

Assessing inbreeding in perennial ryegrass (*Lolium perenne*) as a step towards F1 hybrid breeding

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Abstract

Perennial ryegrass is an important turf and forage grass in temperate regions worldwide. Limited genetic gains have been made with current breeding strategies compared with other grass crops, such as rice and maize, which benefit from Filial 1 (F1) hybrid breeding. One of the largest constraints on hybrid breeding in ryegrass is self-incompatibility preventing inbreeding, as homozygous parental lines are required to develop hybrids with maximal hybrid vigour. Obligate outcrossing in ryegrass has resulted in cultivars with high levels of heterozygosity, lacking trait uniformity across the population. A naturally occurring *self-fertile* (*SF*) locus that overcomes the self-incompatibility system has been identified in a European perennial ryegrass population. This study crossed the *SF* locus into an elite cultivar, producing a self-compatible population that was inbred for several generations. Genotyping-by-sequencing (GBS) was used to assess the population structure and degree of inbreeding in the self-compatible population. Phenotypic analysis indicated that increased homozygosity did not necessarily affect growth and performance. This study concludes that self-compatible ryegrass is a promising tool for hybrid breeding and agronomic improvement of perennial ryegrass.

KEYWORDS

F1 hybrid, forage grass, genotyping-by-sequencing, inbreeding, perennial ryegrass, self-incompatibility

1 | INTRODUCTION

Heterosis, or hybrid vigour, is the phenomenon where filial 1 (F1) offspring grow more vigorously than their parents (Chen, 2013). Charles Darwin was the first to study heterosis, showing that interspecific hybrids tended to be more vigorous than intraspecific hybrids (Darwin, 1876). Heterosis has been observed in plants and animals and has been key to improving the agricultural productivity of many species. For example, the adoption of F1 hybrid cultivars in maize and rice has resulted in yield increases of 20%–50% (Duvick, 1999). The

production of F1 hybrids requires the generation of genetically distinct, near-homozygous parental lines, often through multiple generations of inbreeding. By selecting compatible inbred parental lines, F1 hybrids with high heterosis and consistent, predictable performance can be generated.

Perennial ryegrass is an important turf and forage grass in temperate regions, being widely selected as feed for sheep, beef and dairy cows because of its high metabolizable energy and persistence (Charlton & Belgrave, 1992). Although there has been extensive breeding of ryegrass over the last 80 years, genetic gains have

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remained limited at around 0.25 to 0.7% per year (Harmer et al., 2016; Woodfield, 1999), and current cultivars are highly heterozygous (Faville et al., 2020). There is growing interest in developing F1 hybrid breeding strategies for ryegrass to provide the step-change needed to breed cultivars better adapted to changing environments, with improved yields and stress tolerance (Cropano, Place, et al., 2021a; do Canto et al., 2016; Herridge et al., 2020). One of the current barriers to F1 hybrid breeding is that ryegrass cannot self-pollinate, and thus inbreed, because of a genetic self-incompatibility (SI) mechanism. Ryegrass and other *Poaceae* species have a two-loci (S and Z) SI system, with pollen-tube growth being inhibited when the alleles of both the S and Z loci in the pollen are matched in the stigma (Lundqvist, 1962). SI has evolved to promote outcrossing and survive population bottlenecks, mitigating extinction rates (Takebayashi & Morrell, 2001). However, during breeding, SI prevents the inbreeding required to purge deleterious alleles and generate near-homozygous parental lines (Cropano, Place, et al., 2021a).

A range of approaches to generate homozygous, or near homozygous, *L. perenne* lines has been explored. This includes double haploid induction that has been used to accelerate the breeding of crops such as maize and barley (Begheyn et al., 2016), along with breeding techniques that take the genetic SI mechanism into account. For example, one strategy involves selective crossing based on specific S and Z genotypes, leading to one-way compatibility (Pembleton et al., 2015). Although this approach can produce near-homozygous lines, in field situations, it is easily disrupted by pollen contamination introducing additional S and Z alleles.

Recent genetic characterization of the S and Z loci has also provided an opportunity to generate self-compatible (SC) ryegrass (Herridge et al., 2022). Both loci contain genes that encode two DUF247 proteins and a small pistil-expressed protein (SP or ZP), leading to a model where SP and ZP are secreted from the stigma and, upon interactions with matching DUF247 proteins on the pollen tube, form a complex that signals the arrest of pollen-tube growth (Herridge et al., 2022). Interestingly, most SC grass species have deletions or mutations within the S or Z loci genes (Herridge et al., 2022), including the SC darnel ryegrass (*Lolium temulentum* L.), which contains a frame-shift mutation within an S locus DUF247 gene (Manzanares et al., 2016). Thus, mutating the S or Z loci genes using gene editing tools could be a route to SC *L. perenne* capable of inbreeding (Herridge et al., 2020; Herridge et al., 2022). Such an approach has been undertaken in potatoes, where CRISPR/Cas9 was used to mutate a conserved region of the stigma SI component, enabling self-fertilization (Ye et al., 2018).

