Marker-based paternity test in polycross breeding of timothy (*Phleum pratense* L.)

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Abbreviations: ANOVA, analysis of variance; AWHS, among- and within- half-sib family selection; BLUP, best linear unbiased prediction; COM, competitiveness toward legumes; df, degree of freedom; DIS, averaged disease score; DMY, dry matter yield; FY, forage yield; GCA, general combining ability; HD, date of head emergence; IE, stem ratio of internode elongation in the second harvests; KAES, Kitami Agricultural Experiment Station; LR, lodging resistance; $LSD_{0.05}$, least significant difference at the 5% level; NVs, nutritive values; Ob, low-digestible fiber; OCW, organic cell wall; OW, winter hardiness; RCBD, randomized complete block design; REML, restricted/residual maximum likelihood; SD, standard deviation; SSR, simple sequence repeat; VS, vigor in the second harvests; WSC, water-soluble carbohydrates.

Abstract

Although the polycross is a very useful and cost effective mating design, a lack of paternal pedigree information is a major limitation for polycross breeding in forage grasses such as timothy (*Phleum pratense* L.). This study describes a paternity test for use in timothy breeding using polymorphic data on 27 genomic simple sequence repeat markers. The paternity test is a simple exclusion statistical test with a combination of maternal information. It successfully determined paternity (success rate = 97%) for 112 progeny plants derived from three polycross groups (A, B and D). Indirectly selected paternal parents in polycrosses were inferior to maternal parents directly selected by polycross progeny tests mainly for forage yield. Chi-squared values (χ^2) in goodness-of-fit tests of the frequency distribution of paternal parents compared to the expected probabilities revealed unbalanced selection in polycross B and D (χ^2 = 141.4^{***} and 82.7^{***}, respectively). Significant differences among the maternal and paternal parents in breeding values for competitiveness toward legumes and low-digestibility fiber content indicates that unbalanced paternal selection would result from individual phenotypic selection for these traits. These results demonstrate that implementation of a marker-based paternity test in timothy polycross breeding could significantly improve the selection of superior paternal parents and redress problems of parental imbalance.

Keywords

Paternal selection; *Phleum pratense*; polycross; polyploid; simple sequence repeat.

INTRODUCTION

Pedigree information helps to predict genetic value which has a central role in the genetic improvement of complex traits in plants and animals (Crossa et al. 2010). In particular, animal breeders have used pedigree information to predict breeding values (Piepho et al. 2008). Also in forest tree breeding, pedigree data has been reconstructed using molecular markers for efficient genetic improvement (Wang et al. 2010). Forage grass breeders have often relied on polycrosses especially to create a basic pool of variation in breeding programmes (Posselt 2010). A polycross is defined as an isolated group of plants or clones arranged in some fashion to facilitate random inter-pollination (Sleper and Poehlman 2006). Polycross seeds are often harvested separately from individual plants to produce half-sibs with maternal identity and unknown paternal identity, i.e. maternal lines. In some cases, polycrosses are bulk-harvested as seeds with unknown both parental identity. Although the polycross is a very useful and cost effective mating design, a lack of paternal pedigree information is a major limitation in polycross breeding. A comparison of breeding methods (Posselt 2010) indicates that genetic gain in half-sib family selection program with unknown paternal identity would be less than that in full-sib family selection program with known parental identity under the same selection intensity and population size. Riday (2011) extensively discussed breeding theory and strategies for utilizing knowledge of paternal identity to enhance selection gains.

Molecular markers provide a tool for simplifying a paternity test in several forage grasses and legumes (Riday et al. 2013). Several software programs had been developed for paternity tests in diploid species (Signorovitch and Nielsen 2002; Gerber et al. 2003;

Kalinowski et al. 2007). Riday et al. (2013) developed a paternity assignment program based on simple exclusion analysis for autotetraploid alfalfa (*Medicago sativa* L.) using co-dominant molecular markers with ambiguous dosage, and successfully determined the paternity of progeny from an alfalfa 16-parent polycross. Because the program presupposes the use of co-dominant molecular markers clearly located at one locus, modification is necessary to carry out paternity tests using molecular marker sets including alleles located on multi-loci or showing dominant polymorphisms.

Timothy (*Phleum pratense* L.), a perennial outcrossing hexaploid ($2n = 6x = 42$) grass species with a high degree of self-incompatibility, is extensively used for hay, silage and pasture in Nordic countries, eastern Canada and northern Japan (Tamaki et al. 2010). The genomic composition of hexaploid timothy remains unclear (Cai et al. 2003; Tamaki et al. 2010), but some evidence suggests that its genomic composition contains two doses of the diploid *P. alpinum* L. and four doses of the diploid *P. nodosum* L. (Stewart et al. 2009). Breeding programs for timothy have been active in Hokkaido, Japan, since the 1960s using genetic resources based on local materials and exotic varieties (Ueda 1990). Initially, mass selection or conventional maternal line selection was used in breeding programs in Japan. Subsequently, among- and within- half-sib family selection (AWHS) (Casler and Brummer 2008) has been used in the timothy breeding programs since the 1990s (“maternal line selection combined with a progeny test” described in Tamaki et al. 2010). In this procedure, selection among half-sibs is carried out based on polycross progeny tests in drilled-row plots and individual phenotypic selection within half-sibs is conducted in spaced-plant plots separately and simultaneously.

With regard to molecular marker development, 822 genomic- simple sequence repeat

(SSR) markers have been developed from hexaploid timothy (Cai et al. 2003, 2009). An unsaturated linkage map was constructed for diploid timothy with 226 of all 822 genomic-SSR markers some of which have been localized in multi-positions on the map (Cai et al. 2009). The genomic-SSR makers have been used to assess genetic diversity and hexaploid timothy has proved to be highly polymorphic (Cai et al. 2003; Tanaka et al. 2011; Tanhuanpää and Manninen 2012). Therefore readily available molecular markers for paternity assignment exist should paternity tests be established based on genomic- SSR markers which show dominant polymorphisms in polyploid species.

