Use of molecular marker diversity for forage yield increase in timothy (*Phleum pratense* L.) polycross breeding

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Abstract

The aim of this study was to investigate genetic distances (GDs) based on molecular markers in relation to forage yield improvement in timothy syn1 and polycross progenies. In the first experiment, parental clones with high general combining ability (GCA) from two contrasting syn1 progenies, 'Kitakei 98301' that showed promising high yields and 'Kitakei 98303' that exhibited low yields contrary to expectation, were analyzed. Average GD among the parental clones of 'Kitakei 98301' was higher than that for 'Kitakei 98303'. These results indicate that differences in GD could be a major reason for contrasting yield improvements. In the second experiment using 40 parental clones of a polycross, GD values among the parental clones were partitioned into general genetic distance (GGD) and specific genetic distance components. This study showed a significant correlation between the GGD and GCA for yield and a significant residual mean square for the regression of yield with GGD. These observations reveal the existence of considerable non-additive effects in GCA values and the possibility of partitioning GCA values into additive and non-additive effects.

Keywords: forage yield — genetic distance — *Phleum pratense* L. — polycross — simple sequence repeats marker

Introduction

Timothy is an important perennial forage grass in the Nordic countries, eastern Canada and northern Japan, which have severe winters, as it has high nutritive quality and good winter hardness. Cultivated forage-type timothy (*Phleum pratense* L.) is a hexaploid (2n = 6x = 42) species with self-incompatibility (Tamaki et al. 2010). Its forage yield improvement is regarded as one of the major breeding achievements in Japan. Improvement of self-incompatible forage crops including timothy often relies on the production of synthetic varieties. A variety developed by this method consists of an advanced generation of a population initiated by inter-crossing a limited number of parents selected on the basis of high general combining ability (GCA) using a polycross breeding design. Theoretically, the variance among non-inbred half-sib families, including polycross progeny lines, is a quarter of the additive genetic variance and is equivalent to the GCA (Nguyen and Sleper 1989) of the polycross parents. GCA is defined as the average performance of a genotype in a series of crosses and is measured as the deviation of its progeny from the mean of those crosses. GCA values are especially useful in the prediction of synthetics (Posselt 2010). However, contrary to expectations based on GCA values for forage yield from polycross progeny tests, there have been several synthetic strains that fail to produce high yields in the Japanese timothy breeding programs carried out at Hokkaido prefectural Kitami Agricultural Experiment Station (KAES) since the 1960s (Ueda 1990). There have been a number of reports that attempt to explain these unsuccessful selections. One of the possibilities is the existence of specific combining ability and/or inbreeding depression that can substantially influence the success of improving perennial and self-incompatible forage crop species (Gau et al. 1989; Michaelson-Yeates et al. 1997; Riday and Brummer 2002; Tamaki et al. 2007).

Another convincing explanation is that the GCA value itself may include both additive and non-additive effects (Hayward 1979) and/or be masked by genotype by environment interactions such as for forage yield under spaced plants and swards conditions (Wilkins and Humphreys 2003; Casler and Brummer 2008; Amini et al. 2011).

Today molecular markers are regarded as powerful tools for the analysis of genetic diversity and as criteria for the selection of parents in developing a variety. If diversity at marker loci reflects diversity at other linked loci, then selection for more heterozygosity at marker loci selection should increase the probability of having favorable dominant alleles at linked loci which affect forage yield and produce greater overall complementary gene interaction. Some investigations in maize and oilseed rape have shown that genetic diversity among parents measured with molecular markers significantly correlates with hybrid performance and that molecular markers can be used as a tool to predict heterosis for yield (Smith et al. 1990; Riaz et al. 2001; Betrán et al. 2003; Reif et al. 2003). There are existing studies on molecular marker diversity in relation to forage yield in crops such as alfalfa (*Medicago sativa* L.) (Kidwell et al. 1994, 1999; Tucak et al. 2011), white clover (*Trifolium repens* L.) (Joyce et al. 1999), perennial ryegrass (*Lolium prenne* L.) (Kölliker et al. 2005), tall fescue (*Festuca arundinacea* Schreb) (Amini et al. 2011) and timothy (Tanaka et al. 2011). Some of these studies have revealed promising relationships between forage yields and genetic diversity. The focus of most studies of relationships between molecular diversity and forage yield has been on selecting (and/or pre-selecting) combinations of parents, and not on evaluating the proportion of additive and non-additive effects in the observed yield performance of synthetic varieties. However, Melchinger et al. (1990) partitioned genetic distance (GD) values into general genetic distance (GGD) and specific genetic distance components using a method analogous to