In addition, several European SC *L. perenne* populations have been identified (Arias Aguirre et al., 2014; Cropano, Manzanares, et al., 2021b; do Canto et al., 2018; Slatter et al., 2021). These lines contain a self-fertile (SF) locus that is separate from S or Z loci, mapping to either LG5 (Arias Aguirre et al., 2014; Cropano, Manzanares, et al., 2021b; do Canto et al., 2018) or LG6 (Slatter et al., 2021). The product of the SF loci acts in a dominant manner in the pollen, with pollen containing the SF locus being capable of fertilizing any female regardless of the S and Z alleles present. Although the identity of the

genes at the SF loci and their mode of action has yet to be characterized, the SF loci are another potential source of SC ryegrass.

Although these approaches may provide an avenue to SC ryegrass, a question remains whether inbreeding will be practical in a ryegrass F1 hybrid breeding programme. An increase in homozygosity is often associated with inbreeding depression, where a population loses fitness because of a loss of genetic diversity and an increased burden of harmful recessive alleles that would naturally be selected against (Birchler et al., 2010; García-Dorado, 2012). Severe inbreeding depression would prevent the implementation of F1 breeding, as a high level of reproductive fitness is required in the parental lines for commercial seed production. Although reductions in dry weight (Bean & Yok-Hwa, 1972), as well as germination and seedling survival (Jones & Jenabzadeh, 1981), have been reported in inbred perennial ryegrass, the impact of inbreeding depression appears variable within a population (do Canto et al., 2016), which may relate to the alleles present or differing levels of homozygosity.

Advances in genomic technology have enabled genome-wide analysis of non-model plants such as ryegrass. Although several whole genome assemblies have been reported (Byrne et al., 2015; Frei et al., 2021; Pfeifer et al., 2015), lower coverage techniques enable the analysis of a higher number of plants, with genotyping-by-sequencing (GBS) being a common method used for ryegrass (Faville et al., 2020). GBS provides a reduced representation of the genome through targeted digestion of restriction sites followed by size selection and sequencing. It provides a low-cost method enabling SNP discovery and genotyping of many samples, useful for assessing the relationship between different plants and genomic selection.

In this study, an SF locus was introgressed into elite germplasm followed by selfing, with GBS analysis being used to assess relatedness and calculate an inbreeding coefficient. Analysis after two rounds of selfing revealed phenotypic variation, with some vigorous plants showing a high level of homozygosity. This shows the viability of inbreeding ryegrass, assisted by phenotypic and genotypic selection, as a first step in F1 hybrid breeding. Our work also highlights the usefulness of performing a genomic assessment to monitor the degree of inbreeding.

2 | MATERIALS AND METHODS

2.1 | Crossing and selfing strategy to develop inbred perennial ryegrass (*Lolium perenne* L.) lines

Seeds from 18 SC lines containing the SF locus were obtained from Iowa State University of Science and Technology, USA, whereas elite plants were cv. 'Array' (Barenbrug). Plants for crossing were grown at the Barenbrug New Zealand experimental farm (Courtenay, Canterbury, New Zealand). All seedlings were germinated in seed sowing trays and then transferred to 20 cm-by-20 cm pots. Plants were exposed to either artificial vernalization (4°C cold room for 10 weeks; year 1) or natural vernalization over the New Zealand winter period (years 2 and 3).

Out-crosses and self-fertilization (selfing) were performed in an outdoor environment, either surrounded by wind-netting (year 1) or in a polytunnel (years 2 and 3). Crosses were performed by containing flowering heads from selected parents in pollen-proof bags constructed out of polyester lining material, whereas for selfing flowering heads from a single plant were placed in a pollen-proof bag. Twelve plants from each SC line were grown in rows adjacent to elite plants, and initial crosses between the SC and elite plants were conducted during the 2018/2019 summer (year 1) using adjacent plants that flowered at the same time. Seed heads (containing the F_1 seeds) were harvested only from the elite parent. Selfing of the F_1 plants giving F_1S_1 offspring occurred in the 2019/2020 (year 2) summer, with the F_1S_1 selfed to give F_1S_2 offspring in the summer of 2020/2021 (year 3). F_1S_2 plants were grown for GBS analysis and phenotype analysis in the summer of 2021/2022 (year 4). The SC plants were selfed each season giving S_2 , S_3 and S_4 generations.