Studies on important timothy breeding target traits such as forage yield (FY), lodging resistance (LR), competitiveness toward legumes (COM), seed yield, and nutritive values (NVs) including water-soluble carbohydrates (WSC), low-digestible fiber (Ob) and organic cell wall (OCW) content have elucidated genetic variation, heritability, genetic correlations, and genotype-by-environment interactions. Feasible selection procedures have been developed in polycross breeding for improvement of the target traits (Tamaki et al. 2002a, 2002b, 2004; Ashikaga et al. 2008, 2009, 2010, 2011, 2012; Tanaka et al. 2015). However, there are no reports on the paternity of plants selected by phenotypic selection in polycross breeding. The selection procedures may risk excessively selecting particular paternal parents leading to inbreeding depression in breeding populations and reducing improvement. Knowledge of the performance of indirectly-selected paternal parents for target traits and information on whether selected plants are only derived from a very limited number of pollen parents, could lead to higher genetic gains per selection cycle and/or continuous improvement in timothy polycross breeding. The objectives of this study were: (i) to determine efficacy in an actual dataset where paternity assignment was applied for hexaploid timothy using

multi-loci DNA fingerprinting as described by Riday et al. (2013); (ii) to examine what paternal parents were selected in timothy polycross breeding without paternal information; (iii) to discuss the possibility of genetic improvement by paternal selection from the perspective of major target traits in timothy breeding.

MATERIAL AND METHODS

Plant materials and selection procedures

Four early maturing timothy polycrosses (A–D) consisting of 130 parental clones and 123 individual polycross progeny plants were established at the Kitami Agricultural Experiment Station (KAES), Hokkaido Research Organization, Kunneppu, Hokkaido, Japan (43°47´N, 143°42´E) on a high-humic haplic wet andosol, between 2001–2014 (Tables 1 and 2, Supplemental Tables S1 and S2). Relationships between plants were complex with overlapping parents and progeny in the polycrosses. Parents of polycross A were collected from core breeding materials in terms of COM, LR and NVs. Polycross parents of B were collected from germplasm with high GCA for FYs. Polycross parents of D were selected from core breeding materials based on COM, LR and seed yields, and parents of C were mostly derived from selected progeny plants of polycross D. The breeding scheme used phenotypic recurrent selection based on AWHS (Tamaki et al. 2010) for core breeding materials, and mass selection for well-developed strains and check cultivars. Selections in progenies of polycrosses were conducted with emphasis on FYs, COM in the regrowth after the first harvests, LR in the first harvests, and/or NVs (WSC content, Ob content, and Ob per OCW ratio) of the first harvests (Table 2). The seeds of four polycrosses were grouped into two experiments (I and II),

which consisted of polycross progeny tests as among- half-sib family selection and individual phenotypic selections as within- half-sib family selection. Selection for FYs were conducted in both polycross progeny tests and individual phenotypic selections, although selection for other traits carried out only in individual phenotypic selections. In addition, four experiments (1–4 in Table 3) were established in order to investigate clonally propagated polycross parents, because only polycross progeny plants were examined in this breeding scheme.

Individual phenotypic selections

The individual phenotypic selections of experiments I and II (Table 2) were established in a randomized complete block design (RCBD) with four or six replicates on 25 May 2011 and 25 July 2001, respectively. The timothy seedlings were planted with a spacing of 0.75 × 0.6 m under conditions of competition with simultaneously seeded white clover (*Trifolium repens* L.). Three herbage harvests per year were made in 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 and second harvest.

Polycross progeny tests

Seeds of 64 progenies from polycrosses A and B, check cultivars (‘Natsuchikara,’ ‘Horizon,’ and ‘Aurora’) as comparative controls, and others (total 80 entries) were used in the polycross progeny test of experiment I at the KAES (Table 2). It was established in a RCBD with four replicates on 20 May 2011 at a seedling rate of 20 kg ha^{-1}. Each

plot was drilled in two seeding rows 0.85 m long and 0.25 m apart. Seeding-year management consisted of two harvests without data collection to manage annual weeds with applications of 78 kg N ha^{-1}, 90 kg P_2O_5 ha^{-1}, and 78 kg K_2O ha^{-1}. Three harvests per year were conducted in 2012 and 2013. Plots were fertilized as follows: 81 kg N ha^{-1}, 162 kg P_2O_5 ha^{-1} and 81 kg K_2O ha^{-1} in early spring; 57 kg N ha^{-1}, 65 kg P_2O_5 ha^{-1} and 57 kg K_2O ha^{-1} immediately after the first harvest; and 29 kg N ha^{-1}, 33 kg P_2O_5 ha^{-1} and 29 kg K_2O ha^{-1} immediately after the second harvest. Dry matter yields (DMYs; Mg ha^{-1}) of the entries were evaluated during 2012–2013. Cumulated DMYs over two years were analyzed in the analysis of variance (ANOVA) based on a linear model with entry and block random effect factors. Broad-sense heritability (h_B^2) for the cumulated DMYs was calculated based on ANOVA by the following equation: $h_B^2 = 1 - 1/F$-value.

The polycross progeny test of experiment II (Table 2) is described in a previous study (Experiment 2 in Tanaka et al. 2013). Briefly, cumulated DMYs (Mg ha^{-1}) of 41 progenies from polycross D together with check cultivars (‘Nosappu,’ ‘Aurora,’ and ‘Hokusei’) as comparative controls were evaluated over two years (2002–2003) at the KAES. Seeds were planted in a RCBD with four replicates on 31 August 2000 at a seedling rate of 20 kg ha^{-1}. Each plot was drilled as one row 1.5 m long and 0.6 m apart. Experimental plots were harvested three times per year during 2001–2003. Dry matter yields for each plot comprised the sum of six harvests only (in 2002 and 2003) because missing values in the 2001 data precluded inclusion for analysis. The cumulated DMYs over two years were analyzed using the same procedure as that described in experiment I.

The cumulated DMYs (Mg ha^{-1}) were recalculated as percentages of ‘Aurora’ which

is a common check variety in both experiments I and II. General combining ability (GCA) values of parental clones for cumulated DMY (% of 'Aurora') were calculated in each experiment 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). The values were analyzed together with the results of the marker-based paternity test in order to examine what paternal parents had been indirectly selected in the timothy breeding programs without paternal information at the KAES.

Evaluating polycross parents for target traits

Four sets of experiments (Table 3) were carried out at the KAES during 2008–2013 to evaluate clonally propagated polycross parents for date of head emergence (HD), winter hardiness (OW), COM, averaged disease score (DIS) caused by *Scolecotrichum graminis* Fuchel and/or *Cladosporium phlei* (Gregory) de Vries, lodging resistance (LR) in the first harvest, and NVs (WSC content, Ob content, and Ob per OCW ratio) of the first harvest.

Experiment 1 evaluated the 62 parental clones of polycrosses A and B for traits shown in Table 3 over two years (2012–2013). The parental clones were transplanted into the fields in a RCBD with two replicates on May 2011. Seeds of white clover 'Makibashiro' were sown among timothy plants in July 2011 at a seeding rate of approximately 10 kg ha^{-1} in order to investigate COM. Planting-year management consisted of four harvests without data collection to manage annual weeds, with total applications of 120 kg N ha^{-1}, 138 kg P_2O_5 ha^{-1}, and 120 kg K_2O ha^{-1}. Three harvests per year were conducted in late June, mid-August, and early October in 2012–2013.