Griffing's Model I of Method 4 (Griffing 1956), where GGD was contrasted with GCA in a diallel cross design. This could be a tool to extract contributions of non-additive effects from GCA values obtained in polycross progeny tests, as a large positive GGD value is thought to indicate that a genotype possesses many alleles with low frequency (Melchinger et al. 1990). However, there are no published reports concerning relationships between the GGD and GCA based on polycross progeny tests in forage crops.

The objectives of this study were (i) to evaluate the effects of GD among parental clones on forage yield of their first generation of synthetic (syn1) and polycross progenies, and (ii) to investigate whether the GD values measured with molecular markers are useful for improving forage yield in timothy polycross breeding, by dissecting the performance of polycross progenies into additive and non-additive component effects.

Materials and methods

All field tests were carried out at KAES (43°47´N, 143°42´E; currently Kitami Agricultural Experiment Station, Hokkaido Research Organization) on a wet andosol.

Experiment 1 (synthetic varieties) – Plant materials & field tests design

Eleven early-maturing timothy clones were included in this experiment. Five of the clones were the parents of 'Kitakei 98301' and the remaining six clones were the parents of 'Kitakei 98303'. Both of the synthetic strains were developed at KAES. The parental clones were selected up to 1998 based on high forage yields in polycross progeny tests (Tables 1 and 2). Seeds were planted in a randomized complete block design with four

replicates in May 1999. The seeding rate was 200 g m^{-2}. Plots were 2.5m by 1.2m with two drilled rows. Seeding-year management consisted of two harvests without data collection to manage annual weeds and an application of 70 kg N ha^{-1}, 80 kg P_2O_5 ha^{-1} and 70 kg K_2O ha^{-1}. The experiment was managed with three harvests per year in 2000 and 2001. 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. Plots were clipped to a 10cm stubble height. Dry matter determinations were made on random 300g to 500g forage samples and were used to adjust plot yields to a dry matter basis. Dry matter yields (DMYs) for each plot were summed over all six harvests. Total DMYs for the two years were subjected to analyses of variance (ANOVA) according to a randomized complete block design.

Experiment 2 (polycross progenies) – Plant materials & field tests design

Forty-one timothy polycross progenies and their parental clones were used in this experiment. The 41 progenies, which were derived from a polycross corresponding to the third cycle of 'Maternal line selection combined with a progeny test' (Tamaki et al. 2010), were investigated in the following field tests. All parental clones apart from one clone ('27thPC-05') were analyzed using 28 simple sequence repeat (SSR) markers. The '27thPC-05' clone died during vegetative propagation of the germplasm collection in KAES.

Total DMY for the forty-one polycross progenies together with three check varieties including 'Nosappu' (Ueda et al. 1977) were evaluated over two years. The polycrosses with twelve replicates were produced in 1999 to 2000. The seeds were planted in a

randomized complete block design with four replicates in August 2000. Plots were 1.5m by 0.6m with one drilled row. 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 harvests. The experiment was managed for three harvests per year in 2001 through 2003. Fertilizer application and seeding rate were equivalent to Experiment 1. DMYs for each plot were summed over all six harvests in 2002 and 2003 (three harvests per year for two years). Data of harvests in 2001 were excluded because of some missing values. The total DMYs were analysed using ANOVA according to a randomized complete block design.

Genotyping protocol & statistical analysis

Outlines of the protocol used were described in a previous study made at KAES (Tanaka et al. 2011). Briefly, 28 SSR primer pairs screened out of 55 SSR primer pairs were used. Among this set, at least three SSR loci were located on each linkage group of the diploid timothy map, and the mean distance between neighboring markers was approximately 13.9 cM. Details of the SSR loci and their map locations are given in the report of Cai et al. (2009).