2.2 | Genotyping-by-sequencing

GBS analyses were conducted on leaf material collected at Barenbrug NZ. Material for a first run was collected in year 3 and contained nine SC, nine elite, 31 F_1 and 75 F_1S_1 plants. Material for the second run was collected in year 4 and consisted of 10 elite plants (separate from the initial elite plants), 221 F_1S_2 and 34 S_4 plants. Leaf material was freeze-dried at -80°C before being transferred to AgResearch, Invermay, New Zealand. gDNA was extracted and digested with the restriction enzymes PstI and MspI. Sequencing libraries were prepared with a Pippin Prep (SAGE Science, Beverly, Massachusetts, USA) with fragments in the size range of 193–500 bp selected. Single-end sequencing was then performed on an Illumina HiSeq2500 utilizing v4 chemistry.

2.3 | Relatedness assessment

To assess population structure and relatedness, the Kindship with Depth adjustment (KGD) pipeline from AgResearch (<https://github.com/AgResearch/KGD>; 29) was applied to the GBS data. A reference-free variant calling approach was used to provide an unbiased representation of the whole genome that may be missed with a reference-based approach. HapMap files were made following the TASSEL 3.0 Universal Network Enabled Analysis Kit (UNEAK) pipeline.

2.4 | Calculation of the inbreeding coefficient

Fastq files from GBS were aligned to the Kyuss reference genome (27) using BWA-MEM, and variant calling was conducted using GATK to generate a gvcf containing all sequenced samples (Frei et al., 2021). Inbreeding coefficients were calculated per population using VCFtools to calculate SNP-based estimates and used the equation inbreeding coefficients = $(O - E)/(N - E)$, where O is the number of observed

homozygous sites, E is the expected number of homozygous sites and N is the total number of genotyped loci (Danecek et al., 2011). The inbreeding coefficients for each sample were grouped per population and plotted as a box plot using ggplot2 from the tidyverse package in R studio. Statistical differences between populations were calculated using ANOVA testing in base R.

2.5 | Phenotypic analysis

Phenotypic traits were measured in the F_1S_2 population at Barenbrug NZ, using standard phenotyping practices in year 4. Overall vigour was assessed by eye, the level of rust was measured on a scale of 0–6, leaf width was measured in millimetre and the heading date was measured relative to the average heading date for the cultivar ‘Nui’. The correlations between phenotypic measurements and inbreeding coefficients were calculated using Pearson's correlation coefficient (r).

3 | RESULTS

3.1 | Development of inbred perennial ryegrass lines

To assess the impact of inbreeding in ryegrass containing diverse and elite genetics, SC plants containing the *SF* locus on LG5 with a European background (Arias Aguirre et al., 2014; Cropano, Manzanares, et al., 2021b; do Canto et al., 2018) were crossed with elite plants from the cv. ‘Array’, and plants in subsequent generations inbred. Figure 1a illustrates the crossing and selfing strategy. In the first year, SC and elite plants were crossed by placing flowering heads from adjacent plants in pollen-proof bags (Figure 1b). As the *SF* locus acts in the pollen, seedheads were only harvested from the elite plants to avoid selfed-seeds. Seeds harvested from 10 plants produced F_1 offspring. In year 2, F_1 plants were selfed, and selfed-seed germinated to give 234 F_1S_1 (F_1 , selfed generation 1) offspring. Offspring from four families were further selfed in year 3, resulting in 241 F_1S_2 (F_1 , selfed generation 2) offspring, analysed in year 4.

The original European SC plants were also selfed for three generations (Figure 1a). Eighty-two S_2 (selfed generation 2) plants germinated from 20 original SC plants. These plants were selfed the following summer; however, only a few produced viable seeds with 16 S_3 (selfed generation 3) plants from two initial SC plants germinating. These plants were selfed again during year 3 producing 45 S_4 (selfed generation 3) plants all from one initial SC plant.

3.2 | Inbreeding was established in families

To gain a genetic overview of the families, genetic relatedness was determined based on GBS data (Dodds et al., 2015). Initially, this was performed in year 3 on the SC and elite plants used as parents for crosses along with F_1 plants and their selfed, F_1S_1 offspring, from