Spaced-plant 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; and 60 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1}, and 60 kg K_2O ha^{-1} immediately after the first harvest. Size of spaced-plant plots was the same as in experiment I.

Experiment 2 was carried out to screen germplasm for high forage quality genetic resources over two years (2009–2010) using 145 early maturing clones. Of the 145 clones, 44 were the parents of polycrosses A–D in this study and were assessed for traits shown in Table 3 in 2010. The clones were planted in a RCBD having two replications in May 2009. White clover ‘Sonja’ was sown as in experiment 1 immediately after transplanting the timothy plants. Planting-year management consisted of three harvests without data collection to manage annual weeds. The experimental plots were harvested three times (late June, mid-August, and early October) in 2010. Size of spaced-plant plots and amounts of fertilizer applied in 2010 were the same as in experiment I and II.

Experiments 3 and 4 were conducted for pre-selecting parental candidates for polycrosses A and B from germplasm in the KAES over two years (2008–2009 and 2007–2008, respectively) focusing on LR, COM, and high WSC content. The 108 and 23 parental clones, respectively, of polycrosses A–D were used in experiments 3 and 4 (Table 3). Timothy plants were planted in a RCBD with two replications in May 2008 and July 2007. White clover ‘Sonja’ was sown and planting-year management was the same as in experiment 2. Size of spaced-plant plots and amounts of fertilizer applied were the same as in experiment 1. The experimental plots were harvested twice (early July and early September) in 2009 and 2008.

In the experiments, HD in each plot was recorded as the day after 31 May when the third panicle had emerged from the boot leaf. OW was based on observation of crown

health and amount of regrowth scored on a scale of 1 = poor to 9 = excellent in early spring. COM was evaluated based on vigor (VS) and stem ratio of internode elongation (IE) in the second harvests under mixed sward conditions with white clover (Tamaki et al. 2002b). VS and IE were scored on a 1 = poor to 9 = excellent scale and on a 1 = few to 9 = many scale, respectively. DIS was calculated as the average infection value due to *Scolecotrichum graminis* Fuchel and/or *Cladosporium phlei* (Gregory) de Vries scored on a 1 = healthy to 9 = severe in each harvest of the experiment year. LR was degree of lodging scored on a 1 = non to 9 = severe scale in the first harvest. The harvest samples for investigations of NVs of the first harvests were dried in an oven at 70°C for 48hr, and were milled and passed through a 0.75-mm screen. WSC content, Ob content and Ob per OCW ratio were analyzed by calibration equations for prediction (Ashikaga et al. 2016) using near-infrared reflectance spectroscopy (FOSS NIRSystems Model 6500, Laurel, MD, USA).

Breeding values as random effect for these traits were estimated by best linear unbiased prediction (BLUP) according to the mixed effect model of $y = X\beta + Z\alpha + \varepsilon$, where y is the vectors of examined traits, 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, using the “lme4” package in R statistics software (GNU General Public License). The fit of the model by an iterative process gives estimates of the variance components, a prediction of the random vector and a solution for the fixed effect vector. Heritability (h^2) 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 restricted/residual maximum likelihood (REML) process. The breeding values for the examined traits were analyzed together with results of the marker-based paternity test

similar to the analysis of the GCA values for FYs.

Genotyping protocol

Twenty-seven primer pairs (Supplemental Table S3) were chosen for the paternity test from genomic-SSR markers which have been localized on the unsaturated diploid timothy (*P. nodosum* L.) map (Cai et al. 2009). Tissue from 152 out of the 153 polycross parent plants and all non-overlapping progeny plants maintained in a greenhouse was collected for SSR analysis. One parent plant in polycross D died during clonal propagation for the experiment. Genomic DNA was extracted from the samples using a modified CTAB method (Tamura et al. 2009). Fragment sizes of PCR products by SSR markers were evaluated by capillary electrophoresis (GenomeLab GeXP; Beckman Coulter, Brea, CA, USA) using the M13-tagged forward primer method (Cai et al. 2005). The PCR amplification reactions were performed in a 10 μl reaction volume containing 100 ng of genomic DNA, 2 μl of 5× Colorless GoTaq reaction Buffer (Promega, Madison, WI), 2.5 μM of each dNTP, 0.5 mM of the dye-labelled M13-forward primer with Beckman Coulter dye (D3 or D4), 0.1 mM of the M13-tagged forward primer, 0.5 mM of the reverse primer and 0.20 units of GoTaq polymerase (Promega) on the GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). The cycling regime for the PCR amplification consisted of an initial denaturation step of 4 min at 94°C, 20 cycles touch down: 30 sec at 94°C, 30 sec at 65 to 55°C (decreasing 1°C each cycle) and 1.5 min at 72°C, final extension of 7 min at 72°C, and a holding step at 4°C. Electrophoresis was performed following the procedure described in the GenomeLab GeXP manual. Fragment size determinations were performed using the default fragment analysis parameters of the auxiliary software, allowing the precise determination of

fluorescent DNA fragments resulting in an electropherogram and a fragment summary list. Allele phenotypes of the plants were visually scored using a binary code (1/0) for the presence or absence of allele peaks without knowing the allele dosage.

Paternity test with a known maternal parent

For each progeny plant from polycrosses A, B, and D with maternal parent information, alleles were compared to their respective maternal alleles and alleles not observed in the maternal parent were recorded as paternally-derived. For a given progeny plant paternally-derived alleles were compared to alleles of paternal parent candidates i.e. parents of the polycross not including the maternal parent. For each paternal parent candidate a "matching score" for paternal determination was calculated as follows: $M_{i,pj} = (N_{i,pj} - N_{i,mi,pj}) / (N_i - N_{i,mi})$, where $M_{i,j}$ is the "matching score" of paternal parent candidate *pj* for progeny plant *i*, $N_{i,pj}$ is the number of common alleles between progeny plant *i* and paternal parent candidate *pj*, $N_{i,mi,pj}$ is the number of common alleles among progeny plant *i*, respective maternal parent *mi* and paternal parent candidate *pj*, N_i is the number of alleles for progeny plant *i*, and $N_{i,mi}$ is the number of common alleles between progeny plant *i* and the respective maternal parent *mi*, respectively. The "matching scores" range between "0–1" for paternal parent candidates and the "matching score" of the true paternal parent should be "1" if clearly paternally-derived alleles are detected without missing data. In the highly polymorphic and complex SSR analysis produced by hexaploid timothy in this study, the "matching score" of the true paternal parent should be significantly higher than that of the others. The "matching scores" of paternal parent candidates for a given progeny plant were analyzed by the Smirnov-Grubbs test for one outlier employing the function "grubbs.test" in the R

statistical package “outliers” (GNU General Public License). If the highest “matching score” of a paternal parent candidate was a significant outlier it was identified as the true paternal parent of the progeny plant.

