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Multi-model GWAS reveals key loci for horticultural traits in reconstructed garden strawberry

Attiq ur Rehman[1,2] | Terhi Iso-Touru[1] | Jakob Junkers[3] | Marja Rantanen[1] | Saila Karhu[1] | Daniel Fischer[1] | Muath Alsheikh[4,5] | Stein Harald Hjeltnes[6] | Bruno Mezzetti[7] | Jahn Davik[3] | Alan H. Schulman[1,8,9] | Timo Hytönen[8,10] | Tuuli Haikonen[1,8]

[1]Natural Resources Institute Finland (Luke), Finland

[2]Doctoral Program in Plant Sciences, University of Helsinki, Finland

[3]Norwegian Institute of Bioeconomy Research, Ås, Norway

[4]Graminor AS, Norway

[5]Faculty of Life Sciences, Department of Plant Sciences, Norwegian University of Life Sciences, Ås, Norway

[6]Njøs Fruit and Berry Centre AS, Norway

[7]Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy

[8]Viikki Plant Science Centre, Finland

[9]Institute of Biotechnology, University of Helsinki, Finland

[10]Department of Agricultural Sciences, University of Helsinki, Finland

Correspondence
Tuuli Haikonen,
Email: tuuli.haikonen@luke.fi

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Abstract

The cultivated garden strawberry (*Fragaria* × *ananassa*) has a rich history, originating from the hybridization of two wild octoploid strawberry species in the 18th century. Two-step reconstruction of *Fragaria* × *ananassa* through controlled crossings between pre-improved selections of its parental species is a promising approach for enriching the breeding germplasm of strawberry for wider adaptability. We created a population of reconstructed strawberry by hybridizing elite selections of *F. virginiana* and *F. chiloensis*. A replicated field experiment was conducted to evaluate the population's performance for eleven horticulturally important traits, over multiple years. Population structure analyses based on Fana-50 k SNP array data confirmed pedigree-based grouping of the progenies into four distinct groups. As complex traits are often influenced by environmental variables, and population structure can lead to spurious associations, we tested multiple genome-wide association study (GWAS) models. GWAS uncovered 39 quantitative trait loci (QTL) regions for eight traits distributed across twenty chromosomes, including 11 consistent and 28 putative QTLs. Candidate genes for traits including winter survival, flowering time, runnering vigor, and hermaphrodism were identified within the QTL regions. To our knowledge, this study marks the first comprehensive investigation of adaptive and horticultural traits in a large, multi-familial reconstructed strawberry population using SNP markers.

1 | INTRODUCTION

The cultivated garden strawberry (*Fragaria* × *ananassa*) is an allo-octoploid species (2n = 8x = 56), which first emerged in the early 1700s as the result of spontaneous hybridization between two octoploid species, *Fragaria virginiana* and *Fragaria chiloensis* in Western Europe. This hybridization happened shortly after *F. chiloensis* was brought to France from Chile in year 1714; it was originally discovered by French botanist Antoine Nicolas Duchesne (1747–1827) (Duchesne, 1766; Darrow, 1966). The phenotypic superiority of these

early interspecific hybrids over their octoploid parents for various horticultural traits became the main driver of the *F.* × *ananassa* domestication. The first systematic breeding of strawberries was started in 1817 in England (Darrow, 1966). Similar breeding work was initiated in North America during the mid-1800s with a restricted group of European *F.* × *ananassa* cultivars and native *F. virginiana*, later also including North and South American *F. chiloensis* (Darrow, 1966). This initial germplasm resource played a major role in many public and private breeding programs for the next century (Pincot et al., 2021).

Genetic diversity has been the basis of crop improvement since the earliest days of plant breeding because it increases the possibilities of identifying sources for superior traits and better climatic adaptation (Swarup et al., 2021). In general, because crop wild relatives have not faced the strong bottleneck of domestication and breeding, they can exhibit valuable traits not found among cultivars and contribute adaptations to diverse environmental conditions (Renzi et al., 2022). Strawberry improvement by backcrossing *F.* × *ananassa* with its wild parental species has thus far re-introduced new sources of important traits, including perpetual flowering, resistance to aphids and red stele, tolerance to drought and salinity, and winter-hardiness (Barritt and Shanks, 1980; Sjulin and Dale, 1987; Galletta et al., 1989; Daubeny, 1990).

For expanding the adaptive and yield trait diversity within the *F.* × *ananassa* gene pool, a two-step strawberry reconstruction approach was introduced and implemented by Hancock et al. (2010): first, desirable trait combinations of each parental species were created by intraspecific crosses; second, the superior individuals from these progenies were selected and used for interspecific reconstruction crosses. This method creates a much higher adaptive diversity than traditional backcrossing since the second step reconstruction crosses can utilize different parallel combinations of highly heterozygous selections from the first step, providing a genetically wide pool for selecting desirable individuals (Hancock et al., 2010; Stegmeir et al., 2010).

Extensive collection and preservation of wild and landrace *F. virginiana* and *F. chiloensis* germplasm has been carried out in North and South America to increase the available genetic diversity (Luby et al., 1992; Cameron et al., 1993; Dale et al., 1993; Luby and Stahler, 1993). Several thousand wild plants from collections were evaluated in previous studies (Dale et al., 1993; Hancock et al., 2001a, 2001b, 2002, 2003, 2005). Based on these evaluations, accessions representing a broad natural diversity and inheriting superior horticultural traits and strong vigor were identified and crossed to develop elite *F. chiloensis* and *F. virginiana* selections that subsequently were used to demonstrate the reconstruction approach (Dale et al., 1993; Luby et al., 2008; Hancock et al., 2010). Among the reconstructed families created, one exceptional family, FVC11 of pedigree (F 9 × LH 50–4) × (SC × 2 MAR 1A), was extensively evaluated for various strawberry traits (Stegmeir et al., 2010). A genome-wide association study (GWAS) was carried out to uncover genetic associations for the runner plant number, inflorescence number, inflorescence height, crown production, flower number, fruit size, yield, internal color, soluble solids, fruit firmness, and plant vigor (Hancock et al., 2016).

Despite the potential of reconstructed progenies for breeding (e.g., for new environments), the wide-scale integration of reconstructed materials, particularly in conjunction with newly available genomic tools, has remained limited. To bridge this research gap, our study aimed to: (1) create a diverse breeding resource through a crossing schema with multiple reconstruction crosses; (2) evaluate the adaptive performance of the newly created population for horticulturally important traits; (3) adjust subsequent genetic analyses for any pedigree-derived population structure; (4) potentiate the integration of reconstructed materials into breeding by identifying genomic regions and markers for horticulturally important traits.

To this end, we established a diverse multiparental population of Re-Constructed (ReC) strawberry by crossing several previously improved *F. virginiana* and *F. chiloensis* elite selections. We evaluated it for a range of traits, including winter survival, plant and runnering vigor, flowering earliness, male and female fertility, fruit characteristics, and productivity. The genetic dissection of these traits was performed with a multi-model GWAS approach using Single-Nucleotide Polymorphisms (SNPs) from Fana-50 k Affymetrix array (Hardigan et al., 2020). We identified 39 QTLs for eight traits and highlighted promising candidate genes for future breeding use and research.

2 | MATERIALS AND METHODS

2.1 | Plant material and field trial

Clones of *F. virginiana* and *F. chiloensis* superior individuals selected from intraspecific crosses in USA (Hancock et al., 2005, 2010) were sent to Norway (Bioforsk, Jahn Davik) in 2006 (Table S1). Interspecific reconstruction crosses were made to create 13 biparental families, named PPPS_01 to PPPS_13 (Figure 1). The pedigree connections were visualized using Helium (Shaw et al., 2014) and an accessible color scheme, "light" (Tol, 2021). In all families except one (PPPS_08), *F. chiloensis* served as the mother and *F. virginiana* as the father (Table S2). The PPPS_08 family was created by crosses of wild (RH 30) and landrace (CFRA 24) accessions having no known prior improvement, while the PPPS_02 family was created by a cross between a complex improved hybrid (RC-8) and a wild clone (F 9).

The seeds were sent to the Natural Resources Institute Finland (Luke). In total, 319 F1 seedlings were germinated, propagated through runners, and subsequently planted in a field experiment at Luke's Piikkiö Horticultural Research Station in Kaarina, southwest Finland (60°23′ N, 22°33′ E) in August 2019. Raised beds with black plastic mulch and a trickle irrigation line (spacing of emitters 20 cm) were used with 40 and 140 cm spacing between plants and beds, respectively. Two replicated plots with four clonal plants per plot were established, except for nine individuals present only in Replicate 1 (Figure S1). A control panel of commercial cultivars (four plants each) was planted within each of the 22 rows: 'Lumotar', 'Ria', 'Korona', 'Polka', and 'Honeoye'. Plots with additional controls 'Senga Sengana' and 'Kent' were included in at least one row in both