General combining ability (GCA) values were calculated from percentages of 'Nosappu' for total DMYs in polycross progenies tests using the following equation: $GCA_i = [(p - 1) / (p - 2)] (X_i - \mu)$, 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 p is the parental number of the polycross, respectively. Genetic distance (GD) estimates were calculated from SSR data for all possible pairs of parental clones 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, $N_{i,j}$ is the total number of bands common to clone i and j, and N_i is the total number of

bands present in *i*. This is equal to one minus the genetic similarity coefficient originally devised by Dice (1945) and was first used for molecular diversity by Nei and Li (1979). Principle coordinate analysis (PCOA) was carried out on the matrix of GD estimates by using the function 'pcoa' in the R statistical package 'ape' (Paradis et al. 2004). General genetic distance (GGD) estimates within parental clones of the polycross 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. 1990): $GGD_i = [(p - 1) / (p - 2)] (GD_{i,.} - GD_{.,.})$, where $GD_{i,.}$ is the average of GD estimates between clone *i* and the other parental clones of the polycross, $GD_{.,.}$ is the average of GD estimates between all possible pairs of parental clones of a polycross and *p* is the parental number of the polycross, respectively. The data set including GGD and DMY of 40 polycross progenies was analyzed using linear regression.

Results

Experiment 1 (synthetic varieties)

Significant differences were detected among the DMY in 2000 and total DMY over 2 years (2000-2001) for the four entries (Table 3). 'Kitakei 98301' yielded 18%, 5% and 12% more than a check variety 'Nosappu' for DMY in 2000, in 2001 and in total (2000-2001), respectively (Table 3). On the other hand, 'Kitakei 98303' was comparable to 'Nosappu' and lower than 'Kitakei 98301' for DMY (Table 3). The GD for all pairs among the 11 parental clones ranged from 0.614 to 0.782 and the average GD for all pairs was 0.701. The GD estimates among parental clones for each synthetic strain, ranged from 0.677 to 0.782 with an average GD of 0.744 for 'Kitakei 98301' and from 0.614 to 0.752 with an average GD of 0.677 for 'Kitakei 98303'. Thus the average GD among

parental clones of ‘Kitakei 98301’ was clearly higher than that of ‘Kitakei 98303’. The PCOA based on 28 SSR markers, where the first two principle coordinates termed PC1 and PC2 explained 16.1% and 14.9% of the variation respectively, could not separate the eleven parental clones into groups corresponding to the synthetic strains (Fig. 1). The distribution of the five parental clones of ‘Kitakei 98301’ (closed symbols in Fig. 1) tended to be more widely dispersed than that of ‘Kitakei 98303’ (open symbols in Fig. 1) in the PCOA scatter plot.

Experiment 2 (polycross progenies)

The 41 polycross progenies showed large variation for several traits, except for heading date (data not shown), and ranged from 93 to 115% of ‘Nosappu’ for DMY (Table 4). The ANOVA produced an *F*-value of 2.95 ($p < 0.001$) for the total DMY of 44 entries over two years. GCA values based on total DMY in the progeny test ranged from –10.0 to 11.4 (Table 4). The GD estimates of all possible pairs among the parental clones of the polycross, not including ‘27thPC-05,’ ranged from 0.588 to 0.908 (data not shown). The GGD estimates calculated from the GD estimate matrix for all possible pairs of 40 entries ranged from –0.0244 to 0.0394 (Table 4). The relationship between genetic diversity and performance in polycross progenies for forage yield was evaluated by calculating the Pearson product-moment correlation coefficient between GCA values for total DMY and GGD estimates, not including ‘27thPC-05’. The correlation coefficient for this relationship was 0.45 ($p < 0.01$) (Fig. 2). Analysis of variance of regression with GGD (Table 5) showed that the mean square values for ‘among polycross progenies’ (*F*-value of 2.40), ‘regression with GGD’ (*F*-value of 9.67) and ‘residual’ of the regression (*F*-value of 1.96) were significant for total DMYs of the 40 polycross progenies not

including ‘27thPC-05’.

