.

Selection simulations of RRS with CSS development were performed based on the results in Chapter 2–5; the variation of BLUP values (Chapter 2), the heritability among half-sibs (Chapter 2), the heritability among individuals within half-sibs (Chapter 2), the variation of GDs among current breeding materials (Chapter 3 and 4), and the prediction models for yields of top-cross progenies and CSS strains (Chapter 5). On the assumptions that (i) only two (putative) heterotic groups are identified by genetic diversity on SSR makers and (ii) an initial PP was not improved because no evidence for the third heterotic group and feasible development of PP candidates was shown in this study, three patterns of RSS schemes combined procedures of either top-cross or polycross progeny tests were compared (patterns A–C in Fig. 6.3 and Table 6.1). Genetic gains for cumulative DMYs over two

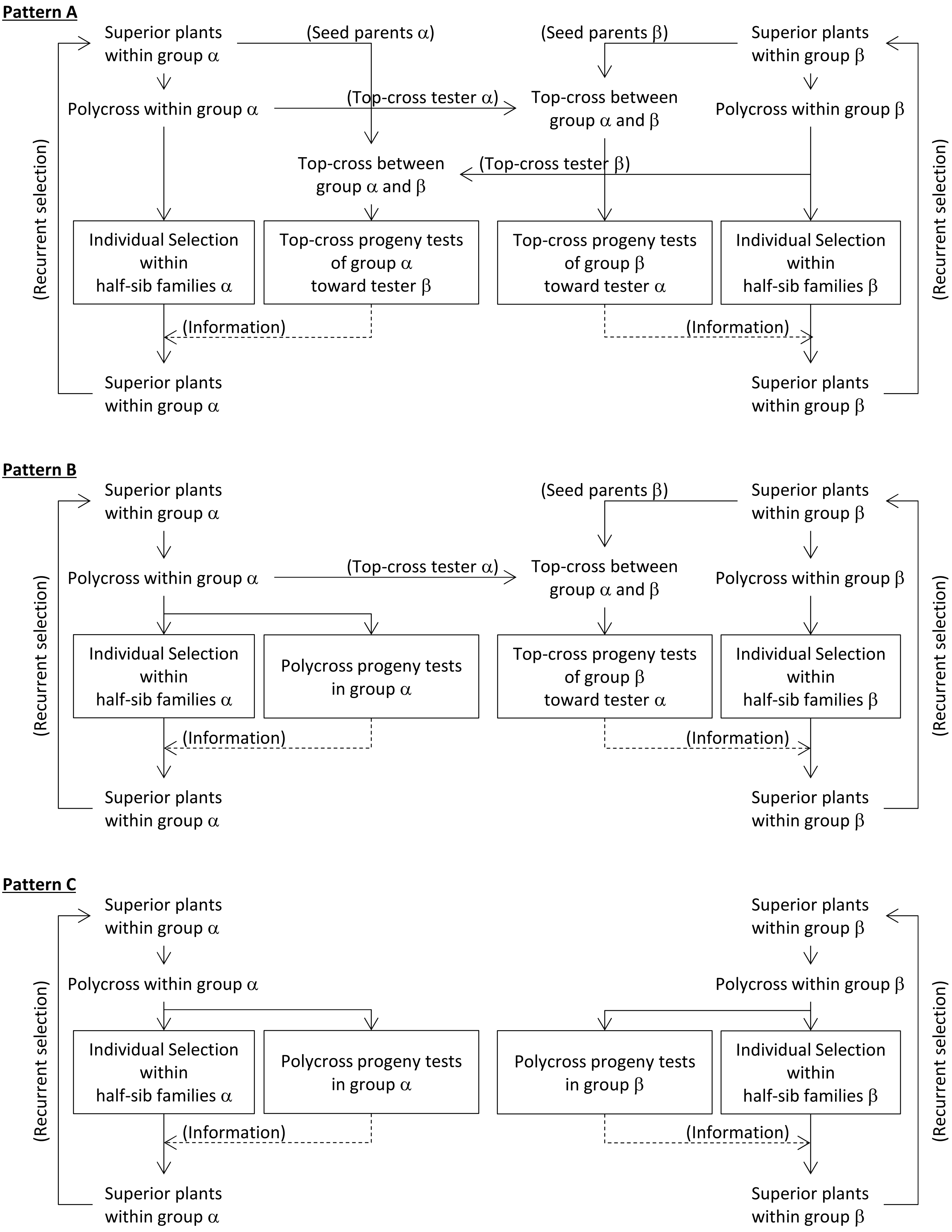

Figure 6.3 Patterns (A–C) of individual selection within each heterotic group with evaluations of combining ability by test-cross and its polycross progeny test in reciprocal recurrent selection (RRS) procedures. Pattern A (upper part in the Figure) shows procedures adopted for top-cross progeny tests in each group. Pattern B (middle part in the Figure) shows procedures adopted for polycross and top-cross progeny tests in groups a and b, respectively. Pattern C (lower part in the Figure) shows procedures adopted for polycross progeny tests in each group.