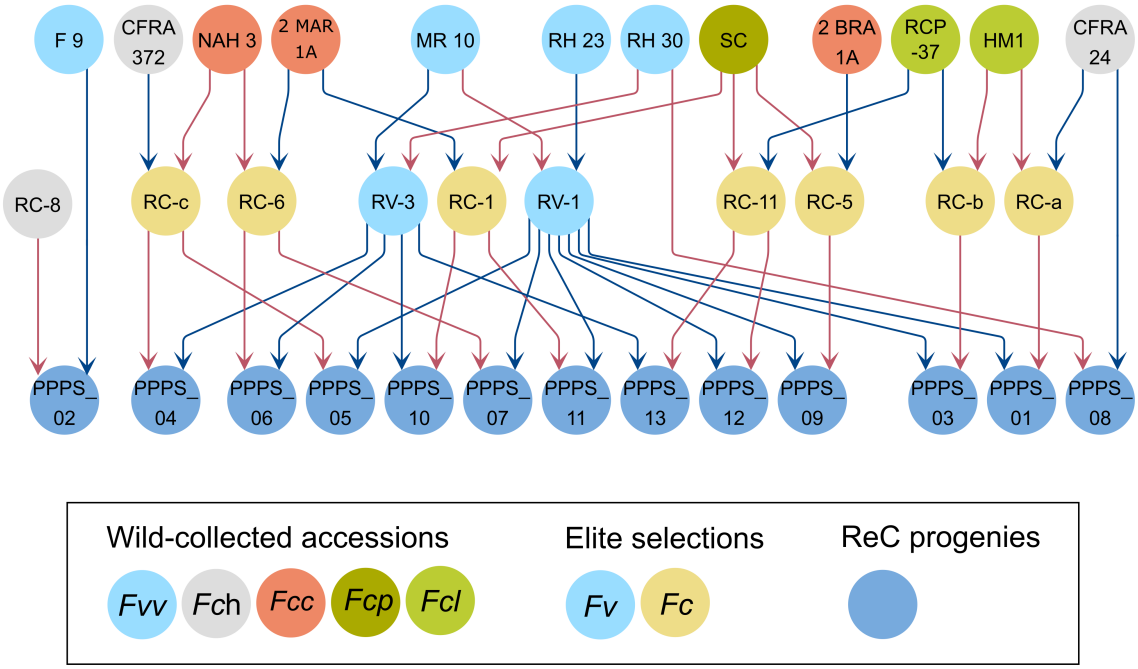

FIGURE 1 Pedigree of the reconstructed strawberry (ReC) population. Blue arrows indicate a male parent, while red arrows indicate a female parent. The uppermost colored circles represent grandparental founder accessions, while the middle circles with RC and RV represent improved elite selections of *Fragaria chiloensis* and *Fragaria virginiana*, respectively. Bottom-most circles represent progenies (PPPS) from 13 reconstruction cross combinations between the two parental species. Acronyms: *Fcl*, *Fragaria chiloensis* sp. *lucida*; *Fcc*, sp. *chiloensis*; *Fcp*, sp. *pacifica*; *Fch*, *F. chiloensis* hybrid with admixed taxonomy; *Fvv*, *F. virginiana* sp. *virginiana*.

replicates. To minimize the border effect, guard rows were established on each side of the experiment and a guard plant at the end of each bed.

Standard local recommended nutrient levels for strawberry according to soil nutrient analysis and soil type (clay loam with 6–12% organic matter) were maintained by fertigation at key growth phases of each season: main flowering; fruit ripening; after fruiting. Integrated pest management was implemented during the three-year trial period. Outside the growing seasons, the plants were protected by a nonwoven polypropene floating row cover (23 g m^{-2}).

2.2 | Phenotypic evaluation and data analysis

Eleven traits were recorded in 2020–2022 using the descriptors agreed to by the European network for strawberry cultivar evaluation within the COST Action 836 programme (Navatel and Krúger, 2004) after modifications (Table 1). Male fertility was assessed as a binary trait, and a four-point scale for female fertility was modified from Goldberg et al. (2010). Data visualizations, descriptive statistics and Pearson's correlation coefficients between the traits were computed in R software using custom script or MVApp (Julkowska et al., 2019; R Core Team, 2020). A Linear Mixed Model (LMM) was developed to control spatial and environmental variation of the quantitative traits and Best Linear Unbiased Predictor (BLUP) values (Dataset S1) were extracted using the *lme4* R-package (Bates et al., 2014). The genotype, control cultivars, year, replicate, row, and column were treated as random variables in the LMM model. Additionally, the interactions of these variables with replicate, row, and column were also included as random variables. Broad-sense heritability (H^2) was calculated by the method of Cullis (Cullis et al., 2006) in the *lme4* R-package. Before conducting GWAS for plant vigor, individuals with low winter survival (<25%) were removed; to carry out GWAS for productivity, staminate and weakly hermaphroditic individuals were removed.

2.3 | Genotyping

Young, non-expanded leaves were sampled (~50 mg/sample) in 96-well DNA extraction racks (Corning® 96 well PP 1.2 mL cluster tubes), frozen at −80°C, and freeze-dried. DNA was extracted and samples were genotyped with the Axiom TM Strawberry FanaSNP 50 k Genotyping Array at the SGS Institut Fresenius GmbH, Trait Genetics Section, Gatersleben, Germany. Genotypes were assigned to the samples with the R-package *fitPoly* (Voorrips et al., 2011). After genotype assignment, the SNP calls for the 50 K loci were filtered based on minor allele frequency (MAF) < 0.01, resulting in a final set of 20,779 SNPs that were used for the subsequent GWAS. We used the subgenome- and haplotype-resolved Royal Royce V1 reference genome for physical positions and chromosome (chr) nomenclature (Hardigan et al., 2021a) (Dataset S2). For population structure analyses, an LD-pruned SNP dataset (2615 SNPs, Dataset S2) was also generated by applying LD threshold of 0.2 in *SNPRelate* package in R (Zheng et al., 2012).

TABLE 1 Plant and harvest traits phenotyped in the ReC strawberry population field trial. Observations were done per plot except for winter survival where plot averages were calculated from plant-wise scores.

Trait	Phenotyping years	Trait evaluation	GWAS Input
Winter Survival (WS)	2021, 2022	Percentage (%) of surviving crown branches of all crown branches per plant.	BLUPs
Plant Vigor (PV)	2021, 2022	Categorical scale 1–9 with low scores corresponding to weak and high scores to vigorous plants.	BLUPs
Male Fertility (MF)	2020, 2021, 2022	Binomial scale 0–1 with poor or no stamens (0); normal stamens with anthers and pollen (1).	Raw scores
Female Fertility (FF)	2020, 2021, 2022	Categorical scale 0–3 with no fruit developed (0); enlarged receptacle with severe malformations (1); 5–10% fruit set with a few primary and rarely secondary flowers producing fruits (2); regular fruit set (3).	Raw scores
Runnering Vigor (RV)	2021, 2022	The average number of runners per plant. Categorical scale 0–5 with less than 4 (0); 5–10 (1); 10–20 (2); 30–40 (3); 40–50 (4); more than 50 runner chains per plant (5). The observation was done thrice in 2021 and once in 2022.	BLUPs
Flowering Time (FT)	2021, 2022	Categorical scale 0–5 with very early (1); early (2); mid (3); late (4); very late (5). Categories were formed based on 15 (in 2021) and 14 (in 2022) bi-weekly observations of the number of plants with open flowers per plot.	BLUPs
Berry Color (BC)	2021	Categorical scale 1–3 with red (1); pink (2); white (3).	Raw scores
Berry Size (BS)	2021	Size classification of primary berries: Diameter of >25 mm (1); 18–25 mm (2); <18 mm (3).	Raw scores
Berry Neck shape (BN)	2020, 2021	Categorical scale 0–2 with no neck (0); short-necked shape (1); long-necked shape (2).	Raw scores
Berry Appearance (BA)	2020, 2021	Categorical scale 1–5 with very unattractive (1); unattractive (2); both unattractive and attractive types present (3); attractive (4); very attractive (5).	Raw scores
Productivity (PR)	2021, 2022	Categorical scale 1–9 with low scores corresponding to low and high scores to high yield.	BLUPs

2.4 | Marker distribution, linkage disequilibrium and population structure

The density of SNPs along the chromosomes was visualized with a 1 Mb window size using *rMVP* package in R (Yin et al., 2021). The pair-wise linkage disequilibrium (LD) between SNP markers was calculated as r^2 in TASSEL version 5.0 (Bradbury et al., 2007). Genome-wide and chromosome-wise LD was calculated with the full matrix option, which considers a complete set of SNP markers present on each chromosome to calculate respective r^2 values. The maximum and half decay were estimated as physical distance and an LD decay curve was fitted with a smoothing spline regression line (Hill and Weir, 1988; Remington et al., 2001). Results were plotted by using a custom R-script.

Population structure was investigated by a Bayesian model-based quantitative assessment of the LD-pruned dataset in the STRUCTURE software version 2.3.4 (Pritchard et al., 2000) using 10,000 as the burn-in iteration followed by 50,000 Markov-chain Monte Carlo replications, assuming an admixture model for eight (K = 1–8) hypothetical subpopulations. Three independent analyses were performed for each K-value. The most reasonable K value was identified by using a parsimony index in KFinder (Wang, 2019) (K = 4). A comparison was also performed with the K values described by Evanno's methods (Evanno et al., 2005) (K = 2). Structure bar-plots were constructed using Structure Plot v2.0 (Ramasamy et al., 2014). Expected Heterozygosity (He) (Nei and Roychoudhury, 1974), and Fixation index (Fst) (Weir and Cockerham, 1984) values for each subpopulation were derived from the STRUCTURE results.

Population structure was further examined by using Principal Component Analysis (PCA), Neighbor-Joining (NJ) phylogenetic analysis, and Kinship (K) matrix calculation in Genome Association and Prediction Integrated Tool (GAPIT) version 3.0 (Wang and Zhang, 2021). PCA plots were created with *ggplot2* package in R (Wickham, 2016).

2.5 | Genome-wide association analysis

To identify Marker-Trait Associations (MTAs), while considering the varying genetic complexity of the studied traits, we conducted GWAS using a single-locus Mixed Linear Model (MLM) and two multi-locus models, FarmCPU (Fixed and Random Model Circulating Probability Unification) and BLINK (Bayesian-Information and Linkage-Disequilibrium Iteratively Nested Keyway), executed in GAPIT version 3.0 (Wang and Zhang, 2021). MLM integrates both population structure as a fixed effect and individual relatedness as a random effect to enhance control over false positive variants. We included the first five principal components (Figure S2) and a

kinship matrix (VanRaden, 2008) to account for covariance between population structure and phenotype (Yu et al., 2006). The FarmCPU model iteratively uses both random and fixed models to estimate pseudo-quantitative trait nucleotides and uses them as covariates when testing marker associations by binning procedure (Liu et al., 2016), whereas the BLINK model replaces the binning method of FarmCPU with linkage disequilibrium to increase statistical power and to decrease the computation time (Huang et al., 2019). For all the tested GWAS models, we applied a conservative Bonferroni threshold of $-\log10(p) = 5.61$ to control the false discovery rate and identify significant MTAs (Benjamini and Hochberg, 1995). To calculate the Phenotypic Variation Explained (PVE) we used the option "random.model = TRUE" in GAPIT v1.0 (Lipka et al., 2012).