For each plant in each polycross, the number of indirectly-selected paternal parents was counted. The goodness-of-fit of the frequency distribution of indirectly selected paternal parents to the expected probabilities in each polycross was tested by the Pearson’s chi-squared test with simulated *p*-value based on 10000 replicates employing the function “chisq.test” in the R statistical package “stats” (GNU General Public License). In terms of the GCA for FYs and the breeding values for target traits, the indirectly-selected paternal parents and the respective maternal parents were compared in each polycross by a paired *t*-test in the R statistical package “base”. Parental combinations with an undetermined paternal parent or a missing breeding value were not included.

Paternity test with both parents unknown

For each progeny plant without information on the maternal parent in polycross C, alleles were compared to all possible pairs of parental combination. For each parental combination candidate, a “matching score” was calculated as follows: $M_{i,j,k} = (N_{i,j} + N_{i,k} – N_{i,j,k}) / N_i$, where $M_{i,j,k}$ is the “matching score” of parental combination candidate *j* and *k* for progeny plant *i*, $N_{i,j}$ is the number of common alleles between progeny plant *i* and parent candidate *j*, $N_{i,j,k}$ is the number of common alleles among progeny plant *i*, parent candidates *j* and *k*, and N_i is the number of alleles for progeny plant *i*, respectively. The “matching scores” without maternal parent information are in the range “0–1” for parental combination candidates and the “matching score” of a true

parental combination is “1” where there is no missing data. In practice, as in the paternity test with known maternal parent information, “matching scores” should be significantly higher for true parental combinations. A parent combination candidate showing a significant higher value for each progeny plant was determined as a true parental combination by the Smirnov-Grubbs test for one outlier. For the progeny plants of polycrosses A, B, and D, the “matching scores” were also calculated and parental combinations were determined on the assumption that there was no information of maternal parent for the progeny plants. The results of the parent tests with and without maternal information for each progeny plant were compared in order to confirm the correctness of the results of the tests without maternal information.

RESULTS

GCA values for forage yield and breeding values for target traits in polycross parents

The polycross progeny test of experiment I showed a significant ($p < 0.001$) difference among all entries for cumulated DMY over two years. The GCA values for the cumulated DMY (percentage of ‘Aurora’) based on the polycross progeny test of experiment I ranged from –10.4 to 10.1 in polycross A and from –9.9 to 11.9 in polycross B (Table 4, Supplemental Table S2). The polycross progeny test of experiment II showed a significant ($p < 0.001$) difference among all entries for cumulated DMY over two years. The GCA values for cumulated DMY (percentage of ‘Aurora’) over two years based on the polycross progeny test of experiment II ranged from –12.0 to 13.7 in polycross D (Table 4, Supplemental Table S2). The experiments

were similar in values of broad-sense heritability, coefficient of error variation, yield levels of polycross progenies compared to ‘Aurora,’ and the standard deviation (SD) of the progenies for cumulated DMYs (Table 4). These indicated that the experiments were conducted under similar test accuracy and variation.

There were significant random effects for clone entries in the mixed effect models for HD ($p < 0.001$), OW ($p < 0.05$), VS ($p < 0.01$), DIS ($p < 0.05$), LR ($p < 0.001$), and WSC ($p < 0.05$), but not for IE ($p = 0.06$), Ob content ($p = 0.1$), and Ob per OCW ratio ($p = 0.1$). Heritability in the mixed effect models for all nine traits ranged from 0.13 to 0.59 (Table 5). For HD and NVs, a genetically meaningful range of breeding values was observed and the heritability values (0.38–0.59) indicated that a considerable genetic effect contributed to variation in the traits. Regarding the differences among polycrosses A, B, and D, the SD for VS, IE, and LR in polycross B was larger than that in the other polycrosses, although differences in the means for all nine traits were not significant (Table 6).

Paternity test with a known maternal parent

The analyses based on 27 genomic-SSR markers detected a total of 570 alleles (Supplemental Table S3) and 62–112 alleles per genotype. For progeny plants in polycrosses A, B, and D with maternal information, numbers of paternally-derived alleles per plant ranged between 27–58, 24–59, and 28–66, respectively. The “matching score” for all parent candidates ranged between 0.06–0.79 in polycross A; 0.03–0.85 in polycross B; and 0.02–0.95 in polycross D. For paternal parent candidates showing a highest “matching score” in each progeny plant, the scores ranged between 0.40–0.79 in polycross A [statistical value (G) of the Smirnov-Grubbs test = 2.91–5.01]; 0.39–0.85 in

polycross B (G = 2.90–4.55); and 0.36–0.95 in polycross D (G = 2.01–4.99) (Supplemental Table S1). The paternity test for three progeny plants in polycross D failed to detect a highest score as a significant outlier at the 5% level. For the other 112 polycross progeny, the paternity test detected an outlier with highest score.

For the 112 polycross progeny plants, 34 polycross parents had been selected as their maternal parents from 105 candidates based on FYs in the progeny tests in the scheme of AWHS (Table 2 and Figure 1). Meanwhile, 20, 11, and 17 paternal parents were indirectly selected through AWHS in polycrosses A, B, and D, respectively (Figure 1). Among the 48 indirectly-selected paternal parents, 29 (15, 7, and 7 in polycrosses A, B, and D) parents were not selected as the maternal parents (Figure 1), i.e. 40% of paternal parents were selected on different selection criteria compared to the maternal parents. Chi-squared values in goodness-of-fit test of the frequency distribution of indirectly-selected paternal parents (closed bar in Figure 1) compared to expected probabilities were 49.1 (simulated p-value = 0.05729), 141.4 (simulated p-value < 0.001), and 82.7 (simulated p-value < 0.001), in polycross groups A, B and D, respectively. Serial nos. “1,” “7” and “13” of parents in polycross B and nos. “18” and “41” of parents in polycross D were indirectly selected with a high frequency as paternal parents (Figure 1).