Table 6.1 Forage yield and additive effect for yield (mean ±SD) with selection cycles† of reciprocal recurrent selection (RRS) with "clone and strain synthesis (CSS)" development in timothy (*Phleum pratense* L.) early maturing populations by computer simulation‡ based on the dataset in this study

Pattern of RRS schemes§	Forage yields of CSS strains¶				An additive effect for forage yields of breeding populations#			
	Cycle 0†	Cycle 1†	Cycle 2†	Cycle 3†	Cycle 0	Cycle 1	Cycle 2	Cycle 3
RRS (Pattern A)	23.0 ±0.3	24.1 ±0.4	25.9 ±0.6	28.1 ±1.1	0.000	0.271 ±0.046	0.521 ±0.070	0.747 ±0.104
RRS (Pattern B)	23.2 ±0.2	24.2 ±0.3	25.6 ±0.5	27.4 ±1.0	"	0.286 ±0.045	0.573 ±0.077	0.847 ±0.125
RRS (Pattern C)	23.2 ±0.2	24.0 ±0.2	24.7 ±0.3	25.4 ±0.5	"	0.303 ±0.042	0.609 ±0.076	0.915 ±0.126

† Number of selection cycles was settled to three cycles (Cycle 0 – Cycle 3) in the simulations.

‡ Selection simulations were performed with 100 replicates on the assumption that only two putative heterotic groups of seed parent candidates of CSS can be identified and that an initial pollen parent was not improved.

§ RRS schemes of combined procedures of progeny tests where either a top- or polycross progeny test was adopted (refer to Fig. 6.3).

¶ Cumulative annual dry matter yields (Mg ha^{-1}) over two years were estimated by prediction models of yields of CSS strains in Chapter 5 (refer to Table 5.6) when nine (= 3 × 3) strains were developed three SPs from each group.

Initial values (mean and variance) of the additive effect in Cycle 0 were settled as following the best linear unbiased prediction values calculated in Chapter 2.

years through three selection cycle in the three schemes were 5.1 Mg ha^{-1} (22.3%) in pattern A, 5.1 Mg ha^{-1} (19.3%) in pattern B, and 5.1 Mg ha^{-1} (10.6%) in pattern C (Table 6.1). Patterns A and B acquired twice the genetic gains as among- and within-half-sib family selection did, attaining one selection cycle shown in Chapter 2. Pattern C was not so much compared with among- and within-half-sib family selection (Fig. 6.4). The increase of the additive effect through three selection cycles in the population of the pattern A established top-cross progeny tests (0.747 Mg ha^{-1}; Table 6.1) was less than that in among- and within-half-sib family selection (1.19 Mg ha^{-1} through four selection cycle in Chapter 2), whereas the increase in the pattern C established polycross progeny tests (0.915 Mg ha^{-1}; Table 6.1) was nearly equal to that in among- and within-half-sib family selection. For pattern B, one group in the procedure of top-cross progeny tests was similar to that of pattern A. Another group in polycross progeny tests was equaled to that of pattern C. The results of the selection simulations indicated that the pattern B of RRS is a more feasible scheme for a rapid yield increase avoiding the loss of accumulating additive effects within the populations.

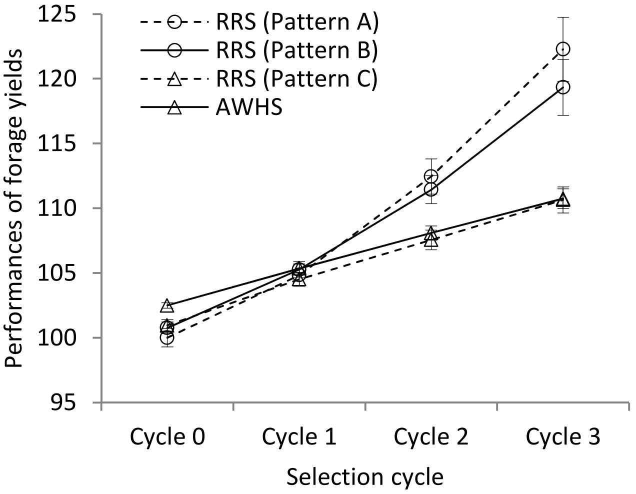

Figure 6.4 Transition of forage yields with selection cycles of three patterns (A, B and C) of reciprocal recurrent selection (RRS) with CSS development and among and within half-sib selection (AWHS) in timothy (*Phleum pratense* L.) by computer simulation based on the dataset obtained in Chapter 2–5. Performances of forage yields (PY) in RRS were calculated as the yield of Cycle 0 in the pattern A was equal to 100 (refer to Table 6.3). PY in AWHS was calculated the yield of Cycle 0 was equal to 100 (refer to Fig. 2.3) on the assumption that selection cycle in AWHS had advanced one selection cycle than that in RRS, i.e. Cycle 1 in Fig. 2.3 corresponds to Cycle 2 in this figure.