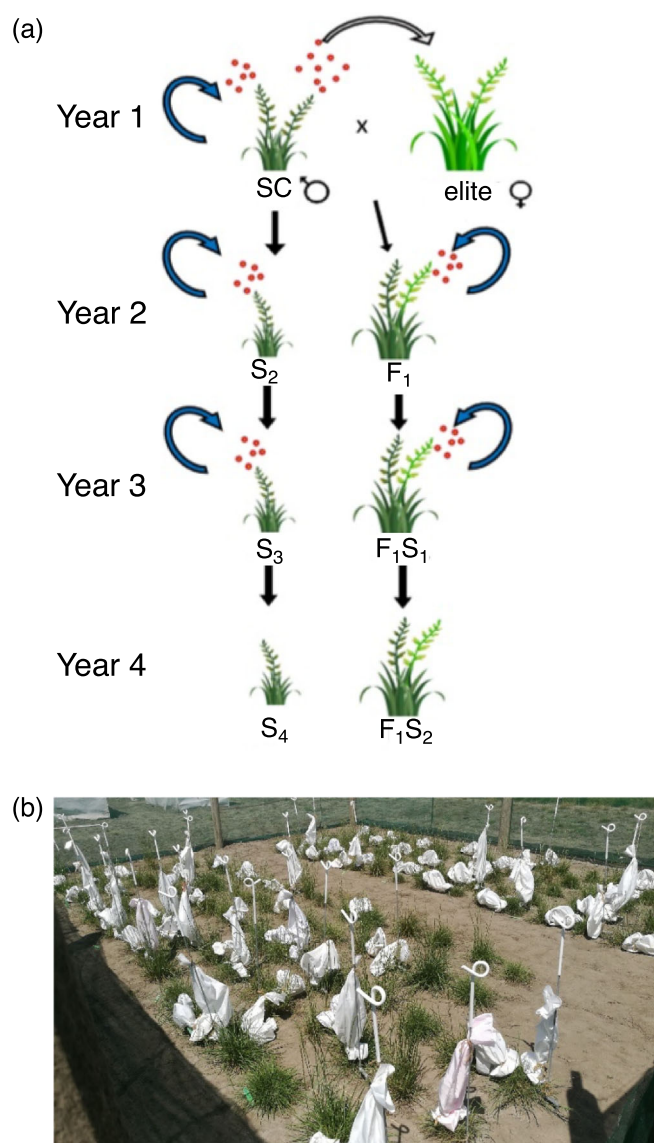


FIGURE 1 Crossing and selfing strategy. (a) self-compatible (SC) ryegrass plants were either selfed three times, giving selfed generations S_2 , S_3 and S_4 (left) or crossed with elite ryegrass plants (right). F_1 seeds were harvested from the elite plant and selfed for two generations giving F_1S_1 and F_1S_2 generations (right). Blue arrows represent selfing, and the grey arrow shows the first cross. (b) Crossing of SC and elite plants in year 1 with flowering heads from adjacent plants contained in pollen-proof bags

three initial crosses (each referred to as a family) (Figure 2a). The SC plants, including those who were a parent for the three families, clustered together with a red colour in the heatmap, showing they are closely related to each other. Additionally, the elite plants that were not a parent of the three families cluster together, but the lack of red in the heatmap suggests they are not closely related, which is not surprising given the outcrossing nature of ryegrass. On the other hand, the elite parent, the F_1 plants and the F_1S_1 offspring within each family cluster together, forming distinct red/orange squares in the heatmap. Thus plants within a family are more closely related to one

another than plants from other families. Furthermore, some closely related subgroups within the F_1 and F_1S_1 families (e.g., boxed regions; Figure 2a) contain an F_1 plant along with their direct F_1S_1 offspring. This indicates that selfing was occurring within the families and there was not widespread pollen contamination, a possibility with a wind-pollinated species such as ryegrass plants when grown outdoors despite the use of pollen-proof bags.

As inbreeding was occurring, the next generation of selfed plants, the F_1S_2 generation were also assessed for relatedness (Figure 2b). The analysis included an outcrossed group of 10 elite plants not used in the crosses, 33 of the S_4 plants descended from one SC plant and 221 plants from the four families that were taken to the F_1S_2 (this included family 1 in Figure 2a and three additional families). As expected, the S_4 plants and plants within each F_1S_2 family generally cluster together and show a high level of relatedness, and plants descended from the selfing of a single F_1 plant often cluster together, forming red boxes on the heatmap (Figure 2b). Family 6 and the S_4 plants are descended from the same SC plant and form a related cluster. However, there were a few cases where plants did not cluster with the expected groups. Two plants thought to belong to F_1S_2 families clustered most closely to the elite plants (Figure 2b), suggesting these two plants were a product of pollen contamination. Three F_1S_2 plants did not cluster with their expected families, as indicated by the coloured breaks in the familial blocks in Figure 2b. One of these F_1S_2 plants had the same SC parent as the S_4 plants and clustered within the S_4 family. As the other two F_1S_2 plants are not closely related to the plants they cluster with (family 5), it is likely that they are a result of mislabelling rather than pollen contamination. These plants were removed from further analyses. Overall, the strength of the relationships between parental plants and selfed offspring indicated that selfing had been largely successful in the families, and the *SF* locus had been successfully introduced into the population.