Paternity test with both parents unknown

For progeny plants without maternal information including those in polycrosses A, B, and D on the assumption of no maternal information, the highest “matching scores” ranged from 0.63–0.91 in polycross A (G = 3.32–5.30); 0.60–0.94 in polycross B (G = 3.52–4.99); 0.73–0.99 in polycross C (G = 3.09–4.07, Supplemental Table S1); and

0.62–0.98 in polycross D (G = 3.35–5.72). The paternity tests failed to detect a highest score parental combination based on the Smirnov-Grubbs test for one outlier at the 5% level for six progeny plants in polycross A, one plant in polycross B, three plants in polycross C, and four plants in polycross D. Among the parental combinations showing a significantly highest "matching score" for the 104 progeny plants in polycrosses A, B, and D, five parental combinations differed from the results in the paternity tests with maternal information. The parental assignments at the 1% level were also conducted, because erroneous determination in paternity tests at the 5% level was suspected. The results for the five parental combinations at the 1% level showed no significant highest score as an outlier. The number of detected parental combinations based on paternity tests at the 1% level decreased to 93 (76%) from 109 (89%) for all 123 progeny plants.

Characteristics of indirectly selected paternal parents

The means of GCA for FYs of the maternal parents were significantly higher than that of the indirectly selected paternal parents in all polycrosses (Table 7). Significant differences among maternal and paternal parents were detected for two traits (VS and WSC content) in polycross A, seven traits (HD, OW, VS, IE, DIS, Ob content, and Ob per OCW ratio) in polycross B, and one trait (Ob per OCW ratio) in polycross D out of all nine examined traits (Table 7). In particular, meaningful differences were detected for HD, VS, IE, and Ob content in polycross B (Table 7). Plants "1," "7" and "13" in polycross B and plants "18" and "41" in polycross D, were selected as paternal parents with a high frequency (Figure 1). Their GCA values (% of 'Aurora') for FYs were 4.8, 1.5, 0.1, –0.5, and 9.4, respectively, and thus included both superior and moderate plants. Breeding values for VS were 6.0, 6.4, 6.7, 6.0, and 5.0 respectively, for IE 5.3, 6.0, 6.1,

5.7, and 5.1 respectively, and for Ob content 53.9, 55.2, 52.3, 54.4, and a missing value respectively.

DISCUSSION

Success for the marker-based paternity test in this study depends on the existence of the true parents in the analysis, a satisfactory number of clearly paternally-derived alleles, and the number of parental candidates. Despite the SSR analysis being complex with some missing data or genotyping errors, a test including maternal information based on 27 genomic-SSR markers, successfully identified paternity for 97% (112/115) of the polycross progeny plants. The three progeny plants that failed the paternity test were derived from polycross D where one of the parental clones was excluded from the analysis due to withering during the experiment. The paternal parents of the three progeny plants might not be among parent candidates examined in this study, and/or information of maternal parents of that would be misrecorded. These results indicate that the paternity assignment test used in this study could be an efficient way of using genomic-SSR markers in hexaploid timothy breeding. In general, polycross breeding in timothy is carried out using 40–50 parental clones, and selecting 20–50 progeny plants from 4,000–8,000 individuals in each polycross based on AWHS or mass selection (Tamaki et al. 2010). The successful results obtained in this study show that a simple exclusion analysis for paternity assignment is practically feasible for timothy breeding in Japan.

The “matching score” for paternal parents was not equal to the theoretical value of “1.” This is probably due to errors in allele calling or binning caused by some problems

in SSR genotyping such as stuttering bands, split peaks and null alleles (Guichoux et al. 2011), as well as complex allele composition in a hexaploid. Strict selection of SSR markers producing a sufficient number of clearly paternally-derived alleles would reduce these errors and improve the matching score up to "1."

For paternity tests without maternal information, the efficiency of assignment at the 5% level in the Smirnov-Grubbs test for outliers declined from 97% (for paternity tests with maternal information) to 89% in polycrosses A, B, and D with mismatches for five progeny plants at the 5% level. In polycross C, the efficiency declined to 63% (5/8). Possible reasons for the decline in efficiency and the mismatches are (i) multiple candidate parental combinations might be detected by the paternity test especially among closely related polycross parents, and (ii) an increase in sample number with consequent gain of statistical power may result in an overestimation of statistical significance since the number of all possible combination candidates in paternity tests without parental information are larger than the number of paternity parent candidates in paternity tests with maternal information. Such misjudgments could be avoided by tightening the *p*-value (from the 5% level to the 1% level) in the Smirnov-Grubbs test for outliers. Reconsideration of judgement criteria (Chakraborty et al. 1988) or the use of polymorphism in chloroplast DNA with maternal inheritance (Kumar et al. 2007) would be needed for more precise and efficient paternity assignment without maternal information. However, it is important to note that this study shows that paternity tests are considerably affected in a negative way by loss of maternal information due to bulk-harvest.

The indirectly-selected paternal parents through AWHS were clearly inferior to the maternal parents in GCA for FYs in polycrosses A, B, and D (Table 7). Moreover, the

paternal parents with a high frequency did not always exhibit high GCA values for FYs. These results indicate possibilities for reasonable genetic gains by paternal selection before seed production of the next generation and/or developing new strains through AWHS.

Individual phenotypic selection, which correspond to within- half-sib family selection in AWHS timothy breeding, had achieved improvement for traits with high heritability and environmental stability such as COM (Tamaki et al. 2002b) and NVs of the first harvests (Ashikaga et al. 2008, 2009, 2010, 2012). Individual phenotypic selection might indirectly affect the selection of paternal parents in the breeding scheme. With regard to breeding values for the traits considered in this study, the paternal parents included superior plants for traits associated with COM (VS and IE) or Ob content of the first harvest. Phenotypic selection may risk excessively selecting particular paternal parents if selection response is good for the target trait. Contrary to our concern, this study did not reveal similar trends across three polycross groups in differences among maternal and paternal parents for all nine examined traits and in the frequency distribution of indirectly-selected paternal parents (Table 7 and Figure 1). This suggests that AWHS can select superior progeny plants not only from mating combinations among superior parents but also from various other parental combinations. Marker-based paternity tests can redress the imbalance of selected parents, although individual phenotypic selection for traits with a high heritability do not always produce unbalanced paternal selection. In this study, the goodness-of-fit test in polycross B (simulated p-value for the chi-squared value < 0.001) clearly showed that indirect paternal selection in polycross B resulted in unbalanced paternal selection (Figure 1). This was probably caused by individual phenotypic selection *per se* for target traits such