6.2: Development of clone and strain synthesis strain and use of molecular marker diversity in actual timothy breeding at KAES

Forty-seven CSS strains with extreme-early to late maturing were developed using the primary procedure described in Tamaki *et al.* (2009) at KAES. Among them, 13 extreme-early maturing CSS strains are attained in most advanced breeding stages, where multi-location yield trials were operated at three locations in Hokkaido: KAES (43°47′N, 143°42′E), the National Agricultural and Food Research Organization Hokkaido Agricultural Research Center (NAROH), Sapporo (43°00′N, 141°24′E), and the Tokachi test field of HFAC, Obihiro (42°50′N, 143°07′E). Annual DMYs in 2013 (Table 6.2 and Fig. 6.5) showed that ranking of the entries differed at respective locations. Two CSS strains (Nos. 10 and 13) were superior to a check variety 'Kunpu' as their PP for annual DMY at all locations (Table 6.2 and Fig. 6.5). In such multiple environment trials, the occurrence of GEI is often unavoidable, especially in the location environment. Reportedly, both biotic and abiotic factors are said to be the main contributors for GEI and yield instability in plants, although these known factors can only partly explain most of the GEI in multiple environment trials (Ferreira *et al.* 2006; Asfaw *et al.* 2009). Nevertheless, it has been reported that SCA tended to be unstable to environments rather than GCA for yield performance of hybrids in maize (de Souza *et al.* 2009; Devi and Singh 2011). Although relations between yield stability and relative contributions of genetic effects in CSS strains call for additional investigation, some (but not all) well-adapted and high-yielding CSS strains at multi-environments have been found in primary CSS breeding (Fig. 6.5).

Table 6.2 Annual dry matter yields (Mg ha^{-1})† of 13 extreme-early maturing timothy (*Phleum pratense* L.) “clone and strain synthesis (CSS)” strains and a check variety ‘Kunpu’ in yield trials in 2013 at the three location: Kitami Agricultural Experiment station (KAES), Hokkaido Research Organization, Kunneppu, Hokkaido, Japan, National Agricultural and Food Research Organization Hokkaido Agricultural Research Center (NAROH), Sapporo, Hokkaido, Japan, and Tokachi test fields of the Hokuren Federation of Agricultural Cooparatives (HFAC), Obihiro, Hokkaido, Japan

Entry	Location		
	KAES (Kunneppu)	NAROH (Sapporo)	HFAC (Obihiro)
CSS strain			
No. 1	11.32	11.35	12.61
No. 2	11.52	11.75	11.93
No. 3	10.69	12.20	12.31
No. 4	10.84	11.44	11.71
No. 5	10.63	11.38	12.50
No. 6	11.20	11.72	11.81
No. 7	11.46	11.07	12.55
No. 8	10.76	12.33	12.08
No. 9	11.55	11.26	12.02
No. 10	11.47	11.67	13.18
No. 11	11.02	11.57	11.50
No. 12	12.03	11.36	11.62
No. 13	11.39	12.23	12.75
Kunpu	11.11	11.31	12.18
LSD (0.05)‡	0.79	NS§	NS
CV (%)¶	4.9	6.6	5.5

Underlined digits are the maximum yield values at each site.

† Experimental plots were established in 2012 and harvested three times in 2013.

‡ Least significant difference at the 0.05 probability level.

§ NS, not significant.

¶ Coefficient of error variation.