4 | THE LEVEL OF INBREEDING INCREASES IN EACH GENERATION

The reason to inbreed plants is to increase the level of homozygosity within the genome, thus fixing many alleles. To confirm that the inbreeding in each generation increased the level of homozygosity, the GBS data were used to calculate an inbreeding coefficient for each plant (Figure 3). The inbreeding coefficient measures the probability that two loci at a position are identical by descent using the Hardy–Weinberg equilibrium to compare the level of observed and expected heterozygous genomic positions for each plant within a generation (Crow, 1970; Slate et al., 2004). The greater the difference between observed and expected levels of heterozygosity (the higher the number of homozygous sites), the higher the inbreeding coefficient; a score of 1 indicates the sample is entirely homozygous, zero indicates that the sample is in Hardy–Weinberg equilibrium and a negative score indicates an excess of heterozygous alleles, most likely due to mapping errors arising from the GBS data.

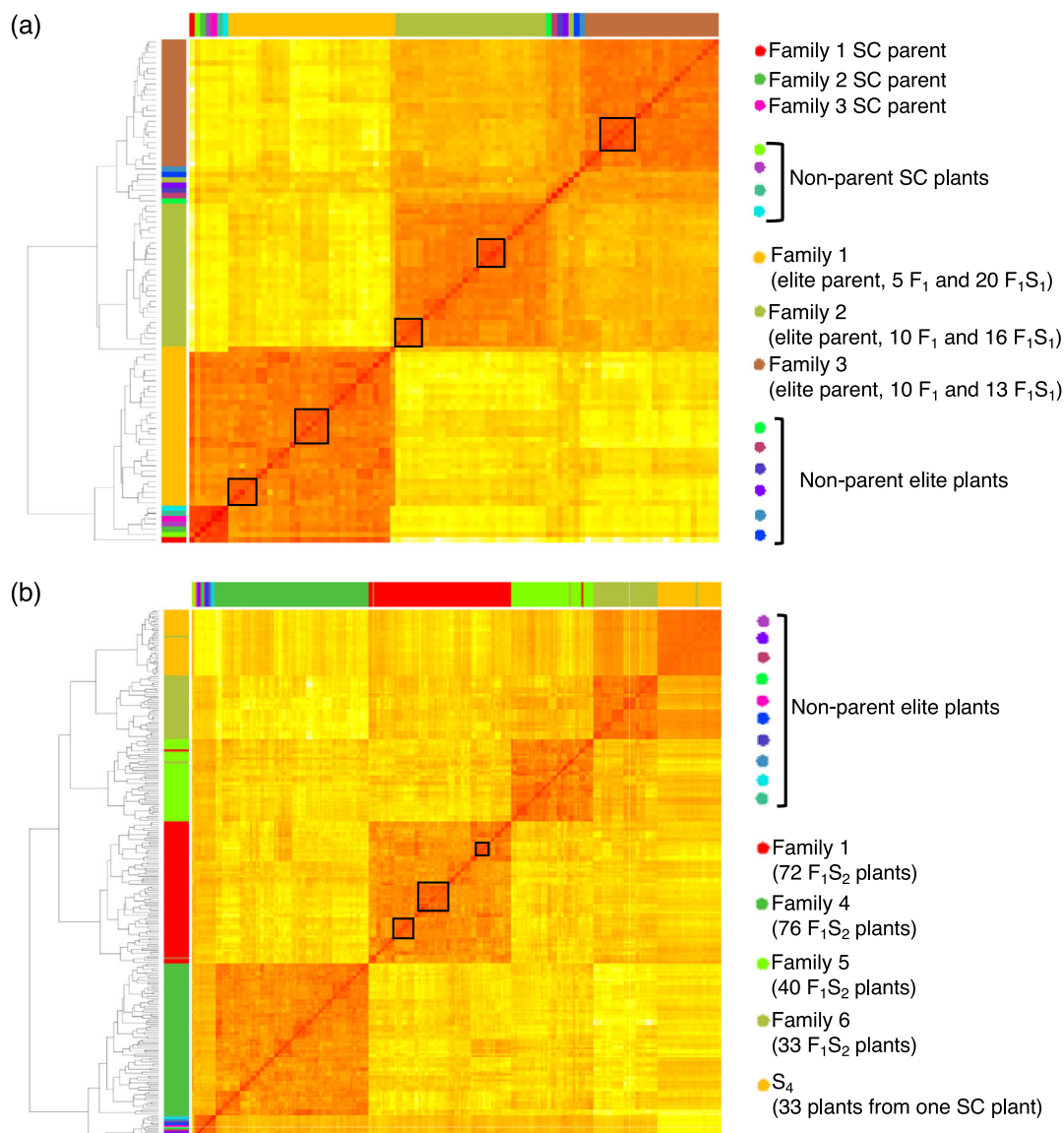


FIGURE 2 Relationship matrices of key inbred families. The relationship matrix was determined from genotyping-by-sequencing (GBS) performed in years 3 (a) and 4 (b). The plants included are indicated to the right, with individual plants from the inbred families (same self-compatible [SC] and elite parents) indicated by the same colour. Dark red on the matrices represents a high level of relatedness, and yellow a low level of relatedness. The tree on the X and Y axes indicates the relationships between the different plants. Black boxes indicate examples where an F_1 plant and its direct offspring show a close relationship [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