2.6 | QTL assignment, allele stacking analysis, and candidate gene search

The physical distance (bp) at which the LD decayed to the critical value of $r^2 = 0.2$ was used as the threshold for assigning MTAs to distinct QTLs on each chromosome. QTLs were assigned unique names consisting of three components: a stem indicating the trait; the name of the population (Rec in our case) linked to the respective chromosome name in the Royal Royce genome assembly v1.0; an integer separating multiple QTLs on the same chromosome based on LD threshold. The markers from previously published studies in octoploid *Fragaria* spp. were re-mapped against the Royal Royce v1.0 reference genome using the nucleotide BLAST tool in Rosaceae.org.

The additive effect of positive alleles was determined based on the most significant marker in each identified QTL. The relationship between the number of favorable alleles and the corresponding variation in the phenotype BLUPs was analyzed using linear regression in the R software. Mosaic plots were produced using *vcd* package in R and the residual shading was applied after a Chi-square test of independence (Zeileis et al., 2007).

Gene searches were performed in the 'Royal Royce' reference genome version 1 (https://phytozome-next.jgi.doe.gov/info/FxananassaRoyalRoyce_v1_0) within the LD half-decay distance to top SNPs of QTLs consistent over tested GWAS models. The following criteria were used to choose candidate genes based on the obtained annotations: (i) genes or orthologs in *F.* × *ananassa* and *F. vesca* with functions associated with the trait of interest; (ii) homologs in Arabidopsis or other closely related plant species with functions associated with the trait.

3 | RESULTS

3.1 | Analysis of phenotypic data and trait correlations

The ReC strawberry population of 319 seedlings in 13 F1 progenies (PPPS_01 to PPPS_13) was created by interspecific crossings between seven *F. chiloensis* and two *F. virginiana* improved elite selections (Hancock et al., 2005, 2010), two superior wild *F. virginiana* accessions (F 9 and RH 30), and two hybrids with earlier reconstruction or admixed backgrounds (RC-8 and CFR 24) (Figure 1, Tables S1, S2). We evaluated clonally propagated ReC F1 individuals in a replicated field experiment focusing on 11 adaptive and horticultural traits [winter survival (WS), plant vigor (PV), flowering time (FT), runnering vigor (RV), male fertility (MF), female fertility (FF), berry color (BC), berry size (BS), presence or absence of berry neck (BN), overall berry appearance (BA), and productivity (PR), Table 1]. The ReC population showed a wide range of phenotypic variation for most traits, highlighting its potential for genetic studies and breeding (Table 2, Figures 2 and S3). Winter survival in the ReC population averaged 91% after the second winter and 64% after the third winter of the field trial. Among the surviving plants, a positive correlation between the phenotyping years was found for the quantitative traits PV, FT, RV, and PR (Figure 2), indicating reasonable stability for the multi-year phenotypic data. The genotype-by-year effect was also found to be significant for these traits (Table 2) and, together with other environmental factors, was included in the statistical model that was applied to calculate adjusted phenotypic values, Best Linear Unbiased Predictors (BLUPs), from the raw phenotypic data.

Pearson's correlation analysis suggested weak associations ($p < 0.01$) between winter survival and plant vigor ($r = 0.29$), runnering vigor ($r = 0.28$), and productivity ($r = 0.36$). Plant vigor was moderately correlated ($p < 0.01$) with runnering vigor ($r = 0.43$) and weakly with productivity ($r = 0.25$), but productivity was moderately correlated with runnering vigor (0.42). Furthermore, a moderate negative association ($p < 0.01$) was found between runnering vigor and flowering time ($r = -0.48$) (Figure S4).

The ReC population consisted of 75.5% male-fertile and 23.5% male-sterile individuals. For the remaining 1% (n = 3), sex determination was not possible due to the absence of inflorescences (Figure 3A). Female fertility was assessed as a categorical trait, determined by fruit set. Among the ReC individuals, 6.6% were classified as infertile (score 0), while the majority (80.6%) had normal berry development (score 3). The remaining 12% displayed rare fruit set or severe fruit malformations, as indicated by scores 1 and 2 (Figure 3B). The ReC individuals were therefore categorized into three floral sexual phenotypes: staminate (n = 17) for male-fertile individuals with a female fertility score of 0; hermaphrodite (n = 223) for male-fertile individuals with a score of 1 or higher for female fertility; pistillate floral (n = 76) for male-sterile individuals (Figure 3C). The proportions of these floral phenotypes varied between progenies (Figure 3D).

3.2 | Population structure and linkage disequilibrium

We utilized the Strawberry Fana-50 k SNP array originally developed from genomic data for *F.* × *ananassa*, *F. virginiana* and *F. chiloensis* accessions (Hardigan et al., 2020) and obtained 20,779 high-quality SNPs for 298 ReC individuals. This resulted in an average SNP density

TABLE 2 Descriptive statistics for five quantitative traits recorded in the ReC population. The number of observed ReC individuals (N) is followed by the number of observed replicate field blocks in the parentheses, while other acronyms refer to Standard Deviation (SD), Coefficient of Variation (CV), and broad-sense Heritability (H^2).

Trait	Year	N	Mean	SD	Range	CV	Genotype	Genotype × Year	H^2
Winter Survival (%)	2021	319 (1)	90.62	16.34	0–100	0.18	$p < 0.01$	$p < 0.01$	0.55
	2022	318 (1)	64.26	24.18	0–97	0.38			
Plant Vigor (1–9)	2021	627 (2)	5.88	1.57	1–9	0.27	$p < 0.01$	$p < 0.01$	0.83
	2022	532 (2)	6.35	1.30	2–9	0.21			
Flowering Time (1–5)	2021	720 (2)	2.92	0.94	1–5	0.32	$p < 0.01$	$p < 0.01$	0.79
	2022	346 (1)	2.68	1.20	1–3	0.45			
Runnering Vigor (0–3)	2021	736 (2)	1.63	0.80	0–3	0.49	$p < 0.01$	$p < 0.01$	0.78
	2022	735 (2)	1.40	0.83	0–3	0.59			
Productivity (1–9)	2021	616 (2)	3.35	1.60	1–8	0.48	$p < 0.01$	$p < 0.01$	0.73
	2022	348 (1)	4.36	1.81	1–9	0.41			

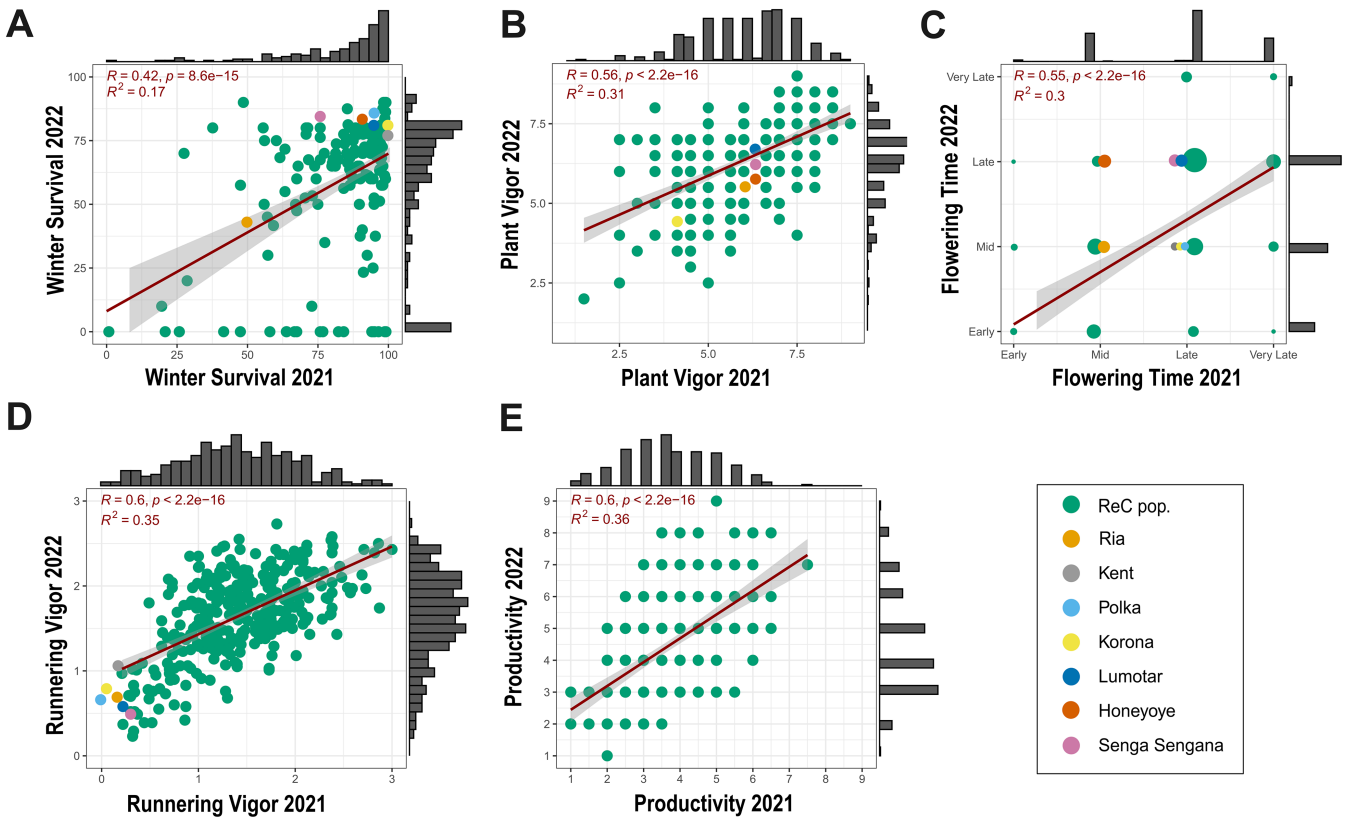

FIGURE 2 Marginal plots comparing winter survival (A), plant vigor (B), flowering time (C), runnering vigor (D), and productivity (E) of the ReC population and control cultivars across two years. In panel C, the relative size of the points reflects the frequency of ReC individuals per each flowering time category over two years. Productivity values of cultivars are not shown in panel E, because they are not easily comparable with the values of ReC individuals due to major differences in fruit size. Linear regression lines with R-squared values are shown.