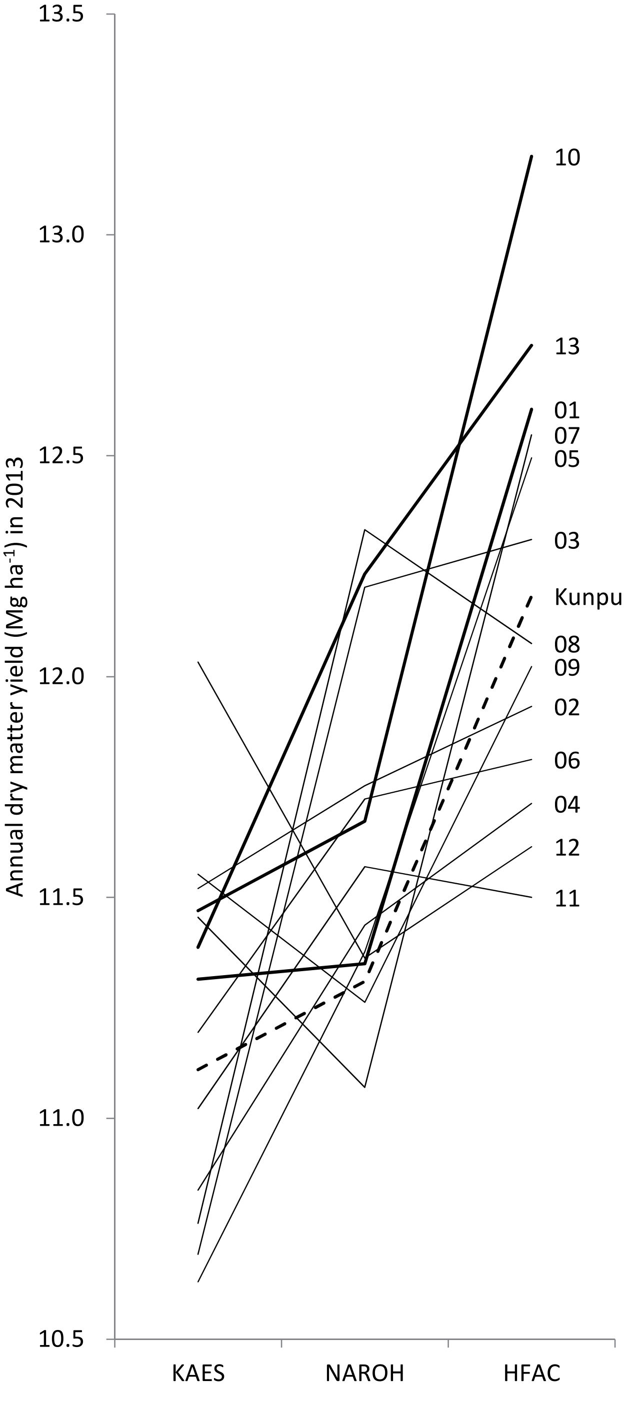

Figure 6.5 Annual dry matter yields (Mg ha^{-1}) of 13 extreme-early maturing timothy (*Phleum pratense* L.) “clone and strain synthesis (CSS)” strains and a check variety ‘Kunpu’ in yield trials in 2013 at the three location: Kitami Agricultural Experiment station (KAES), Hokkaido Research Organization, Kunneppu, Hokkaido, Japan, National Agricultural and Food Research Organization Hokkaido Agricultural Research Center (NAROH), Sapporo, Hokkaido, Japan, and Tokachi test fields of the Hokuren Federation of Agricultural Cooparatives (HFAC), Obihiro, Hokkaido, Japan. Abbreviations of entries showed as follow: “1” – “13” are clone and strain synthesis strains.

Results of this study suggest that genetic diversity on DNA markers has been used for the following: (i) identification of putative heterotic groups in a current breeding population, (ii) pre-selecting materials for an introgression of new resources such as top-crosses among different maturing groups, and (iii) parental selection in conventional synthetic varieties. In actual timothy breeding at KAES, some relative attempts were operated as follow: (i) early maturing 96 individuals selected from the current breeding base population were divided into putative heterotic groups based on 28 SSR markers in 2013 (Figs. 6.6 and 6.7), (ii) materials were pre-selected for top-cross between early maturing single plants and medium varieties based on results of cluster analysis of six current reading varieties in Hokkaido in 2012–2013, and (iii) experimental strains were synthesized based on a GD matrix of 64 parental candidates in 2012–2013. These materials are anticipated for use and examination for development of high-yield varieties in timothy breeding at KAES. Especially, the 96 individuals of putative heterotic groups are crossed in top- and polycross mating designs. Operations of the RRS with top-cross and polycross progeny tests (pattern B in Fig. 6.3) will be started in 2015. Consequently, the operations of RRS with CSS development are just launched in timothy breeding at KAES. Such approaches using genetic diversity on SSR markers might enhance improvement of forage yield by exploiting heterosis. In the future, these approaches are expected to achieve long-term rapid yield improvement in timothy. Developing high-yielding timothy varieties engenders increased productivity in renovated grasslands and earlier recovery of costs for grassland renovation. Moreover, these might provide solutions for conversion to dairy systems that are dependent solely on self-sufficient feed by enhancing grassland renovation and a reduction in environment burden of dairy systems depending on imported feed. Finally, the author hopes this work can support the continued development of environment-friendly agricultural system through increased production of high-quality feed that can be produced with domestic self-sufficiency.

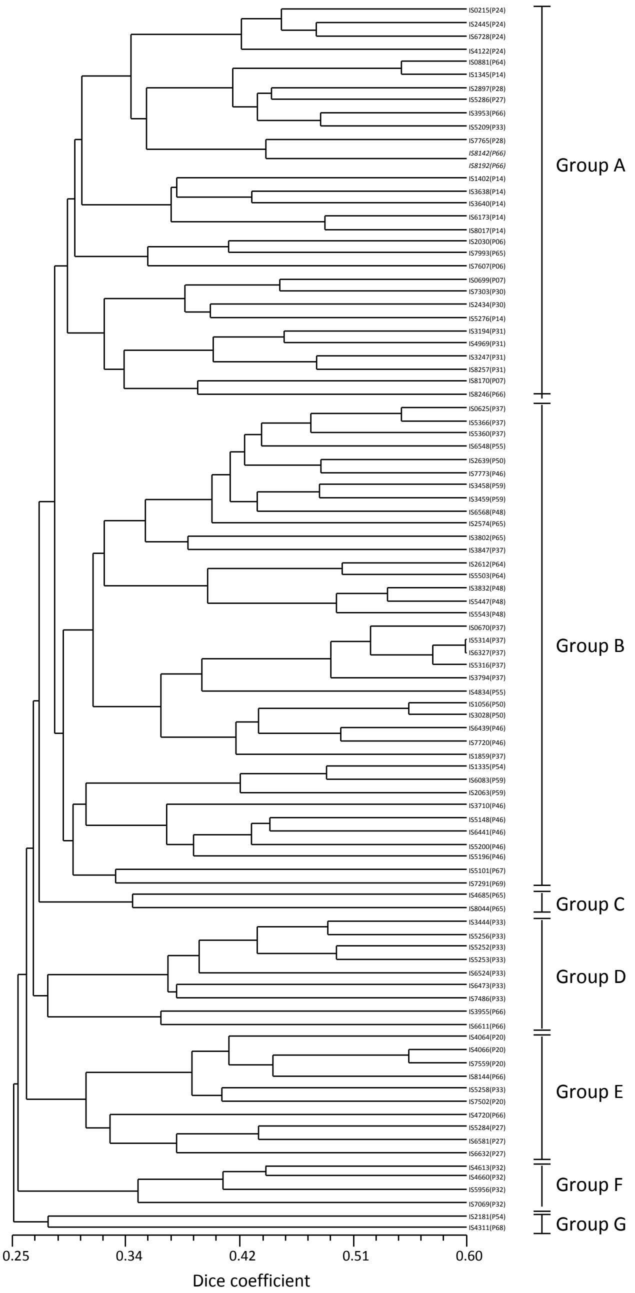

Figure 6.6 Unweighted pair group method using arithmetic average (UPGMA) clustering of 96 timothy (*Phleum pratense* L.) genotypes using a matrix of Dice coefficients by marker-based diversity. The genotypes were early maturing individuals selected from the breeding base population during 2011–2013 at Kitami Agricultural Experiment Station. Words between round brackets in names of genotypes represent their seed parental clone ID except for P65–69.