Discussion

The synthetic strains ‘Kitakei 98301’ and ‘Kitakei 98303’, both derived from parental clones with high GCA, exhibited contrasting forage yield levels (Table 3). Compared with ‘Kitakei 98303’, which showed lower DMY contrary to expectations based on GCA estimates, the parental clones of ‘Kitakei 98301’ had larger GD values among themselves which spread wider in the PCOA scatter plot (Fig. 1). The results from SSR analyses clearly show that genetic diversity among the parental clones of ‘Kitakei 98301’ was higher than for ‘Kitakei 98303’. Differences in molecular diversity among parental clones could be a major reason for contrasting yield performance between the two synthetic strains because of non-additive gene effects and/or inbreeding depression. These observations agree with a previous study where high molecular marker diversity led to a significantly increased DMY in synthetic varieties of perennial ryegrass (Kölliker et al. 2005). The results support a hypothesis that the selection of polycross parents with high molecular diversity is a possible way to exploit heterosis and avoid inbreeding depression. However, there is no conclusive proof that populations derived from parents with high molecular marker diversity invariably result in high performance for forage yield. There is a report of unsuccessful selection experiments using molecular marker diversity for forage yield in alfalfa (Kidwell et al. 1999). Kidwell et al. (1999) pointed out the importance of additive gene effects as well as dominance effects and complementary gene interactions for improving forage yield. Thus both additive and non-additive effects must be considered when investigating the relationship between molecular marker diversity and forage yield in the performance of polycross progenies. Experimental

polycross parent selections based on GD together with an evaluation of additive and non-additive genetic effects are necessary to elucidate contrasting synthetic performance.

Although estimates of GCA are generally useful in the prediction of synthetic performance (Posselt 2010), there are unsuccessful selection experiments based on GCA values such as the timothy synthetic strain ‘Kitakei 98303’ in this study (Tables 2 and 3) and the tall fescue syn1 progenies (HGCA) described by Amini et al. (2011). It is true that a polycross can provide satisfactory discrimination between the breeding values of genotypes when both additive and dominance effects are present with equal gene frequencies. However, as Hayward (1979) points out, unequal gene frequencies are likely to arise as a consequence of selection and here the discrimination among genotypes would be more difficult. The present study showed that differences in GGD among the parental clones of polycrosses correlated with their respective GCA values for DMY based on polycross progeny tests (Fig. 2) and that the mean of square for regression with GGD was significant for DMY (Table 5). These results indicate that the GCA values included a genetic effect associated with genetic diversity in addition to additive effects, as suggested by Hayward (1979). This situation might have arisen from the low levels of additive genetic variation remaining after three cycles of selection. ‘Maternal line selection combined with a progeny test’ allows breeders to conduct individual selection and a polycross progeny test simultaneously. Although genetic variation among individuals within timothy populations and their interrelationships have usually been assessed using morphological and agronomic traits, these are not sufficient to provide good estimates of genetic relatedness among timothy populations. Molecular markers provide useful tools to assess the genetic relatedness of breeding material when selecting within genetically broad-based populations. In addition, it is interesting that

both the mean square for the residual of regression and that for regression with GGD were significant (Table 5). It shows that the former residual mean square includes considerable genetic effects unrelated to genetic diversity, i.e. additive effects. Therefore, these approaches will result in a rigorous evaluation for yield performances in polycross progeny tests dividing the GCA values into additive and non-additive effects based on molecular marker diversity, and lead to more efficient improvement of forage yield in timothy polycross breeding.

In conclusion, GD values based on molecular marker diversity were related to the forage yield of progenies. These investigations support the hypotheses that selection of polycross parents for high molecular diversity is a possible way to exploit heterosis or to avoid inbreeding depression and that molecular diversity can be useful for understanding the contribution of both additive and non-additive effects to GCA values in polycross progeny tests. Our results confirmed the usefulness of molecular markers in the selection of parents for forage yield in polycross breeding with selection strategies that capitalize on both additive and non-additive genetic variation.

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Table 1: General combining ability (GCA[1]) for forage yields of five parental clones of a synthetic variety, 'Kitakei 98301', based on total dry matter yields (DMY) in a polycross progeny test over three years (1991-1993)

Parental clones names	DMY in a polycross progeny test		
	(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[2]	18.98	103	5.7
Mean of entries[3]	17.98	97	0.0
Nossapu[4]	18.48	100	

[1]GCA calculated by the following equation: $GCA_i = [(p - 1) / (p - 2)] (X_i - \mu)$, where p is 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.