of 24 SNPs per Mb across the genome, with variations ranging from 18 SNPs per Mb (chr 6B) to 30 SNPs per Mb (chr 7A) (Table S3, Figure S5). Linkage disequilibrium (LD) decay was similar across the four sub-genomes, but variation between individual chromosomes was found (Figure S6). Using an LD-pruned dataset of 2615 SNPs, population structure was comprehensively analyzed using four different approaches (STRUCTURE, PCA, NJ-clustering, and Kinship). We found four pedigree-based population clusters (Figures S7-S10) having significant genetic divergence, as indicated by fixation index values consistently greater than 0.15 (Table 3), a threshold commonly considered significant for differentiated populations (Frankham et al., 2002). The expected heterozygosity provided an estimate of the gene diversity based on the observed allele frequencies within the groups, which in the ReC population was 0.24 or higher for all four groups, with the

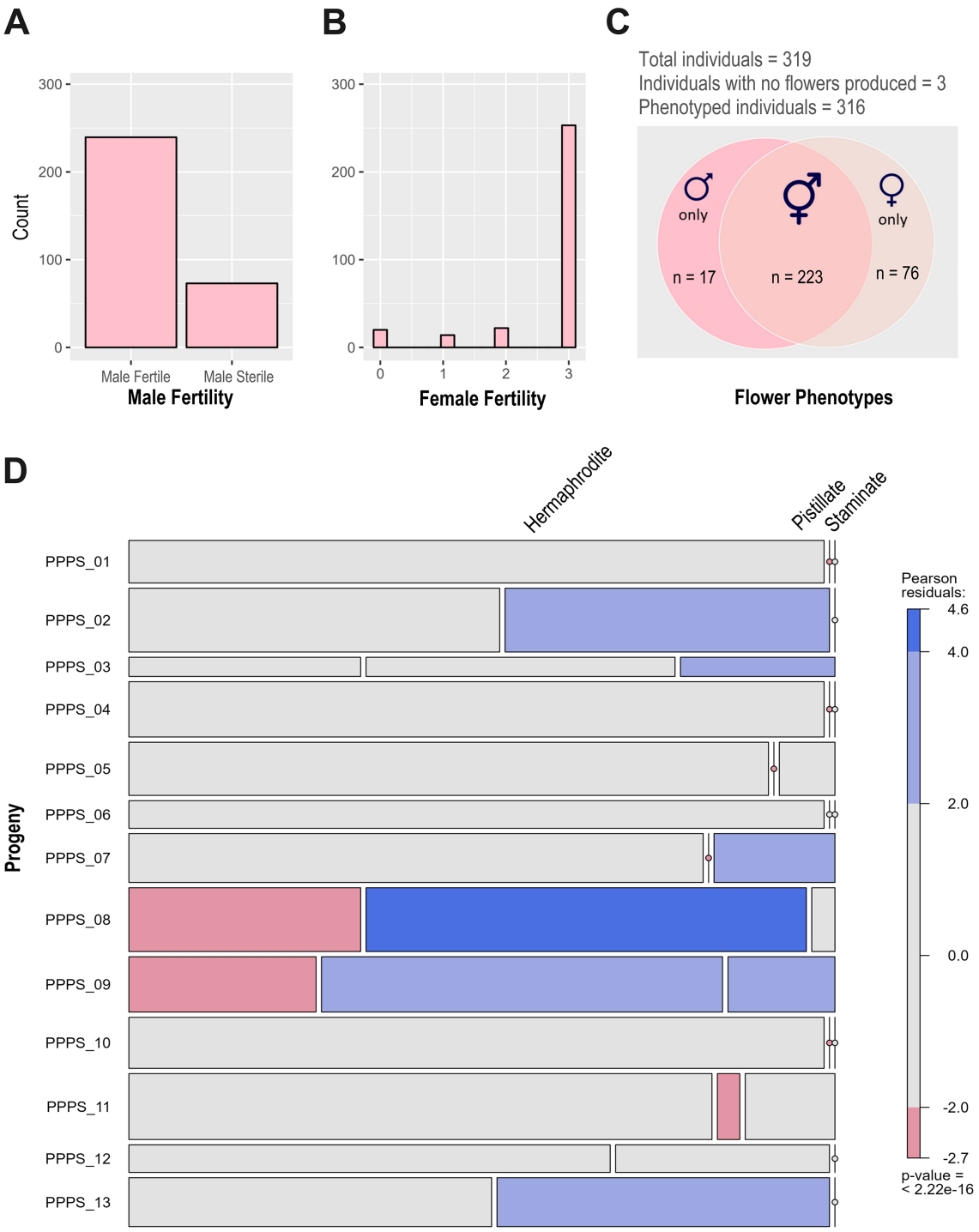

FIGURE 3 Segregation of fertility phenotypes in the ReC strawberry population: (A) male fertility, (B) female fertility, and (C, D) three different flower phenotypes (staminate, hermaphrodite, and pistillate) shown with a Venn diagram for the whole population (C) and with a mosaic plot per progeny (D). The mosaic plot box heights indicate the proportion of individuals in that progeny, while box widths reflect the proportion of individuals with that specific floral phenotype. The color of each box (or dot in the case of 0 observations) shows if the observed frequency deviates from the expected frequency under the assumption of independence (Chi-square test). Blue boxes mean more individuals than expected, red boxes mean fewer individuals than expected, and gray boxes mean no significant difference based on Pearson residuals.

highest value of 0.30 attributed to group 1 (PPPS_02) (Table 3). The distinct clustering of PPPS_02 and PPPS_08 could be attributed mainly to the limited sharing of parentage with other progenies. On the contrary, the rest of the progenies clustered together and displayed admixtures in accordance with their shared parents and grandparents (Figure 1). Overall, these findings confirmed the presence of substantial heterozygosity between and within population clusters, with potentially unique alleles in ReC population.

TABLE 3 Fixation index (Fst) and expected Heterozygosity (He) values for four groups identified by STRUCTURE analysis.

Group	Fst	He	No. of individuals
I	0.34	0.30	30
II	0.34	0.27	76
III	0.38	0.24	162
IV	0.37	0.26	30

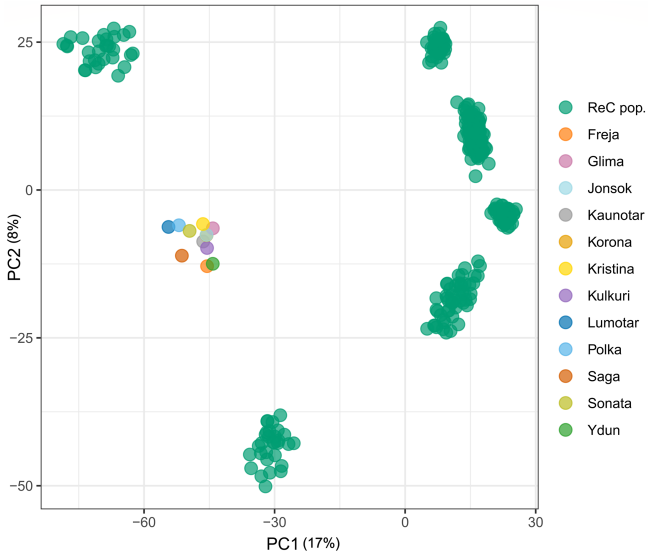

FIGURE 4 Scatter plot of the first two principal components from the PCA for 298 ReC strawberry genotypes and a set of twelve cultivars based on 20,779 SNP markers.

Furthermore, a combined PCA analysis incorporating the ReC population and a set of 12 cultivars confirmed the differentiation within the ReC population, while cultivars clustered together despite their diverse pedigrees (Figure 4, Table S4).

3.3 | Association analyses and candidate genes

The association analyses were performed with 20,779 high-quality SNP markers using single-locus (MLM) and multi-locus (FarmCPU, BLINK) models. The observed versus expected distribution of LOD (−log10(p)) values in quantile-quantile (QQ) plots displayed significant deviations from the expected distribution, providing clear evidence of the presence of significant MTAs for most traits (Figures 5 and S11-S13). In total, we identified 78 significant MTAs for eight of the eleven studied traits, surpassing the Bonferroni corrected threshold for significance (-log10(p) ≥ 5.61). Specifically, there were 15 significant MTAs for WS, 2 MTAs for PV, 30 MTAs for MF, 17 MTAs for FF, 5 MTAs for BA, 2 MTAs for RV, 6 MTAs for FT, and 1 MTA for PR. Most MTAs were identified only by the MLM model, but several consistent (high-confidence) MTAs were detected across more than one GWAS model (Table S5).

MTAs originating from the same chromosomal regions were assigned to Quantitative Trait Loci (QTLs) according to their physical distances and by applying LD decay as a critical threshold value, giving 28 putative and 11 high-confidence QTLs associated with 8 traits located on 20 chromosomes (Table 4). The total number of MTAs ranged from 1 to 11 per identified QTL (Table S5), and the Phenotypic Variation Explained (PVE) by the most significant MTAs per QTL ranged from <1 to 83% for different traits (Table 4). Most of the identified ReC QTLs were novel; however, some were found in close proximity to QTLs from earlier mapping studies in *F.* × *ananassa*, *F. virginiana*, or in the reconstructed FVC11 family (Table 4). To further identify candidate genes underlying the ReC QTLs, we conducted searches within the strawberry genome and reviewed existing literature for regions proximate to the most significant MTAs. The comprehensive list of genes is presented in Dataset S3. Subsequently, we narrowed down the pool of potential candidate genes through an examination of their putative functions, listed in Dataset S4.

3.4 | QTLs for winter survival and plant vigor

We identified a total of six winter survival QTLs on chromosomes 1A, 3A, 3C, 4C, and 7C. Among these QTLs, two QTLs on chr 3C (q.WS.Rec-3C.2) and 4C (q.WS.Rec-4C.1) were considered high-confidence QTLs, as they contained MTAs consistently identified by two multi-locus GWAS models (Figure 5A, Table 4). We found 19 plausible candidate genes characterized by annotations associated with cold or freezing tolerance. Among these were: four genes homologous to alcohol dehydrogenase (*Fxa1Ag102514.1*, *Fxa3Ag104340.1*, *Fxa4Cg200253.1*, *Fxa7Cg103062.1*); four containing a predicted hAT dimerization domain (*Fxa1Ag103043.1*, *Fxa3Ag104138.1*, *Fxa3Ag104305.1*, *Fxa7Cg102635.1*); two with a NAC domain (*Fxa1Ag102350.1*, *Fxa7Cg102969.1*); one homologous to *CTR1* (*Fxa3Ag103481.1*); one homologous to *BIDIRECTIONAL SUGAR TRANSPORTER SWEET3* (*Fxa7Cg102846.1*) (Dataset S4). For plant vigor, we identified two QTLs on chr 2A and chr 3B of which the latter (q.PV.Rec-3B.1) was consistent between models (Figure 5B). Within the LD distance of the ReC QTL on chr 2A, Antanaviciute (2016) also reported vigor-associated SNP markers in a mapping population 'Redgauntlet' × 'Hapil' (Table 4).