as COM and/or Ob content, as this study detected meaningful differences in traits between maternal and paternal parents in polycross B (Table 7). The goodness-of-fit tests showed contrasting results in the random paternal selection in polycross A and the unbalanced selection in B, although selection in both polycross groups was carried out using the same selection criteria regardless of polycross group or maternal derivation. Reasons for unbalanced paternal selection may include factors affecting genetic gain through individual phenotypic selection, such as heritability of the target traits, diversity of the base population, and/or intensity of selection. The most compelling reasons are high heritability and wider diversity in a polycross population as illustrated by the larger SD for VS, IE, and LR in polycross B compared to that in polycross A (Table 6). Intensity of selection as a cause of unbalanced paternal selection is indicated by a difference in the selected proportion of within- half-sib selection (Table 1, 0.013–0.067 in polycross B vs. 0.020–0.090 in polycross A). Applying the same selection criteria in both polycrosses would lead to a more stringent paternal selection for Ob content in polycross B, since the maternal parents selected, based only on GCA for FYs in polycross B, were inferior compared to the mean Ob content of the polycross population (Tables 6 and 7). Another reason for unbalanced selection may be that only progeny plants derived from a particular parental combination tend to be selected due to affinity between maternal and paternal parents exhibiting specific combining ability and/or inbreeding depression. The significant correlation between yield performance of polycross progenies and marker-based genetic distances shown in a previous study using polycross D (Tanaka et al. 2013) supports this as a cause of unbalanced paternal selection in polycross D. However, this cannot apply to polycross B (data not shown). Unequal polycross mating may also cause unbalanced frequency of paternal parents in a

polycross population. This was not elucidated further in this study although unbalanced paternal selection was not found in polycross A which was carried out under similar mating conditions to polycross B.

In this study, we have demonstrated the value of paternal selection of superior plants in polycross breeding, in terms of higher genetic gain per selection cycle and balanced selection of paternal parents, to ensure continuous improvement based on phenotypic recurrent selection. Paternal assignment using genomic-SSR markers is easily applied in conventional polycross breeding without excessive time and labor constraints. Forage grass breeders should proactively adopt paternal selection based on paternal parent assignment using molecular markers in order to acquire higher genetic gain per selection cycle in polycross breeding especially for traits with low heritability and small selection response such as FYs. However, intensity of paternal selection should be weaker than that for maternal selection. Therefore, it is necessary to prepare a larger number of entries for marker-based paternal selection than the number of selected individuals based on conventional AWHS. With regard to DIS, LR, OW and WSC content, it is not clear whether individual phenotypic selection would influence indirect selection of paternal parents, even though good selection responses for these traits is expected, as is the case with COM or Ob content (Tamaki et al. 2002b; Ashikaga et al. 2008). Genotype-by-environment interaction in a broad sense may mask some relationships. DIS in this study was a composite disease score based on *S. graminis* and/or *C. phlei* infection. LR depends on growth stage, although heritability in each growth stage is high (Tamaki et al. 2002a). OW included several factors such as cold tolerance and snow mold resistance and the relative contribution of the factors differs by year. Different conditions of overwintering in each experiment may also make

interpretation difficult as undesirable correlations between WSC content and OW were observed in previous study (Ashikaga et al. 2016).

Further work is needed to take account of genotype-by-environment interaction and dominance effects in this novel use of a paternity test in timothy polycross breeding. Paternity assignment using molecular markers provides detailed paternal pedigree data in polycross breeding. Further study of polycross breeding in forage grasses should include BLUP models with pedigree data, originally developed in animal genetics and breeding (Piepho et al. 2008). BLUP animal models can include pedigree information, dominance effects, genetic correlations among target traits, and/or flexible variance-covariance structures for genotype-by-environment interaction. These approaches will help breeders efficiently improve forage grasses with greater genetic gains in polycross breeding.

CONCLUSION

Marker-based paternity tests using three polycrosses of timothy revealed unbalanced numbers of selected parents and inferior paternal parents in GCA for FY. The imbalance of selected paternal parents was produced by individual phenotypic selection for target traits such as COM and/or Ob content. These results indicate the possibility of using paternal selection in timothy polycross breeding in terms of removing inferior paternal parents and redressing the imbalance of selected parents.

Conflict of interest

The authors declare that they have no conflict of interest.

Supplemental Material Available

Supplementary material for this article is available online. The supplementary data consists of the following tables. Table S1: Maternal and paternal parents of progeny plants identified by paternity tests using 27 genomic-SSR markers in four polycrosses A–D. Table S2: General combining ability values for forage yields and the breeding values for target traits of parents in four polycrosses A–D. Table S3: Genomic-SSR markers used in paternity tests for four polycrosses.

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Table 1 Four polycrosses used in early maturing timothy (*Phleum pratense* L.) breeding at Kunneppu, Hokkaido, Japan

Polycross designations	Outlines of derivation of polycross parents†	Mating design			
		Number of parental clones	Replicates	Harvested year	Preservation of seeds
A	Collection from core breeding materials in terms of COM, LR, NVs	36	9	2010	Half-sib seeds
B	Collection from germplasm with high GCA for FYs	28	9	2010	Half-sib seeds
C	Progeny plants of polycross D	25	1	2005	Bulked seeds
D	Selected plants from core breeding materials based on COM, LR, and seed yields.	41	12	2000	Half-sib seeds

† COM, LR, NVs, and GCA for FYs denote competitiveness toward legumes, lodging resistance, nutritive values, and general combining ability for forage yields, respectively.

Table 2 Progeny plants selected by individual phenotypic selection in early maturing timothy (*Phleum pratense* L.) breeding focused on forage yield (FY), competitiveness toward legumes (COM), lodging resistance (LR), and nutritive values (NVs) at Kunneppu, Hokkaido, Japan

Experiment designations	Experiment year	Derivation of seeds†	Number of entries (individuals, half-sibs)	Selection results				
				Number of selected plants	Breeding objectives	Selection method	Selected proportion‡	
							within half-sib or population	among half-sib
I	2011–2014	Polycross A	3600, 36	44	FY, COM, LR, NVs	AWHS§	0.020–0.090	0.31
		Polycross B	4200, 28	36	FY, COM, LR, NVs	AWHS	0.013–0.067	0.29
		Polycross C	150, -	8	COM, LR, NVs	Mass¶	0.053	-
II	2001–2004	Polycross D	6100, 41	35	FY, COM, LR	AWHS	0.005–0.042#	0.37

† Polycross designations were referred to Table 1.

‡ Proportion of the number of selected plants or half-sibs relative to the number of entries.

§ Among- and within- half-sib family selection.

¶ Mass selection.

The numbers of individuals within half-sibs differed depending on entries (95-215 individual plants per half-sib).

Table 3 Evaluation of clonally propagated early maturing timothy (*Phleum pratense* L.) polycross parents at Kunneppu, Hokkaido, Japan

Experiment designations	Experiment year	Number of entries of polycross parents			
		Polycross A†	Polycross B	Polycross C	Polycross D
1	2011–2013	35	27	0	0
2	2009–2010	8	12	15	9
3	2008–2009	28	17	23	40
4	2007–2008	8	15	0	0
Total‡		36	28	24	40

† Polycross designations were referred to Table 1.