The elite and the SC plants used in the original crosses had mean inbreeding coefficients of 0.28 and 0.31, respectively, indicating that both populations are not highly inbred. Although this was not surprising for the outcrossing elite plants, it suggested that the SC plants had not been extensively inbred immediately prior to this work. As expected, given the difference in the background of the parents, the F_1 plants also had a low mean inbreeding coefficient of 0.27. There was a wide spread of values in the individual F_1 plants, including several plants with higher inbreeding coefficients (Figure 3). This could be due to the inherently low coverage of GBS, meaning that in some plants, sites could be called homozygous as only one allele has been sequenced at a sufficient level (Wang et al., 2020). Following each

round of selfing of the crossed material, there was a significant increase in the mean inbreeding coefficient in the subsequent generation, with mean inbreeding coefficients of 0.46 and 0.65 for the F_1S_1 and F_1S_2 plants, respectively. Thus, there was an increase in the level of homozygosity in each generation, confirming that inbreeding was occurring.

There was also a significant increase in the inbreeding coefficients in S_4 plants compared with the SC plants, with a mean inbreeding coefficient of 0.47 (Figure 3). This value was lower than for the F_1S_2 plants with one less round of selfing. This, along with the ability of only one family of SC plants to survive to this generation, suggests that the SC plants may contain a high burden of deleterious alleles,

and only those plants with high proportions of heterozygous alleles maintained sufficient levels of fitness to survive. This suggests that the addition of elite genetics, and material adapted to New Zealand, may provide beneficial alleles that enable a higher degree of homozygosity with fewer deleterious effects in a New Zealand environment.

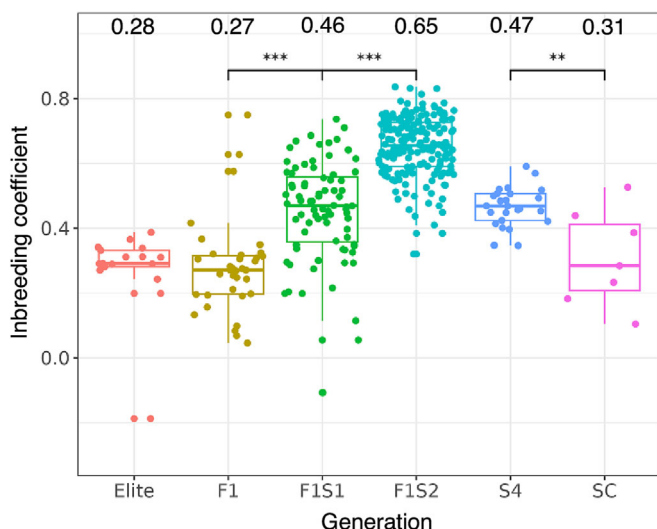


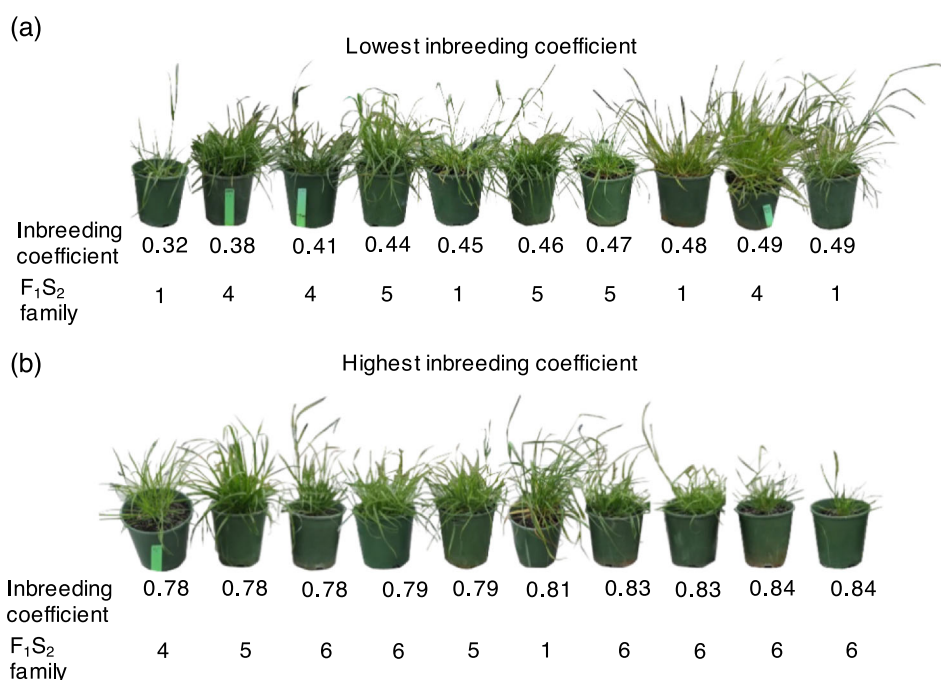
FIGURE 3 Inbreeding coefficients for plants from each generation. Inbreeding coefficients were determined from genotyping-by-sequencing (GBS) data. A score of 1 indicates 100% homozygous genomic sites while a score of zero indicates that the sample is in the Hardy–Weinberg equilibrium. Box plots show a median with the 25th and 75th quartiles. Means are indicated above each boxplot. *** ($p < 1 \times 10^{-7}$) and ** ($p < .02$) indicate a significant difference between the generations (ANOVA testing)