3.5 | QTLs for male and female fertility

We identified nine significant QTLs for male fertility on six different chromosomes, including three high-confidence QTLs on chromosomes 3D, 6A, and 6D, and a total of 11 QTLs for female fertility on nine different chromosomes, including one high-confidence QTL on chr 6A (Table 4, Figure S11). Interestingly, we observed two overlapping QTL regions for both male and female fertility, where the ones on chr 6A (q.MF.Rec-6A.1 and q.FF.Rec-6A.2) shared four MTAs, while the QTLs on 6D (q.MF.Rec-6D.2 and q.FF.Rec-6D.2) shared only one MTA. The positions of these QTLs overlapped with the positions of the main sex-determining region (SDRs) of the wild progenitor

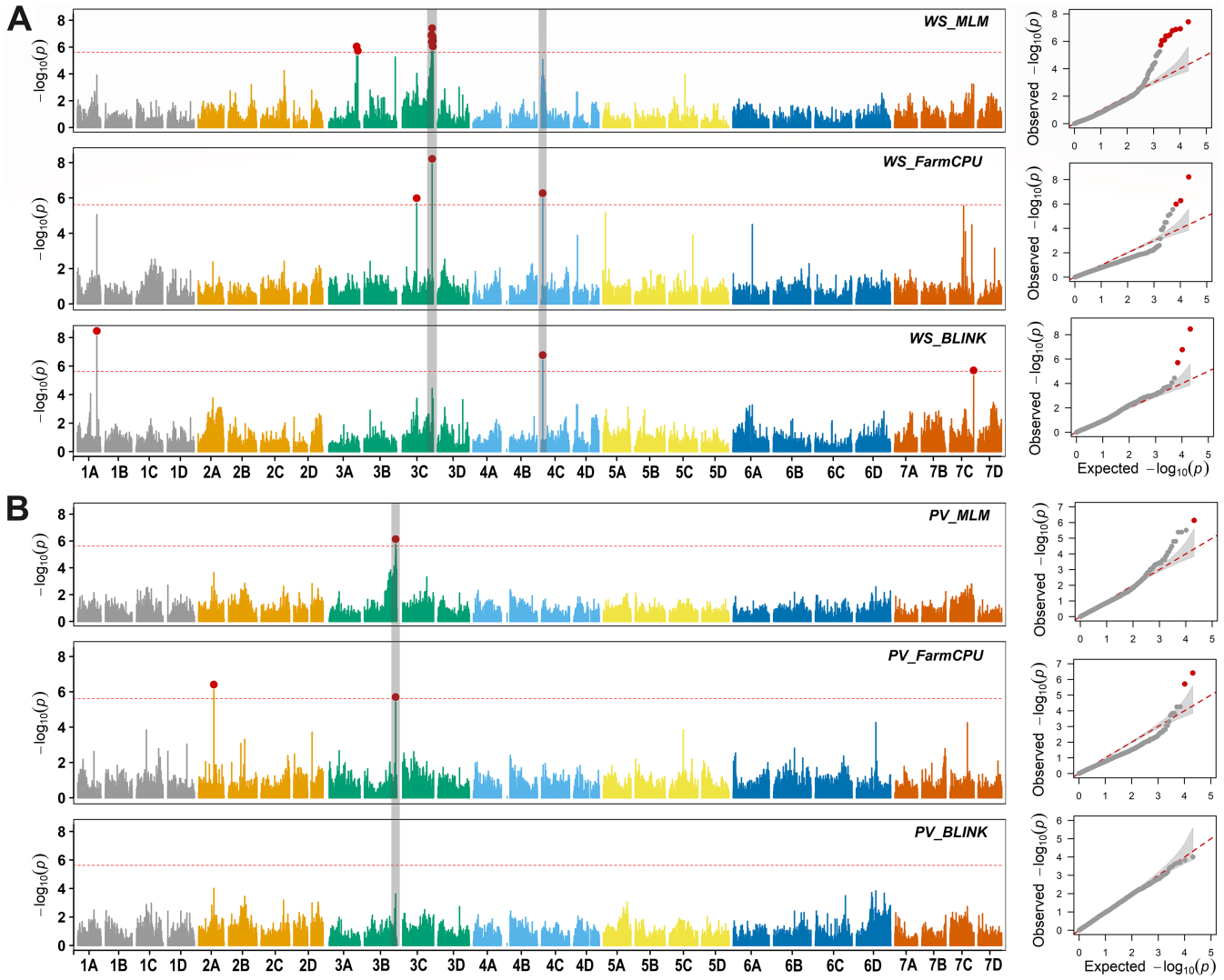

FIGURE 5 Stacked Manhattan plots and QQ plots for: A) Winter Survival and B) Plant Vigor in the strawberry ReC population. Left: Manhattan plots using MLM, FarmCPU, and BLINK models. Right: QQ plots of observed vs. expected p-values for each model. The red horizontal lines indicate the genome-wide significance level (−log10(p) = 5.61) and significant SNPs are indicated as red dots. The vertical shaded bands align common SNPs found significant for more than one GWAS models.

species in chr Fvb6-Av of *F. chiloensis* (Cauret et al. 2022) and in Fvb6-B2 of *F. virginiana* sp. *virginiana* (Tennessen et al., 2018), which are homologous to chromosomes 6A and 6D of the Royal Royce reference genome v1.0, respectively (Table 4).

3.6 | QTLs for runnering vigor and flowering time

For runnering vigor, two QTLs were identified on chr 2C and 3B (Table 4, Figure S12). The RV QTL detected on 2C colocalized with a marker for runner number previously detected in garden strawberry (Antanaviciute, 2016). The other QTL for RV on chr 3B (q.RV.Rec-3B.1) was in the vicinity of two runnering-related markers from the *F. virginiana* mapping population (Spigler et al., 2011). Furthermore, it overlapped with one of our ReC plant vigor QTLs, q.PV.Rec-3B.1, the distance between their respective top SNPs being only 0.7 Mb. For RV, we found 15 possible candidate genes, of which the most promising ones code for ent-kaurene oxidase (GA3) (*Fxa2Cg200089.1*), ELF3 (*Fxa2Cg200887.1*), FPF1 (*Fxa2Cg201029.1*), ARF4 (*Fxa3Bg202819.1*), ARF5 (*Fxa2Cg200277.2*) and the GRAS transcription factor LAM (*Fxa3Bg203528.1*) (Dataset S4).

For flowering time, three QTLs on chr 2D, 6D, and 7D were found (Table 4, Figure S12). Of the three FT QTLs, the one on chr 6D overlapped with a flowering time plasticity SNP marker in garden strawberry (Prohaska et al., 2024) and the one on 7D overlapped with a microsatellite marker for flowering time in an *F. virginiana* mapping population (Spigler et al., 2011) (Table 4). For FT, 33 candidate genes were found, including genes coding for ELF3 (*Fxa2Dg202776.1*), FPF1 (*Fxa2Dg202640.1*), transcription factor ASYMMETRIC LEAVES 1 (AS1) *Fxa6Dg101060.1*), APETALA2 (AP2) (*Fxa6Dg101201.1*), TERMINAL FLOWER 1 (TFL1) (*Fxa6Dg101555.1*), and DELLA protein (*Fxa7Dg100589.1*) (Dataset S4).

TABLE 4 QTLs co-segregating with winter survival, plant vigor, runnering vigor, flowering time, male fertility, female fertility, berry appearance, and productivity, their physical position (bp), minor allele frequency (MAF), *p* values, and Phenotypic Variance Explained (PVE) in percentage for the most significant SNP per respective GWAS model. QTL names in bold indicate consistent QTL regions with significant SNP(s) identified by more than one GWAS model.