‡ Total number of parental clones except for overlap between experiments.

Table 4 General combing ability (GCA), mean, standard deviation (SD), and range for cumulated dry matter yield (DMY) based on polycross progeny tests over two years at Kunneppu, Hokkaido, Japan

Experiment designations	Examined years	Polycross group designations	Cumulated DMY (Mg ha^{-1}) over two years					GCA (% of 'Aurora') †	
			n‡	Mean ± SD	Range	Check§	h_B^2 ¶	Mean ± SD	Range
I	2012–2013	Polycross A	36	23.89 ± 0.83	21.77 - 25.94	20.87	0.67	0.0 ± 4.1	–10.4 - 10.1
		Polycross B	28	23.87 ± 1.03	21.88 - 26.27			0.0 ± 5.1	–9.9 - 11.9
II	2002–2003	Polycross D	41	25.31 ± 1.15	22.81 - 28.17	21.35	0.66	0.0 ± 5.5	–12.0 - 13.7

† GCA values were calculated using following equation: GCAi = [(n – 1) / (n – 2)] (Xi – μ), where n is the number of parents in a polycross, Xi is DMY (percentage of 'Aurora') of a polycross progeny i, and μ stands for the means of DMY (percentage of 'Aurora') of all polycross progenies (Griffing 1956).

‡ Number of polycross progenies (number of entries except for check varieties).

§ The check variety 'Aurora' was used as the comparative control.

¶ The broad-sense heritability (h_B^2) was calculated based on ANOVA in each progeny test by the following equation: $h_B^2 = 1 - 1 / \text{F-value}$.

Table 5 Results of mixed effect model for target traits of early maturing timothy (*Phleum pratense* L.) clonal propagated polycross parents evaluated at Kunneppu, Hokkaido, Japan

Traits (Designations)	Unit	No. of data	No. of entry	Grand mean	Breeding values†		Variance components‡		h^2§
					Min	Max	Entry (σ^2_g)	Error (σ^2_e)	
Date of head emergence (HD)	Day after 31 May	269	114	18.1	14.3	22.0	3.509	3.273	0.52
Winter hardiness (OW)	1:poor – 9:excellent	276	114	4.3	3.7	5.0	0.1879	0.9926	0.16
Competitiveness toward legumes (COM)									
Vigor in the second harvests (VS)	1:poor – 9:excellent	266	114	5.6	4.6	6.8	0.4456	1.5833	0.22
Stem ratio of internode elongation (IE)	1:few – 9:many	241	113	5.5	4.7	6.1	0.3627	2.1223	0.15
Averaged disease score (DIS)¶	1:healthy – 9:severe	269	114	2.9	2.2	3.7	0.2485	1.6286	0.13
Lodging resistance (LR) in the first harvests	1:non – 9:severe	207	114	1.5	1.2	3.3	0.2630	0.5886	0.31
Nutritive values (NVs) in the first harvests									
Water-soluble carbohydrate (WSC) content	%DM	117	95	9.0	6.3	14.4	3.127	2.137	0.59
Low-digestible fiber (Ob) content	%DM	117	95	54.2	51.1	61.3	3.749	3.388	0.53
Ob per organic cell wall (OCW) ratio	%	117	95	80.3	78.5	82.3	0.9954	1.5917	0.38

† Breeding values were obtained by the sum of grand mean and best linear unbiased prediction values as entries random effect.

‡ Variance components of entries random effect (σ^2_g) and residuals (σ^2_e) were estimated by restricted/residual maximum likelihood (REML).

§ Heritability (h^2) was calculated based on estimated variance components as follows: $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e)$.

¶ Averaged disease score by *Scolecotrichum graminis* Fuchel and/or *Cladosporium phlei* (Gregory) de Vries.

Table 6 Mean, standard deviation (SD), and range of the breeding values for target traits of timothy (*Phleum pratense* L.) polycross parents evaluated at Kunneppu, Hokkaido, Japan

Trait designations†	Polycross A‡			Polycross B			Polycross D		
	n§	Mean ± SD¶	Range#	n§	Mean ± SD¶	Range#	n§	Mean ± SD¶	Range#
HD	36	18.4 ± 1.79	14.6–22.0	28	17.7 ± 1.64	14.8–21.2	40	17.9 ± 1.33	14.3–20.3
OW	36	4.4 ± 0.24	4.1–5.0	28	4.3 ± 0.29	3.7–4.8	40	4.3 ± 0.17	3.9–4.7
VS	36	5.7 ± 0.35	4.8–6.3	28	5.6 ± 0.60	4.6–6.8	40	5.6 ± 0.34	4.9–6.7
IE	36	5.6 ± 0.26	5.0–6.1	28	5.4 ± 0.41	4.7–6.1	40	5.4 ± 0.25	4.8–6.1
DIS	36	2.9 ± 0.28	2.3–3.4	28	2.9 ± 0.38	2.2–3.7	40	2.9 ± 0.18	2.2–3.6
LR	36	1.5 ± 0.28	1.2–2.3	28	1.7 ± 0.54	1.2–3.3	40	1.5 ± 0.15	1.2–2.0
WSC	33	9.3 ± 1.56	7.5–14.4	23	9.3 ± 1.52	7.6–14.1	33	8.8 ± 1.41	6.8–14.1
Ob	33	53.8 ± 1.22	51.1–56.5	23	54.3 ± 1.38	51.1–56.3	33	54.4 ± 1.82	51.2–61.3
Ob/OCW	33	80.3 ± 0.71	78.5–82.3	23	80.5 ± 0.77	78.5–81.4	33	80.2 ± 0.51	79.1–81.0

† The target trait designations were refer to Table 5.

‡ The polycross were consisted of 36, 28, and 41 parental clones in polycrosses A, B, and D, respectively.

§ The number of parents in each polycross except for a missing value.

¶ The means and standard deviation of parents in each polycross.

The minimum – maximum values.