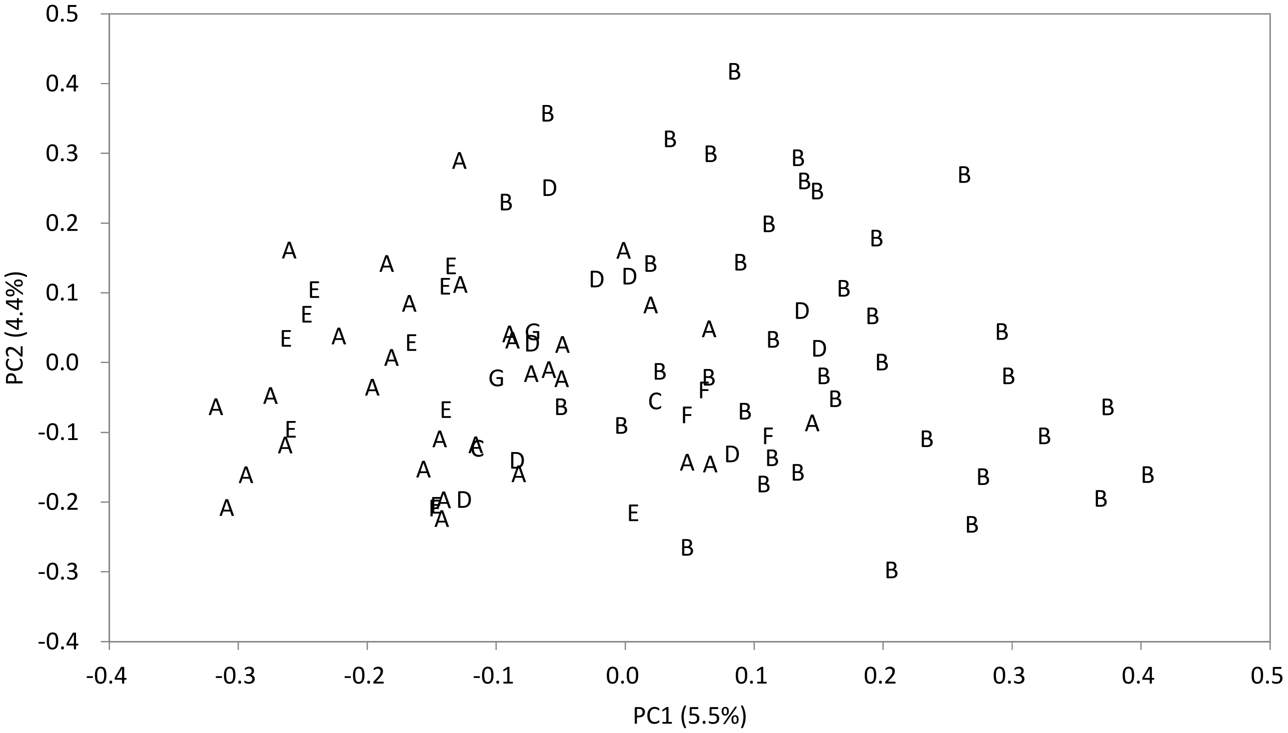

Figure 6.7 Scatter plot of the first two principal coordinate scales of 96 timothy (*Phleum pratense* L.) genotypes selected from a current breeding base population in principal coordinate analysis (PCOA) performed with matrix of Dice coefficients framed by 28 SSR markers. PC1 and PC2 respectively represent the first and second principal coordinates. Symbols A–G correspond to the groups A–G in the unweighted pair group method using arithmetic average clustering (Fig. 6.6).

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Summary

Heterosis—the superiority of a progeny to its parents—underpins the long-term rapid yield improvement in maize (*Zea mays* L.). Historically, attempts of estimations of the genetic distances (GDs) and its predicting heterosis have been reported for several plants. Several studies have indicated that molecular marker-based GDs can provide breeders helpful information for yield improvement. However, breeding of forage grasses and legumes including timothy (*Phleum pratense* L.) has achieved low or nonexistent gains for forage yields, where breeders have mainly emphasized the accumulation of additive effect with a wide array of the other target traits. A potentially effective way to improve forage yield in the future is to capitalize on non-additive gene action by variety development such as "clone and strain synthesis (CSS)" with the aim of capturing heterosis or specific combining ability (SCA). This study was conducted to assess breeding methods for long-term rapid improvement of forage yields with taking up timothy as experimental materials, which occupies approximately 80% of total grassland in Hokkaido. Increased timothy yield is expected to strongly affect the livestock industry and environment around grasslands in Hokkaido.