Trait	QTL Name	Top SNP	Chr	Position	MAF	P value	PVE (%)	GWAS Model	Previously published SNP/gene
Winter Survival	*q.WS.Rec-1A.1*	AX.184091424	1A	19 144 883	0.12	3.44E-09	23.46	BLINK	
	q.WS.Rec-3A.1	AX.184840569	3A	28 438 618	0.21	8.87E-07	<1.00	MLM	
	q.WS.Rec-3C.1	AX.184946485	3C	15 341 787	0.13	1.04E-06	<1.00	FarmCPU	
	q.WS.Rec-3C.2	AX.184506312	3C	30 025 163	0.30	3.84E-08	3.92	MLM	
						6.14E-09	3.92	FarmCPU	
	q.WS.Rec-4C.1	AX.184861474	4C	1 583 556	0.22	5.35E-07	1.23	FarmCPU	
						1.69E-07	18.29	BLINK	
	q.WS.Rec-7C.1	AX.184492583	7C	22 835 463	0.12	2.00E-06	12.68	BLINK	
Plant Vigor	*q.PV.Rec-2A.1*	AX.184258351	2A	14 622 416	0.35	1.35E-07	<1.00	FarmCPU	Vigor, one year AX-89904609 (2.9 Mb) [1] Vigor, one year AX-89876601 (4.4 Mb) [1]
	q.PV.Rec-3B.1	AX.184098865	3B	29 912 198	0.21	7.27E-07	22.13	MLM	
						1.96E-06	22.13	FarmCPU	
Runnering Vigor	***q.RV.Rec-2C.1***	AX.184605169	2C	21 307 006	0.32	2.10E-06	3.64	FarmCPU	Runner number, one year AX-89781773 (4.2 Mb) [1]
						6.48E-07	17.85	BLINK	
	q.RV.Rec-3B.1	AX.184304857	3B	29 058 174	0.36	4.85E-07	<1.00	FarmCPU	Runner length ARSFL028/ CFVCT011, DQ117018 (3.8 Mb) [2] Plantlet number EmFn125, AX-89781773 (7.3 Mb) [2]
Flowering Time	*q.FT.Rec-2D.1*	AX.184456631	2D	27 522 484	0.12	2.15E-06	<1.00	FarmCPU	
	q.FT.Rec-6D.1	AX.184857914	6D	9 377 205	0.47	8.35E-07	5.65	MLM	Flowering time plasticity, AX.184201950 (1.1 Mb) [3]
						1.29E-12	5.65	FarmCPU	
	q.FT.Rec-7D.1	AX.184433867	7D	6 722 913	0.35	3.73E-09	18.49	BLINK	Date of 1st flower UFFa20G06, AJ870457.1 (1.5 Mb) [2]
Male Fertility	*q.MF.Rec-3C.1*	AX.184571580	3C	11 757 203	0.14	9.12E-07	3.37	BLINK	
	q.MF.Rec-3C.2	AX.184384120	3C	24 176 574	0.07	2.87E-08	6.11	BLINK	
	q.MF.Rec-3D.1	AX.184383078	3D	13 948 061	0.41	1.39E-10	9.84	MLM	
						1.73E-12	9.84	FarmCPU	
	q.MF.Rec-3D.2	AX.184158601	3D	16 932 399	0.03	4.13E-09	20.11	FarmCPU	
	q.MF.Rec-6A.1	AX.184409449	6A	33 285 271	0.14	2.81E-11	6.38	MLM	*F. chiloensis* SDR at 37 Mb of Fvb6-Av (<0.1 Mb) [SDR-Av]
						1.71E-13	6.38	FarmCPU	
						3.25E-10	4.99	BLINK	
	q.MF.Rec-6D.1	AX.184685668	6D	5 621 124	0.48	3.26E-08	<1.00	MLM	*F. virginiana* SDR at 1.7 Mb of Fvb6-B2 (3.9 Mb) [SDR-B2]
						3.69E-22	28.12	BLINK	
	q.MF.Rec-6D.2	AX.184244128	6D	30 346 011	0.22	4.13E-10	3.62	MLM	
	q.MF.Rec-7A.1	AX.184915292	7A	2 524 253	0.41	4.40E-09	<1.00	FarmCPU	
	q.MF.Rec-7D.1	AX.184502657	7D	2 548 759	0.08	9.34E-08	7.92	FarmCPU	
Female Fertility	*q.FF.Rec-1B.1*	AX.184682586	1B	25 125 618	0.32	1.36E-10	<1.00	FarmCPU	
	q.FF.Rec-1C.1	AX.184455342	1C	10 643 413	0.36	8.63E-08	<1.00	FarmCPU	Ovule number EMFxaCAD1A, AF320110 (0.6 Mb) [2]
	q.FF.Rec-1D.1	AX.184962399	1D	3 765 480	0.15	8.11E-08	12.03	BLINK	
	q.FF.Rec-2C.1	AX.184062428	2C	4 563 557	0.38	7.89E-07	12.77	BLINK	
	q.FF.Rec-4C.1	AX.184113018	4C	6 075 767	0.08	2.84E-07	<1.00	FarmCPU	

TABLE 4 (Continued)

Trait	QTL Name	Top SNP	Chr	Position	MAF	P value	PVE (%)	GWAS Model	Previously published SNP/gene
	q.FF.Rec-5C.1	AX.184602064	5C	16 901 835	0.34	5.38E-08	6.18	FarmCPU	
	q.FF.Rec-6A.1	AX.184210995	6A	5 242 694	0.23	4.15E-08	<1.00	FarmCPU	
	q.FF.Rec-6A.2	AX.184928439	6A	33 226 646	0.40	6.49E-08	9.02	MLM	*F. chiloensis* SDR at 37 Mb of Fvb6-Av (<0.01 Mb) [SDR-Av]
						2.80E-12	9.02	FarmCPU	
						1.43E-10	23.52	BLINK	
	q.FF.Rec-6D.1	AX.184421788	6D	9 705 519	0.33	1.55E-06	0.79	BLINK	*F. virginiana* SDR at 1.7 Mb of Fvb6-B2 (8.0 Mb) [SDR-B2]
	q.FF.Rec-6D.2	AX.184244128	6D	30 346 011	0.22	6.12E-09	<1.00	MLM	
	q.FF.Rec-7B.1	AX.184244972	7B	7 338 370	0.37	5.46E-09	<1.00	FarmCPU	
Berry Appearance	*q.BA.Rec-1A.1*	AX.184235566	1A	11 113 151	0.18	6.13E-09	17.98	FarmCPU	
	q.BA.Rec-2A.1	AX.184734269	2A	17 801 777	0.03	3.98E-07	<1.00	FarmCPU	
	q.BA.Rec-3A.1	AX.184127104	3A	3 628 143	0.42	1.21E-06	<1.00	FarmCPU	Fruit shape ratio AX-89863436 (0.7 Mb) [4]
	q.BA.Rec-3D.1	AX.184522680	3D	11 394 220	0.04	6.22E-07	83.43	MLM	
						7.27E-10	83.44	BLINK	
	q.BA.Rec-4B.1	AX.184229816	4B	4 378 463	0.02	1.60E-07	<1.00	FarmCPU	
Productivity	***q.PR.Rec-7D.1***	AX.184493289	7D	12 707 590	0.24	1.74E-06	<1.00	FarmCPU	Marketable yield, divergent sweep AX-166508528 (2.7 Mb) [5] Class2 Mass AX-166515770 (6.2 Mb) [6] Class 2 number AX-166527137 (6.2 Mb) [6] Fruit weight AX-89823518 (6.8 Mb) [7] Yield, one-year AX-89802589 (11.6 Mb) [1]
						4.75E-09	31.47	BLINK	

[1-7]Closest SNPs associated with correlated traits as reported by 1) Antanaviciute (2016) in iStraw90k SNP array; 2) Spigler et al., (2011) by microsatellites in a *F. virginiana* mapping population, 3) Prohaska et al., (2024) using Fana-50 k SNP array; 4) Rey-Serra et al., (2021) using iStraw35k SNP array, 5) Fan & Whitaker (2023) using a set of 5895 common markers from iStraw35k and Fana-50 k 6) Cockerton et al., (2021) on iStraw35k, or 7) Hancock et al., (2016) on iStraw90k array in reconstructed FVC11 family, and their distance from the top SNP of the identified QTL in brackets.

[SDR-Av]Homologs to predicted genes bordering the sex-determining region (SDR) in *F. chiloensis* Fvb6-Av: *F-box kelch* (Camarosa *FxaC_21g00450* = Royal Royce *Fxa6Ag104953*) and *arabinogalactan* (Camarosa *FxaC_21g03930* = Royal Royce *Fxa6Ag104954*) (Tennessen et al., 2018), and their mean distance from the top SNP of the identified QTL in brackets.

[SDR-B2]Distance to SDR in *F. virginiana* Fvb6-B2 was determined by its physical map position (Tennessen et al., 2018).

3.7 | QTLs for fruit characteristics and productivity

Five QTLs on chromosomes 1A, 2A, 3A, 3D, and 4B were found for berry appearance (Table 4; Figure S13A), including a single consistent QTL on chr 3D. A previously reported marker for fruit shape ratio was 0.7 Mb distant from the BA QTL in chr 3A (Table 4). However, we were unable to detect any significant associations for the other fruit characteristics, including berry color, berry size, and berry neck. One consistent QTL was discovered for productivity on chr 7D (Table 4; Figure S13B). This QTL region overlapped with multiple yield-related markers reported in several previous publications (Table 4).

3.8 | Allele effect and favorable allele combinations

The effect of the most significant SNP(s) of the consistent QTL(s) on the respective ReC phenotype was found significant for WS, PV, FT, RV, MF, and FF, but not for BA or PR (Figure S14). We further inspected the impact of each significant SNP for the fertility traits MF and FF and visualized the ones that had the strongest influence on the respective fertility trait (Figure 6, Table S6). To better examine the effect of favorable alleles in multiple loci on phenotypes for WS, PV, and FT, we grouped the ReC individuals based on the number of favorable effect alleles they possessed across all QTLs. The individuals exhibited variable grouping, with groups ranging from three to nine favorable alleles for WS, one to four for PV, and two to six alleles for FT. Individuals carrying a greater number of favorable alleles for winter survival (eight or more) illustrated significantly improved winter survival percentage ($r = 0.47$) compared to groups with fewer alleles (Figure 7A). Similarly, there was an additive effect of positive alleles on plant vigor, with plants containing three or more positive alleles demonstrating greater vigor than those with fewer positive alleles ($r = 0.22$) (Figure 7B). For flowering time, we observed a linear trend ranging from late to early flowering ($r = -0.14$) (Figure 7C). Our findings demonstrated the quantitative inheritance of