5 | INBRED RYEGRASS PLANTS CAN MAINTAIN VIGOUR

The inbreeding coefficients indicated that plants in the F_1S_2 generation were inbred, with some variation in the degree of homozygosity (Figure 3). A key aspect of being able to use inbreeding to generate parental lines for F_1 hybrid seed is that plants have high levels of homozygosity while also being sufficiently vigorous to produce large quantities of seeds. We therefore conducted phenotypic analysis on the F_1S_2 plants and compared the phenotype to the inbreeding coefficient (Figure S1). When leaf width, heading date and rust level were measured, there was no correlation between the phenotypic traits measured and the overall inbreeding coefficient. Thus, a higher level of homozygosity was not uniformly negatively impacting the performance of the plants.

We then compared the overall morphology of the 10 F_1S_2 plants with the lowest inbreeding coefficient (least number of homozygous genetic regions) and the 10 plants with the highest inbreeding coefficient (greatest number of homozygous genetic regions; Figure 4). In both cases, there was a range of phenotypes, with some plants showing a minor overall habit, with fewer, shorter leaves, whereas others were more vigorous and produced more, longer leaves. Thus, there is not a clear distinction in phenotypes between the two groups of plants despite having significantly different inbreeding coefficients. Additionally, plants from all four different F_1S_2 families were represented among the various groups (most/least inbred, and most/least vigorous). This indicated that differences in inbreeding coefficient and phenotype likely relate to the alleles that are homozygous in these plants rather than the initial parents. As the inbred plants from different families are likely fixed for different alleles, this indicates that selfing in ryegrass can generate genetically distinct, vigorous inbred plants.

FIGURE 4 Phenotypic assessment of the F_1S_2 plants. The 10 plants with the lowest (a) and the 10 plants with the highest (b) inbreeding coefficients. All plants were photographed at the same time in year 4. The inbreeding coefficient and the family each plant belongs to are indicated below each plant. The inbreeding coefficients between the lowest and highest groups are significantly different (Wilcox test, $p = 1.4e^{-10}$)



6 | DISCUSSION

Developing an F1 hybrid breeding strategy is a major long-term goal of the ryegrass industry to significantly improve genetic gains and cultivar uniformity (Chattopadhyay, 2017; Herridge et al., 2020; Woodfield, 1999). As a first step, F1 hybrid breeding requires the generation of genetically distinct parental lines that are largely homozygous. Crossing such lines to generate F1 hybrids enables both the benefits of heterosis, along with producing offspring with consistent and predictable performance. Inbreeding is commonly used to generate parental lines in other crops such as wheat, rice and maize, but as ryegrass is usually SI, inbreeding has yet to be used in commercial breeding programmes. In this work, we have used SC ryegrass containing an SF locus (Arias Aguirre et al., 2014; Cropano, Manzanares, et al., 2021b; do Canto et al., 2018), elite ryegrass germplasm and genetic and phenotypic analyses to show that inbreeding is a viable route to the generation of near homozygous parental lines for commercial F1 seed production in ryegrass.

To use inbreeding to generate near homozygous parental lines for F1 hybrid breeding, it is essential that inbreeding depression is not severe, as parental lines need to have sufficient vigour and reproductive fitness (Birchler et al., 2010; García-Dorado, 2012). In this study, inbreeding depression was observed within the SC germplasm, with only one line out of 20 generating plants after three rounds of self-fertilization (the S₄ generation), although the fitness of the SC plants may also have been impacted by a lack of adaptation to the New Zealand environment (the SC plants did not have the same longevity as the elite plants). Notably, the mean inbreeding coefficient for the S₄ generation (0.47) was much lower than the expected value (0.86) for a population that has been selfed for three generations (Chattopadhyay, 2017). Although the low sample number may impact this value, it could also indicate that only plants with lower levels of homozygosity were sufficiently fit to be able to reach the S₄ stage.