Table 7 Comparison between maternal and paternal parents of polycross progeny plants selected through among- and within- half-sib family selection (AWHS) in timothy (*Phleum pratense* L.) breeding in the Kitami Agricultural Experiment Station for target traits including forage yields

Trait designations†	Polycross A‡				Polycross B				Polycross D			
	df§	Maternal parents¶	Paternal parents¶	Paired *t*-value#	df	Maternal parents	Paternal parents	Paired *t*-value	Df	Maternal parents	Paternal parents	Paired *t*-value
FY	43	4.8	0.5	6.73 ***	35	6.8	1.4	7.88 ***	31	5.4	2.1	2.72 *
HD	43	18.2	18.5	0.60 NS	35	17.1	16.3	2.80 **	31	18.5	18.2	0.99 NS
OW	43	4.4	4.5	1.27 NS	35	4.4	4.5	2.47 *	31	4.3	4.3	0.06 NS
VS	43	5.6	5.8	2.14 *	35	5.8	6.1	2.45 *	31	5.7	5.6	0.93 NS
IE	43	5.7	5.6	1.58 NS	35	5.4	5.7	4.12 ***	31	5.4	5.5	0.45 NS
DIS	43	2.8	2.8	0.19 NS	35	2.9	2.7	2.26 *	31	2.8	2.9	1.62 NS
LR	43	1.5	1.5	0.82 NS	35	1.5	1.6	0.48 NS	31	1.5	1.5	0.94 NS
WSC	32	10.4	8.9	2.60 *	33	9.4	8.8	1.82 NS	19	8.5	8.9	0.88 NS
Ob	32	53.7	54.2	2.02 NS	33	55.1	53.9	4.40 ***	19	55.6	54.4	1.94 NS
Ob/OCW	32	80.2	80.5	1.85 NS	33	80.8	80.1	6.19 ***	19	80.3	80.1	2.34 *

† FY denotes general combining ability (% of ‘Aurora’) for forage yield (Table 4), and the other target trait designations were refer to Table 5.

‡ The parental combination of the polycross progeny plants in each polycross A, B, and D were analyzed except for parental combinations with an undetermined paternal parent or a missing value for the breeding values.

§ The degree of freedom in paired *t*-test for difference between maternal and paternal polycross parents in each polycross.

¶ The means of maternal and paternal parents in each polycross.

NS denotes not significance at the 5% level (two-tailed test), and single, double, and triple asterisks indicate significant differences at the 5%, 1%, 0.1% levels (two-tailed test), respectively.

Figure legends

Figure 1 Frequency of timothy (*Phleum pratense* L.) selected-parents of polycross progenies through among- and within- half-sib family selection (AWHS) in three polycross groups, A (top), B (middle) and D (bottom), at the Kitami Agricultural Experiment Station. Polycrosses A, B, and D consisted of 36, 28, and 41 parental clones, respectively. The 44, 36 and 32 progeny plants were selected through AWHS from polycrosses A, B, and D, respectively, except for three plants in polycross D for which the paternity test did not determine paternal derivation. The closed bar denotes number of indirectly-selection as a paternal parent. The open bar denotes number of selection as a maternal parent.

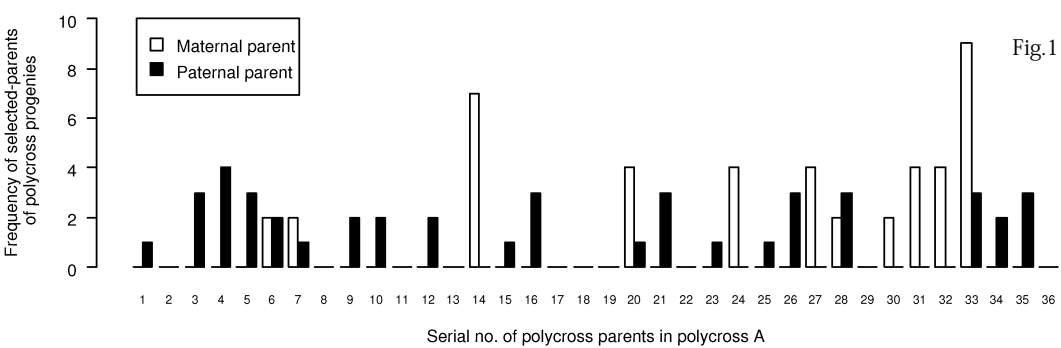
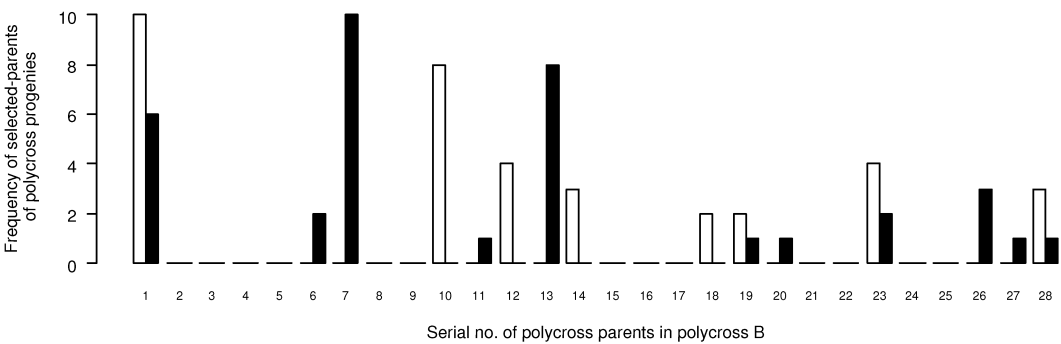
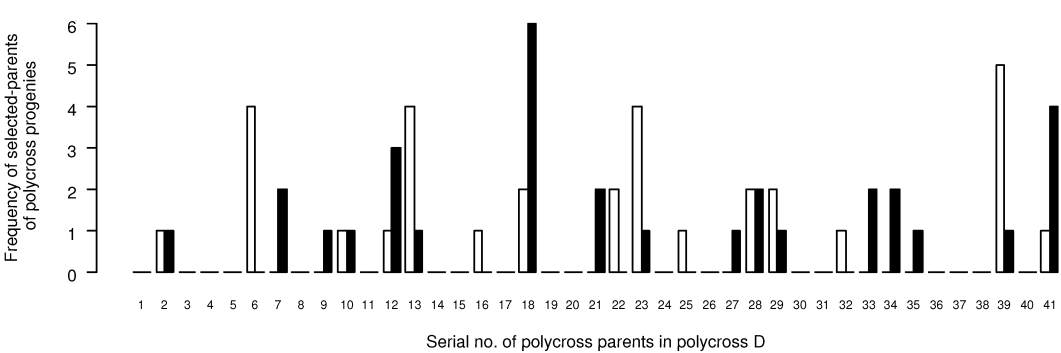

Maternal parent
Paternal parent
Frequency of selected-parents of polycross progenies
0 2 4 6 8 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Serial no. of polycross parents in polycross A

Fig.1


Frequency of selected-parents of polycross progenies
0 2 4 6 8 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Serial no. of polycross parents in polycross B


Frequency of selected-parents of polycross progenies
0 1 2 3 4 5 6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
Serial no. of polycross parents in polycross D