1: Genetic gains and heritability for forage yields of timothy in Japanese breeding programs (Chapter 2)

An initial step of the breeding strategy needs to identify genetic gains or heritability for forage yield in timothy breeding programs in Hokkaido. The aims of this study were to evaluate genetic gains and narrow sense heritability based on datasets of timothy local-adaptability tests in Hokkaido since the 1960s, polycross progeny tests at the Kitami Agricultural Experiment Station (KAES), Hokkaido since the 1970s and a parent–offspring simultaneous test of 15 representatives of early maturing timothy breeding population at KAES. The genetic gains were estimated using the slope coefficients of simple linear regression predicting yield performance of 16 recommended varieties in Hokkaido, where the value of 'Climax' equals to 100, with released year of the varieties. The heritability among half-sib families was estimated by variance components in restricted/residual maximum likelihood of the mixed effect model based on 11 polycross progeny tests. Heritability among individuals within half-sib families was estimated by the correlation coefficient between annual dry matter yields (DMYs) during two years (2011–2012) of 15 seed parents and their polycross half-sib progenies. These results showed small but steady genetic gains (3.1% per decade in early maturing hay type and 6.6% per decade in medium maturing), a medium heritability (h^2 = 0.43) among half-sib families, and a low heritability (h_N^2 = 0.03) among individuals within half-sib families. Significant ($p < 0.05$) difference of genetic gains among early and medium maturing type is expected to depend on their genetically background and breeding stages. Selection simulation in early maturing breeding population showed a potential of genetic gain of 5.4% per decade, and suggested a limitation of exploitation of additive effects and necessary of additionally exploiting non-additive effects for yield improvement.

2: Marker-based genetic distances among timothy genotypes in polycross breeding and its relation to forage yield (Chapter 3)

Molecular marker technologies might provide an important tool to overcome the forage yield plateau in timothy. Therefore, this study was designed to investigate the relation between marker-based GD estimates and yield in polycross mating designs. Genetic distances were estimated using Dice coefficients framed by 28 simple sequence repeat (SSR) markers. In Experiment 1, parents with high general combining ability (GCA) from two contrasting first-generation synthetic strains (exhibiting high and low yields) were compared. Average GDs of each strain were 0.74 (high) and 0.68 (low). These differences indicate that GD might partially constitute the basis for contrasting yields. In Experiment 2, GDs among 40 parents of a polycross were used for calculating the general genetic distance (GGD), which is indicative of their allelic complementation. Analyses revealed a significant ($p <$ 0.01) moderate correlation (r) between GGD and GCA for yield (r = 0.45) and a significant ($p < 0.01$) residual mean square for the regression of yield on GGD, suggesting that considerable non-additive effects were associated with GCA. The results are indicative of the potential use of GD

estimation for yield improvement in timothy.

3: DNA profiling of seed parents and a top-cross tester and its application for yield improvement in timothy (Chapter 4)

Assessment of genetic diversity based on DNA profiling contributes to the selection of superior parents for heterosis. The objective of this study was to investigate how closely the yields of timothy top-cross progenies are related to the genetic diversity between their seed parents and the pollen tester parent ‘Aurora’, and to predict their yield potentials from the index based on SSR marker polymorphisms. Genetic diversity among 67 genotypes, which consisted of 16 plants of timothy variety ‘Aurora’, the pollen parent for top-cross testers, and 51 clones derived from breeding populations was evaluated based on 28 SSR markers carrying 408 alleles. Marker-based GD was estimated for all possible pairs of genotypes. Then the mean of the GD between each seed parent and the top-cross tester (GD_T) was also assessed to ascertain the relation between GD_T and yields of top-cross progenies. The GD_T were 0.65–0.76, and a correlation coefficient between the GD_T and yields of their progenies was 0.68 ($p < 0.001$). These results strongly suggest that GD can be expected to provide reliable information related to the yields of the assumed top-cross progeny lines and that it will help breeders to accelerate yield improvements for a practical breeding program of timothy with the goals of exploiting heterosis or SCA.

4: Development of timothy “clone and strain synthesis” strains by combining top-cross progenies (Chapter 5)

The CSS, where strains are synthesized by combining two top-cross progeny comprising two seed parental clones (SPs) and a pollen parental strain (PP), have the capacity to integrate a reciprocal recurrent selection (RRS) in breeding of forage grasses and legumes including timothy aiming at improvement of both GCA and SCA. In this study, relative contributions of GCA and SCA in the first (Syn1) and second synthetic generation (Syn2) of CSS strains and yield performances of subsequence generations of a CSS strain were demonstrated through development of timothy CSS strains. These approaches can engender proposals for a feasible RRS scheme with CSS development.