The introduction of a broader genetic base and importantly a base adapted to the local conditions, through crossing elite plants with the SC plants, appears to provide some protection against inbreeding depression. Over two rounds of selfing, the mean inbreeding coefficient increased significantly, indicating an increase in the proportion of homozygous genetic regions. Notably, the values obtained (0.46 and 0.65 in the F₁S₁ and F₁S₂, respectively) were only slightly below the predicted levels (0.50 and 0.75, respectively) (Chattopadhyay, 2017) and were higher for the crossed plants after two rounds of selfing compared with the SC material after three rounds of selfing. Importantly, a number of vigorous plants were present in the F₁S₂ population, including plants with high inbreeding coefficients. Thus, producing inbred ryegrass lines with sufficient vigour appears possible in ryegrass. However, as the level of inbreeding depression varies, presumably depending on which combination of alleles becomes homozygous, selecting the best-performing plants with desired traits will be necessary. The crossing, inbreeding and selection process should also be repeated as the introduction of new genetics will likely be important for further improvement, as observed with the continued improvements in maize F1 hybrids (Byers & Waller, 1999; Charlesworth, 2006).

Although phenotypic vigour is an easy selection method, there is the potential to be selecting plants with a lower level of homozygosity that is buffering against inbreeding depression (as may have occurred in the S₄ population). In addition, in an outdoor breeding environment low level pollen contamination is possible and may result in the selection of outcrossed plants, which are likely to show high levels of vigour because of heterosis. Here, we show that GBS is an economical method to support phenotypic selection. By calculating the inbreeding coefficient, plants that are both vigorous and have higher levels of homozygosity can be selected. Further, the relationship between plants can be used to confirm parentage so plants that result from pollen contamination can be removed. Information about the relationship will also aid in selecting genetically distinct parental lines. There is a strong correlation between the amount of genetic difference in the parental lines and the degree of heterosis observed in the offspring, likely due to a lack of interacting dominant genes, which often reduces heterosis (Boeven et al., 2020; Lin et al., 2016). Additionally, using a combination of phenotypic and genetic selection, parental lines can be selected for specific traits, with evidence that improving parental lines leads to increased performance of F1 hybrids (Duvick, 1999; Hochholdinger & Baldauf, 2018; Virmani et al., 1982).

To inbreed plants, this work relied on the presence and function of an SF locus that overcomes the SI mechanism in ryegrass (Arias Aguirre et al., 2014; Cropano, Manzanares, et al., 2021b; do Canto et al., 2018). Using an SF locus may be suitable for commercial breeding, as it has the advantage of producing large populations, enabling the selection of plants. This selection can also occur at each generation, incrementally removing deleterious alleles from the population. This contrasts with other techniques, such as double haploid methods that generate fully homozygous lines in one step (Begheyn et al., 2016). This one-step increase in homozygosity can result in a high burden of inbreeding depression as multiple recessive alleles are fixed in one step, and the low number of plants that can be easily produced through double haploids may limit selection. However, introducing the SF locus into elite material via crossing also introduces genes from the SC plants that may reduce fitness for the local environment or agronomic performance. Although some of these alleles will likely be removed by selection, performing further backcrossing prior to inbreeding to reduce the SC genetic contribution would be advisable. Alternatively, identifying the SI components in ryegrass (Herridge et al., 2022) may enable tools such as CRISPR-Cas site-directed mutagenesis to introduce self-fertility into desired elite plants directly.

The development of ryegrass lines able to self-fertilize over several generations could be incorporated immediately into breeding programmes to purge deleterious alleles. However, the development of an F1 hybrid ryegrass seed would still require the outcrossing of the now self-fertile parental lines. Several strategies have been developed within other self-fertile crops such as maize, rice and wheat that could be adopted to ryegrass (Herridge et al., 2020). This could include methods that use cytoplasmic male sterility (CMS) (Havey, 2004), which has been observed but not well utilized in ryegrass (Islam et al., 2014). The “Seed Production Technology” system developed

for maize (Wu et al., 2016) and adopted for rice (Chang et al., 2016) is a biotechnological approach for reversible male sterility that could also be used in ryegrass. This involves complementing a male-sterile mutant with a transgene during the inbreeding stages using a genetic construct that can be removed via segregation. Male-sterile plants without the complementing construct can then be used as the female for F1 hybrid seed production to ensure outcrossing.

In summary, our research shows that using SC ryegrass germplasm as a starting point for developing inbred ryegrass cultivars is a viable approach and that GBS is a valuable tool to measure the extent of inbreeding.

AUTHOR CONTRIBUTIONS

Caitlin Harris, Ruby Arrowfield and Madison Hall grew plants, performed crosses and self-fertilizations and conducted the phenotypic analysis of the plants; Caitlin Harris and Rowan Herridge performed bioinformatic analysis; Caitlin Harris, Colin Eady, Rowan Herridge, Richard Macknight and Lynette Brownfield designed experiments, analysed the data and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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