[2]Mean of polycross progenies derived from selected five parental clones.

[3]Mean of all thirty-five polycross progenies evaluated in progenies test.

[4]A check variety.

Table 2: General combining ability (GCA[1]) for forage yields of six parental clones of a synthetic variety, 'Kitakei 98303', based on total dry matter yields (DMY) in a polycross progeny test over two years (1996-1997)

Parental clone names	DMY in a polycross progeny test		
	(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[2]	17.96	102	4.2
Mean of entries[3]	17.24	98	0.0
Nossapu[4]	17.66	100	

[1]GCA calculated by the following equation: GCAi = [(p–1) / (p–2)](Xi – μ), where p is number of parents in a polycross, Xi is DMY (percentage of 'Nosappu') of a polycross progeny i, and μ is means of DMY (percentage of 'Nosappu') of all polycross progenies, respectively.

[2]Mean of polycross progenies derived from selected six parental clones.

[3]Mean of all thirty-four polycross progenies evaluated in a progenies test.

[4]A check variety.

Table 3: Dry matter yields (DMY) of synthetic strains in a yield trial over two years (2000-2001)

Entry	DMY (Mg ha^{-1})		
	2000	2001	Total (2000-2001)
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
$LSD_{0.05}$[1]	0.59	NS[2]	1.06

[1]Least significant difference at the 0.05 probability level.

[2]NS, not significant.

Table 4: General combining ability (GCA[1]) for total dry matter yields (DMY) over two years (2002-2003) in a polycross progeny test and general genetic distances (GGD[2]) within the parental clones of the polycross

Parental clone names	Total DMY of polycross progenies			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	–[3]
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[4]	25.31	103	0.0	
Check varieties				
'Nossapu'	24.47	100		
'Aurora'	21.36	87		
'Hokusei'	23.92	98		
$LSD_{0.05}$[5]	2.08	8		

[1]GCA calculated by the following equation: GCAi = [(p–1) / (p–2)] (Xi – μ), where p is number of parents in a polycross, Xi is DMY (percentage of 'Nosappu') of a polycross progeny i, and μ is means of DMY (percentage of 'Nosappu') of all polycross progenies, respectively.

[2]GGD calculated from genetic distances based on molecular marker diversity by methodology analogous to Griffing's Model I of Method 4 (Griffing 1956; Melchinger et al. 1990).

[3]The clone was excluded from the SSR analysis due to its non-existence.

[4]Mean of all forty-one polycross progenies.

[5]Least significant difference at the 0.05 probability level.

Table 5: Analysis variance of regression with general genetic distances (GGD) based on molecular marker diversity for total dry matter yields (DMY) of 40 timothy polycross progenies over two years (2002-2003)

Source of variance	df	Mean of squares	*F*-value	*P*-value
Block	3	72.168	32.91	<0.001
Among polycross progenies	39	5.259	2.40	<0.001
Regression with GGD[1]	1	41.595	9.67	0.004
Residual	38	4.303	1.96	0.003
Error	117	2.193		
Total	159	4.266		

[1]A regression with GGD based on molecular marker diversity among parental clones for DMY of the polycross progenies.

Figure legends

Fig. 1: Scatter plot of the first two principle coordinate scales for eleven parental clones of the strains ‘Kitakei 98301’ and ‘Kitakei 98303’ which were developed by a synthetic variety method. PC1 and PC2 are the first two principle coordinates, respectively. The closed symbols show five parental clones of ‘Kitakei 98301’ and the open symbols show six parental clones of ‘Kitakei 98303’.

Fig. 2: Relationship between general combining ability (GCA) for total dry matter yields (DMY) of polycross progenies and general genetic distances (GGD) of each parental clone within polycross. *r* denotes Pearson’s correlation coefficient. ** indicates significance *r*-value ($p < 0.01$). GCA values calculated by the following equation: $GCA_i = [(p–1) / (p–2)] (X_i – \mu)$, where p is 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 values calculated from genetic distances based on molecular marker diversity by methodology analogous to Griffing’s Model I of Method 4 (Griffing 1956; Melchinger et al. 1990).