4.1: Relative contributions of general and specific combining abilities in timothy “clone and strain synthesis” breeding scheme

Estimating the effects of GCA and SCA helps breeders to allocate resources for parental selections in CSS breeding scheme because various genetic effects such as GCA of parental materials (SPs and PP) and SCA among parental materials are expected to influence the forage yields of Syn1 and Syn2 of CSS strains, respectively. This study was conducted to examine the relative contributions of GCA and SCA to forage yield in CSS breeding scheme by predicting yields of Syn1 and Syn2 with molecular marker and field evaluation data. Multiple linear regression analysis, used to predict yields of top-cross progenies, where standardized partial regression coefficients of GD_T between SPs and a tester as PP and GCA of SPs were 0.68 and 0.35, shows a greater contribution of SCA in yields rather than GCA. These results demonstrate that molecular marker diversity can be anticipated for wider use in the identification of different heterotic groups in a current timothy breeding population. Marker-based GDs among SPs and sum of GCA of two SPs examined using best linear unbiased prediction values were selected as the explanatory variables in the yield prediction models in Syn2, suggesting that utilization of putative heterotic groups as the first step in a RRS approach should be selection based on GCA of SPs and SCA among SPs. An adoption of elite clones as SPs of CSS strains, selected from putative heterotic groups formed by molecular markers, results in the efficient development of high-yielding strains with exploiting SCA among SPs. The CSS development presents some important advantages over conventional cultivar synthesis: RRS with two or three heterotic groups can be established in CSS; also, current leading cultivars are useful as the PP.

4.2: Evaluation of forage yield performance in advanced generations of timothy “clone and strain synthesis” strain

The CSS is similar to variety synthesis to boosting improvement of yield in perennial and self-incompatible forage grasses such as timothy. This study was conducted to investigate the scale of inbreeding depression in advanced generations of CSS. The second to fourth synthetic generations (Syn2–4) of timothy CSS strain, ‘Kitakei 04306’, were compared for cumulative annual

DMYs and some traits of 2009–2011. No significant difference was found among the generations for any examined characteristic, aside from a slight yield decrease (–1.7% for Syn2–4) with the advance of generations. Furthermore, DMY of Syn4, corresponding to a generation propagated as commercial seeds, remained 9.0% higher than that of its PP. These results indicate the yield superiority of ‘Kitakei 04306’. No risk of severe yield decrease in CSS was indicated by results of our limited experiments.

5: Proposal for reciprocal recurrent selection combined with “clone and strain synthesis” using genetic diversity on DNA markers in timothy and its prospects (Chapter 6)

The objective of this study is a proposal for an RRS scheme with CSS development to increase forage yields in timothy. Here the author discussed feasible RRS schemes using genetic diversity based on SSR marker polymorphism with selection simulations judged from the results in Chapter 2–5. The selection simulations of three patterns combined procedures of top-cross or polycross progeny tests were performed on the assumption that only two heterotic groups were identifiable by genetic diversity on SSR markers, and that an initial PP was not improved in selection schemes. Among the three patterns, the RRS scheme, which established top-cross progeny tests in one group and polycross progeny tests in the other group, showed approximately twice the genetic gains (ΔG = 10.3% per decade) for yields as much as that in among- and within-half-sib family selection with avoiding loss of increase of additive effect. In actual timothy breeding at KAES, many extreme-early to late maturing primary CSS strains were developed. They have been examined in yield trials. Thirteen extreme-early maturing CSS strains, which attain the most advanced breeding stage at KAES, have been examined in multi-location yield trials since 2012. Some of them exhibited higher yields than the check variety ‘Kunpu’ as their PP in 2013 at all locations. The first CSS variety might be released in 2018 as the Hokkaido recommended variety. Moreover, putative heterotic groups in the early maturing current breeding population had been identified based on 28 SSR marker diversity, and RRS of the breeding population will be started in 2015. Operations of RRS with CSS development using genetic diversity on SSR markers are just launched in timothy breeding at KAES. In the future, these approaches are expected to achieve long-term rapid yield improvement in timothy. Finally, the author hopes this work can help environment-friendly agricultural system through increased production of high-quality, domestically self-sufficient feeds.

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チモシーにおける収量性改良のための**DNA**多型の利用

田　中　常　喜

要　約

ヘテロシス（後代が両親よりも収量などの形質で優れる現象）を最大化させる F_1 雑種法は，20 世紀後半の農業の発展の象徴のひとつである．この F_1 雑種法を採用し著しく収量が増加したトウモロコシ（*Zea mays* L.）の遺伝獲得量は約 20（%／10 年）と報告される．多くの作物で，系譜や DNA 多型などから遺伝距離を求め，ヘテロシス程度を予測しようとする試みが古くから報告されている．いくつかの報告では，DNA マーカーの利用により，収量性の改良に有益な情報が得られることを示唆している．一方，多年生・他殖性を特徴とする牧草類の品種改良において，収量性の増加は低いことが指摘されている．国内のチモシー（*Phleum pratense* L.）の品種改良を含む牧草育種における収量性の改良は，集団内の相加効果の改良に重点が置かれ，牧草収量の飛躍的な増加を目指す育種戦略において，ヘテロシスの利用が課題である．本研究では，北海道で最も重要な牧草であるチモシーに焦点をあて，ヘテロシスの効果を最大限に発揮して収量性の改良効果をより高めるために，主に早生の育種材料に用いて遺伝解析を行い，DNA マーカー多型を利用した新しい育種法の提案を検討した．