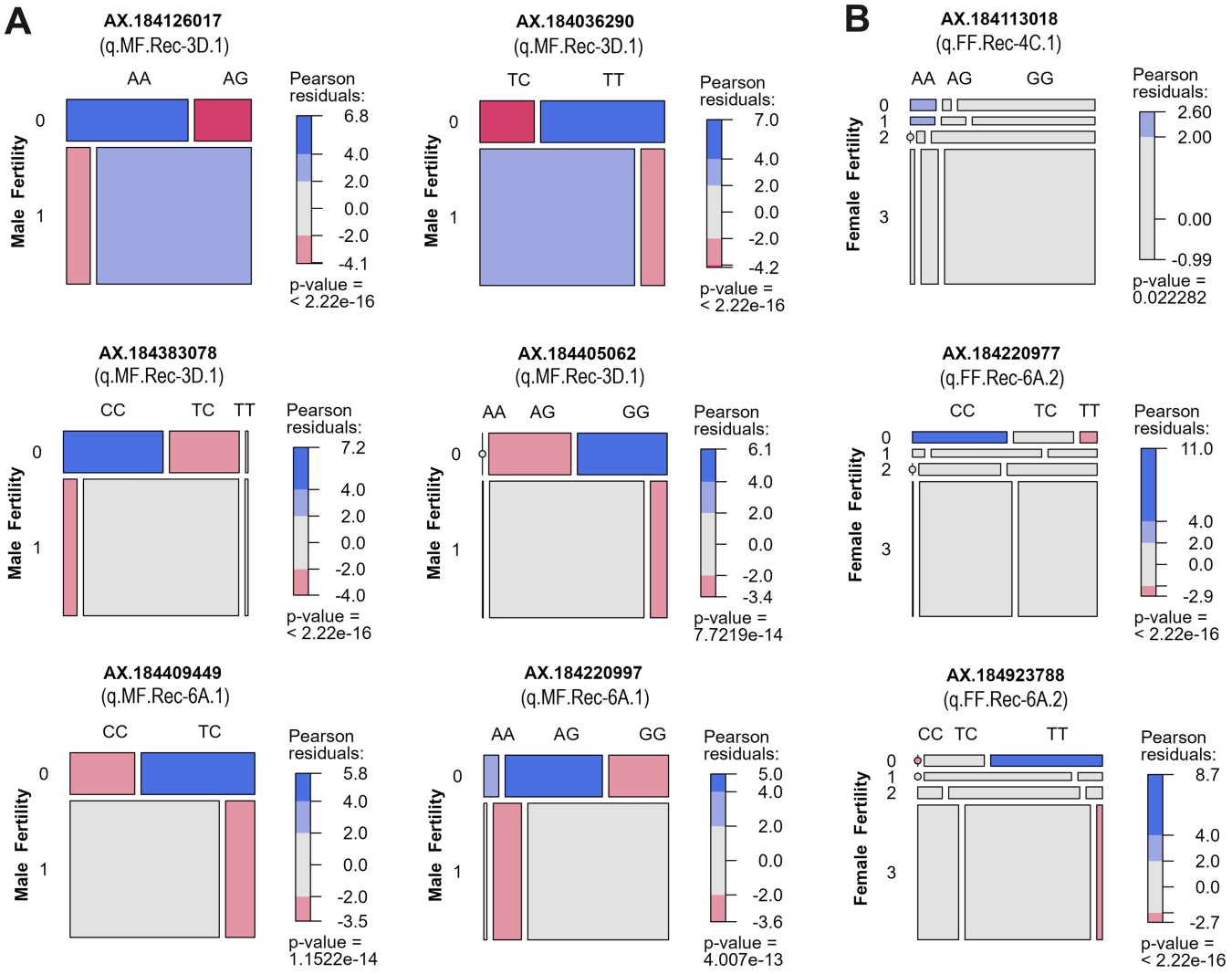

FIGURE 6 Frequency distribution mosaic plots of male fertility (A) and female fertility (B) phenotypes and their respective association with different allelic forms of the selected SNPs. Box height indicates the proportion of individuals in that phenotypic category, while box width reflects the proportion of individuals with that specific allelic form. The color of each box (or each dot in case of 0 occurrences) shows how confidently the observed frequency deviates from the expected frequency under the assumption of independence (Chi-square test). Blue boxes mean more individuals than expected, red boxes mean fewer individuals than expected, and grey boxes mean no significant difference based on Pearson residuals.

these traits and suggested that stacking of favorable alleles from different genomic regions can be used to improve winter survival and plant vigor and to alter flowering time in future germplasm derived from the ReC strawberry population. Furthermore, a recently reported SNP marker (Prohaska et al., 2024) for flowering time plasticity in garden strawberry had a significant allelic effect, suggesting that this marker might be transferable to ReC-derived materials (Figure S15).

4 | DISCUSSION

To obtain new adaptive variation for strawberry breeding in Northern Europe, we created a Re-Constructed (ReC) octoploid strawberry population from elite selections of the Northern American subspecies *virginiana* and three *F. chiloensis* subspecies (*lucida*, *pacifica* and *chiloensis*) from North and South America. We carried out multi-model GWAS on 11 important strawberry traits in the ReC population and identified significant QTL regions for eight of the field-evaluated traits, including winter survival, plant vigor, flowering time, runnering vigor, male fertility, female fertility, berry appearance, and productivity. Previously, a subset of the ReC population was also evaluated in greenhouse hydroponic conditions for root vigor and crown rot susceptibility (Haikonen et al. 2021). Characterization of adaptiveness in this highly diverse reconstructed germplasm will greatly foster its breeding use. The identified QTLs will facilitate the development of marker-assisted selection both for desired and against undesired wild alleles.

A previous study used a single reconstructed family (FVC11) for GWAS (Hancock et al., 2016), whereas our multi-family ReC

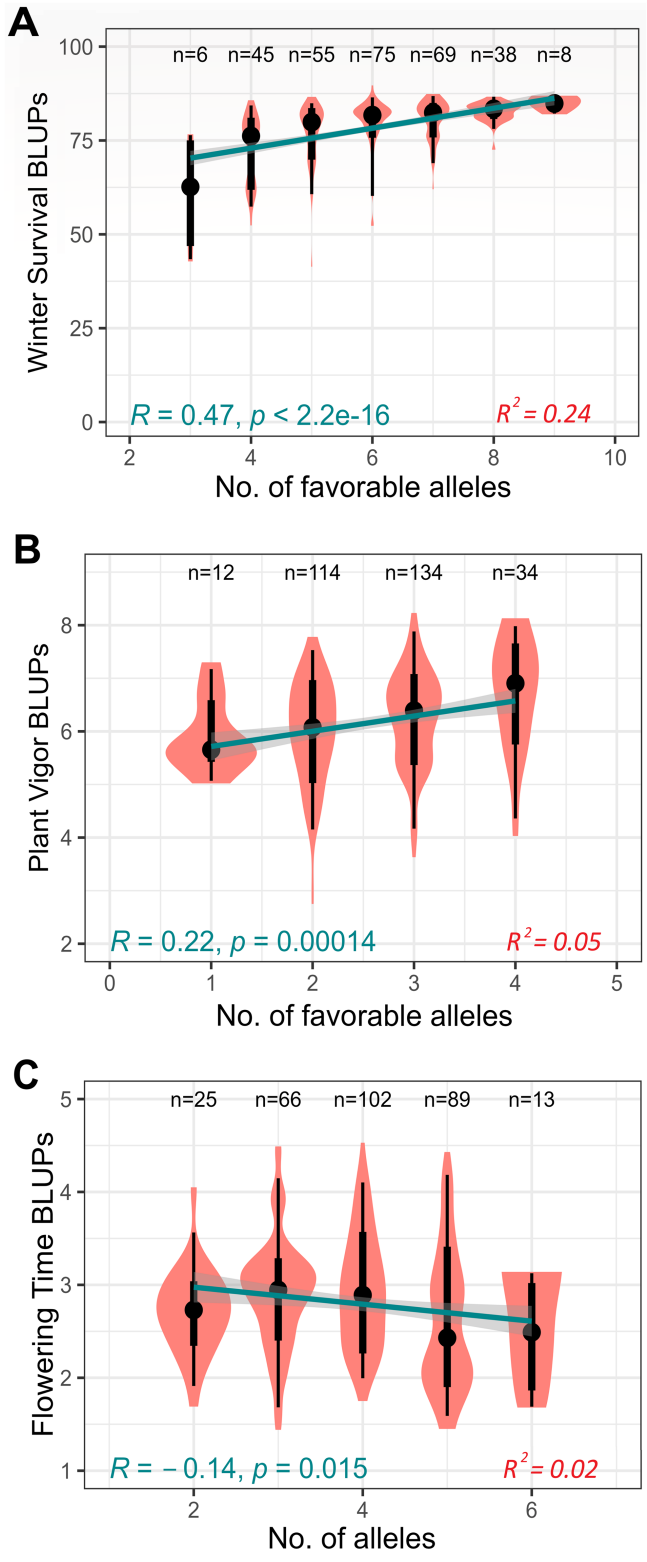

FIGURE 7 Effect of the number of favorable alleles on winter survival (A), plant vigor (B), and flowering time (C) in the ReC strawberry population. Regression lines illustrate the relationships, with slopes indicating effect magnitudes. Pearson correlation coefficients (R) and p-values show strength and statistical significance, respectively. Coefficient of determination (R^2) is represented on the bottom right for each trait.

population encompassed a broader diversity. Three of the four grandparental founders of the exceptional FVC11 were represented in the ReC pedigree. However, most (ten) of our cross combinations were unique, using the superior selections derived from the best-performing *F. virginiana* or *F. chiloensis* wild accessions and landraces from a wide geographic area (Table S1). The *F. chiloensis* selections combined various yield, fruit quality and resistance traits of South and North American accessions (Hancock et al., 2005), which, in our case, descended from three subspecies. To improve climatic adaptation, we selected elite material derived from northern accessions of *F. virginiana* sp. *virginiana* as reconstruction parents because their overwintering success is better than that of southern accessions and far superior to *F. chiloensis* (Hancock et al., 2001b).

Garden strawberry is cultivated predominantly in regions where winters are mild, yet successful overwintering remains vital for cultivation in several areas, where the trait ensures a substantial second harvest in the following spring (Lieten, 2005). In the Nordic countries, where strawberry production relies mainly on open-field perennial systems, changing climatic conditions are increasing the challenges due to temperature fluctuations, reduced snow cover, and higher winter precipitation, which can damage strawberries and other perennials (Sønsteby and Karhu, 2005; Himanen et al., 2013). Therefore, it is crucial to screen for winter-hardiness in pre-breeding materials for the Nordic region. The variation in the field performance of the ReC population was substantial, and a notable proportion of the plants overwintered at levels comparable to those observed in Northern European cultivars. The *F. virginiana* parents of the ReC population, being northern accessions of this subspecies, are the probable source of winter hardiness in the ReC material (Hancock et al., 2001b).

So far, few mapping studies on winter survival or cold tolerance have been made in *Fragaria*. Previously, a single QTL spanning approximately 5.1 Mb on chromosome *Fvb2* was identified in the *F. vesca* mapping population for freezing tolerance under controlled conditions (Davik et al., 2021). Our GWAS identified a total of six QTLs, including two high-confidence QTLs that were consistent across both the single- and multi-locus models tested. In proximity to these individual QTLs, we found multiple candidate genes involved in molecular pathways determining cold tolerance in strawberry or other plant species. The most promising are *ALCOHOL DEHYDROGENASE* (*ADH*) genes (on chr 1A, 3A, 4C, and 7C), which contribute to the synthesis of C1 to C9 alcohols and are induced by cold in Arabidopsis and cereal crops (Lindlöf et al., 2007). Previous studies on both *F.* × *ananassa* and *F. vesca* have shown correlations between ADH protein levels and cold tolerance (Koehler et al., 2012; Davik et al., 2013). Another candidate gene is *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) (chr 3A), which suppresses ethylene-related processes such as fruit ripening, senescence, and stress responses (Ju et al., 2012), and increases freezing tolerance in Arabidopsis (Shi et al., 2012). We also found a bidirectional sugar transporter gene, *SWEET3*, on chr 7C, a member of the *SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER* (*SWEET*) gene family known to have a role in cold responsiveness in Arabidopsis (Le Hir et al., 2015), cabbage (Zhang et al., 2019), and two rose species (Song

et al., 2023). Davik et al. (2021) also observed higher expression of a *SWEET-1*-like homologue in cold-treated *F. vesca* plants, suggesting its role in winter survival of *Fragaria* species.