まず始めに，北海道でのチモシー品種改良における収量性の遺伝獲得量と遺伝率について検討した．1960 年代以降に北海道各地で実施した系統適応性検定試験結果における収量性の推移から，採草用早生品種群における収量性の遺伝獲得量は，3.1（%／10 年）と推定され，その他の牧草類での報告と同様に低い値であった．多交配後代検定 11 試験での年間合計収量を混合効果モデルにより解析した結果，母系間の遺伝率は中程度（$h^2 = 0.43$）であった．親栄養系とその後代系統 15 組を供試した親子同時検定の結果，母系内個体間の遺伝率は極めて低かった（$h_N^2 = 0.03$）．これら結果をもとにした選抜シミュレーションから，相加効果の改良により期待される収量性の遺伝獲得量は，最大で 5.4（%／10 年）であった．ヘテロシスの利用が，牧草収量の飛躍的な改良に必要であることが示された．

DNA マーカーによる多型解析は，牧草の収量性改良の停滞を打破できる可能性がある．そこで，合成品種および多交配後代系統を用いて，SSR マーカー28 ペアの多型から算出した母材間の遺伝距離（GD）と後代の収量性の関係を調査した．親栄養系の一般組合せ能力（GCA）が高いにもかかわらず後代の収量が対照的な 2 つの合成品種を用いた試験では，親栄養系相互間の GD の平均値は，期待通りの多収であった系統で 0.74，収量の低かった系統では 0.68 であった．一つの多交配群内の後代系統とその親栄養系を用いた試験では，後代系統の収量性と親の郡内平均遺伝距離（GGD）の間に有意な正の相関（$r = 0.45$）があった．これらのことから、母材間の GD が後代の収量性と関係することが明らかになった．また，多交配後代系統の収量と GGD の回帰分析の結果，回帰残差項の分散は有意であった．DNA マーカーによる解析から，GD に関連する遺伝効果と残りの遺伝効果，すなわちヘテロシス効果と相加効果を識別できる可能性が示された．

DNA 多型による遺伝変異と表現型値の評価は，ヘテロシスの発揮が期待される優良な親個体の選抜に貢献できる．北海道優良品種「オーロラ」を花粉親，早生育種母集団を種子親としたトップ交配後代系統を用いて，母材間の GD と後代の収量性の関係を調査した．GD と収量性の相関は，0.68（$p < 0.001$）と比較的高かった．一般的な相反循環選抜の行程では，ヘテロティックグループ（HG；群内よりも群間で高い SCA の発揮が期待される集団）間の特定組合せ能力（SCA）の改良にトップ交配後代検定が用いられる．DNA 多型に基づいた遺伝解析とトップ交配後代検定の採用によって，ヘテロシスあるいは SCA を利用した多収性育種を実現できる可能性が示された．

2 つのトップ交配後代系統を組み合わせて合成する CSS（clone and strain synthesis）法は，3 つの母材（種子親 2 栄養系および花粉親 1 系統）により構成され，相反循環選抜での HG の利用に適した合成品種法の変法である．本試験では，CSS 法による系統開発を通じて，(i) 合成 2 代の収量における GCA や SCA の相対的な寄与と（ii）流通種子の世代に相当する後期世代について，収量性を含む CSS 系統の能力の安定性について検討した．トップ交配後代 6 系統を材料に CSS 法により合成した

15（$={}_6C_2$）系統（CSS系統）の合成2代の収量を重回帰に当てはめた結果，トップ交配後代の収量性，種子親のGCA，種子親－花粉親間のGDおよび種子親間のGDの説明変数の候補から，種子親のGCAと種子親間のGDが選択され，それらの標準化回帰係数は，それぞれ0.55，0.53であった．このことから，効率的に多収系統を育成する初期段階として，各HGからCSS系統の種子親栄養系候補を選抜することが妥当であると考えられた．その後，上記15系統のひとつ「北系04306」について，合成2～4代の世代間比較を行った。2か年合計収量に世代間で有意差はなかった．世代経過に伴って，僅かに収量が低下する傾向（－1.7%，合成2～4代）がみられたが，「北系04306」合成4代は，花粉親「オーロラ」に比べ109%と多収であった．CSS系統の流通種子に相当する世代において，近交弱勢が起きる危険のないことが確認できた．

以上の試験結果をもとに実施した選抜シミュレーションから，チモシー多収性品種の開発に向けたDNA多型を利用した相反循環選抜法を提案した．この手法の選抜サイクル当たりの収量性の増加は6.2%であり，選抜サイクルに要する期間を6年と仮定すると，従来法の期待値の約2倍に相当する10.3（%／10年）の遺伝獲得量が見込まれた．また，従来法と比較して，提案法の相加効果の増加は同程度であったことから，系統を合成する段階で，従来法により育成した場合にも収量改良の損失は少ないことが示された．現在の育種事業では，初期に育成したCSS系統について，複数箇所で生産力検定試験を実施している段階にある．最もすすんでいる極早生育成系統については，北海道優良品種として2018年に登録することを目指して，試験を展開中である．これらの系統群の中に，道内3か所で実施した生産力検定試験いずれにおいても，2年目の収量で標準品種を上回る優良な系統を見いだしている．また，28DNAマーカーセットを用いて，早生育種母集団の選抜96個体から，HGの候補を選定した．相反循環選抜法を2015年から育種現場で展開することを目指して，交配試験を実施している段階である．本研究で提案した育種方法を活用することにより，飛躍的に収量性を改良したチモシー新品種開発が加速され，北海道の酪農畜産業における飼料基盤の強化と自給粗飼料を主体とした環境保全型農業の推進に貢献することが期待できる．