The cultivars of garden strawberry are generally hermaphroditic. However, wild octoploid *Fragaria* segregates into individuals with female, male, and fully or partially hermaphrodite flowers, hence being trioecious, challenging their use for breeding (Taghavi et al., 2016). The *F. virginiana* and *F. chiloensis* grandparental accessions of ReC population included a variety of male, female, partially fertile and fully fertile hermaphrodite phenotypes (Hancock et al., 2010). Similarly, we observed considerable variation in the expression of floral sex in the ReC population.

Several studies have shown that sex determination in wild octoploid populations is controlled by a pseudo-autosomal female-specific sex-determining region (SDR). Mapping of SDR in the octoploid Homoeologous Group (HG) 6 has revealed its ancient translocations to alternative sub-genomes in different North-American octoploid taxa, for example, to a position at 37 Mb in Fvb6:Av of *F. chiloensis* and to a position at 1.7 Mb in Fvb6:B2 of *F. virginiana* ssp. *virginiana* (Goldberg et al., 2010; Wei et al., 2017), corresponding to Royal Royce chromosomes 6A and 6D, respectively. The SDR in the HG6 is a gene cassette of up to 31.7 kb, flanked by inverted repeats and present only in female fertile individuals (Cauret et al., 2022). Female fertility is reported as a continuous trait in octoploid wild strawberry (Ashman, 1999) regardless of the major effect SDR. Therefore, the ReC fertility QTLs outside HG6 are also highly relevant for yield and quality improvement in the pre-breeding material.

Male sterility in strawberry is a binary trait that, depending on the population, is either recessively or dominantly inherited (Wada et al., 2020). QTL mapping for male sterility in *F.* × *ananassa* has indicated three recessive regions on chromosomes Fvb4-3 and Fvb4-1 (Camarosa map) (Wada et al., 2020, 2021). In *F. vesca*, Ashman et al. (2015) suggested epistatic dominance of a male sterility determinant in Fvb4 over a recessive locus in Fvb6 (Ashman et al., 2015); a similar epistatic mechanism could occur in octoploid *Fragaria* between the quasi-autosomal SDR in HG6 and other loci. Such *F. virginiana* autosomal QTL for anther number has been reported in chr 3D (Spigler et al., 2011). We identified QTL regions associated with male fertility in multiple chromosomes, implicating the role of more than one genomic region in the expression of this trait also in the ReC population.

Plant vigor represents the overall vegetative fitness of the plant and, similar to winter survival, is an indicator of vegetative adaptation. Large, vigorous plants having strong foliage are preferred by strawberry breeders due to their overall robustness and often high yield. Previously reconstructed F1 individuals were found to be vigorous; their vigor correlated with fruit size and total yield (Luby et al., 2008; Hancock et al., 2010). Similarly, the mean scores for plant vigor in the ReC population were high, commonly exceeding the vigor scores of the control cultivars. We identified two QTLs for plant vigor in our two-year data, one of them within the LD of a QTL previously identified on Linkage Group (LG) 2A in garden strawberry (Antanaviciute et al., 2016), suggesting the stability of this QTL across environments. In contrast, the study on the reconstructed FVC11 progeny identified a common SNP association for plant vigor and number of runner plants in HG6 (Hancock et al., 2016), distinct from our findings.

Early and highly vigorous runner formation was the most striking weedy characteristic of the ReC population compared to control cultivars. There was a tendency for runner growth to begin already before or during flowering in the ReC plants. Runnering vigor and plant vigor shared a QTL region on chr 3B, and a moderate phenotypic correlation between these two traits was apparent. Notably, the scores for flowering time and runnering vigor were negatively correlated, indicating that late flowering was associated with later or less abundant runnering. Despite the contrasting runner growth and flowering responses, a degree of independence between their respective patterns was suggested (Heide, 1977). Previous studies reported several QTLs in multiple LGs associated with abundant runnering in two different *F.* × *ananassa* and one *F. virginiana* mapping populations (Spigler et al., 2011; Antanaviciute et al., 2017; Hossain et al., 2019). We found two runnering vigor QTLs on chr 2C and 3B that were supported by two of these studies (Spigler et al., 2011; Antanaviciute, 2016). In *F. vesca*, two QTLs on the diploid LG4 and LG5 were associated with the number of runners, with an overlapping QTL in LG4 for flowering time (Samad et al., 2017).

The intricate interplay between vegetative and sexual reproduction in strawberries, influenced by environmental cues, holds both biological and economic significance. Strawberry provides a valuable model system for studying these traits due to the distinct origin of runners from axillary meristems and flowers from apical meristems (Andrés et al., 2021). As a candidate gene for runnering vigor, we discovered a gene encoding gibberellin 3-oxidase (*GA3ox*) on chr 2C, which is responsible for converting GA20 or GA9 to bioactive GA1 or GA4, which plays an important role in the formation of runners in strawberries (Hytönen et al., 2009). We also found a homolog of the GA receptor GID1 (on chr 3B), activation of which leads to the degradation of DELLA proteins, repressors of GA responses. Moreover, we found two homologs of auxin response factors (ARFs) that are involved in the runner developmental pattern of *F. vesca* (Qiu et al., 2019). At a distance of 0.5 Mb to the top SNP of the runnering vigor ReC QTL on 3B, we furthermore identified a homolog of *F. vesca LOSS OF AXILLARY MERISTEMS*, which is vital for stamen and runner formation in strawberry (Feng et al., 2021).

Flowering time is affected by sexual dimorphism to a minor degree in *F. virginiana*, male-fertile individuals flowering earlier than non-male individuals (Spigler et al., 2011). Indeed, one of the FT QTLs overlapped with the SDR-associated fertility QTLs on chr 6D; similarly, Spigler et al. (2011) found one of ten flowering time QTLs in *F. virginiana* linked to SDR in chromosome Fvb6-B2. We found genes encoding TFL1 and transcription factors AS1 and AP2 on the chr 6D QTL region. AS1 has both GA-dependent and -independent roles in flowering time regulation of Arabidopsis (Song et al., 2012), while AP2 determines the identity of floral organs in Arabidopsis (Kunst et al., 1989). TFL1 is a floral repressor in *F. vesca* and *F.* × *ananassa*, and is differently regulated between early and late flowering cultivars (Koskela et al., 2012, 2016). On chr 7D, we found a gene encoding a DELLA protein, which controls runnering in octoploid strawberry (Caruana et al., 2018).

We found candidate genes that could pleiotropically be associated with runnering vigor and flowering time. These include homologs of *EARLY FLOWERING3* (*ELF3*), located proximal to the ReC-QTLs for both traits. *ELF3* is a photoperiod pathway gene and plays a role in flowering control in Arabidopsis (Hicks, 2001). It was also differentially expressed in *F. vesca* everbearing and short-day genotypes, although its biological function in *Fragaria* is yet unknown (Mouhu et al., 2009). Similarly, we found homologs of the *FLOWERING PROMOTING FACTOR1*-related (*FPF1*) gene coinciding with QTLs for both runnering vigor and flowering time (Kania et al., 1997).

The berry appearance ReC-QTL in chr 3D was located within a high-confidence male fertility QTL. A physiological connection between these two traits is conceivable because abundant pollen availability is required for fruit set and good fruit appearance. An alternative explanation could be the accumulation of alleles antagonistic to male fertility in the vicinity of SDR, as hypothesized earlier (Tennessen et al., 2016). We also found a previously identified QTL for fruit shape ratio (Rey-Serra et al., 2021) within 1 Mb of our identified QTL on chr 3A, potentially implicating fruit enlargement patterns also as determinants for symmetric fruit appearance.

Yield is a highly polygenic trait having multiple associated chromosomal regions also in strawberry (Cockerton et al., 2021). We identified a single QTL for productivity on chr 7D, near a marketable-yield marker in octoploid strawberry identified earlier (Fan and Whitaker, 2023). These authors found the marker within a divergent selective sweep when comparing different breeding programs, heirloom cultivars and wild octoploid progenitors of strawberry.

5 | CONCLUSIONS

Modern strawberry cultivars, and even wild-collected accessions of parental species, have significantly lower heterozygosity and phenotypic diversity than the oldest extant garden strawberry cultivars (Hardigan et al., 2021b; Fan and Whitaker, 2023). These early hybrids were, however, often the result of random crosses between the limited wild material that was available (Darrow, 1966). The reconstruction approach that we took here has the advantage of generating unique coadapted material with increased heterozygosity and genetic diversity (Hancock et al., 1993, 2005, 2010; Luby et al., 2008). Incorporating wild *F. virginiana* and *F. chiloensis* material into the *F.* × *ananassa* gene pool is beneficial since the number of founders, especially wild ones, is low in many modern breeding populations (Diamanti et al., 2014; Pincot et al., 2021). Furthermore, the genetic contribution of *F. chiloensis* has been under negative selection during the breeding history (Hardigan et al., 2021b). Hence, the *F. chiloensis* elite selections, which in our multi-familial ReC population are derived from multiple subspecies and wide geographic origins, offer a remarkable potential source of novel alleles. Simultaneously, the northern *F. virginiana* parents are expected to ensure the future material's adaptation to harsh winters. Our ReC population showed high genetic and phenotypic variation and allowed us to identify 39 QTLs for eight horticulturally important traits, most of which were novel, demonstrating the value of the population for strawberry pre-breeding.

AUTHOR CONTRIBUTIONS

T.Ha., S.K. and M.R. conceived the project and designed the experiments with seed materials provided by J.D. A.R., T.Ha. and M.R. performed the experiments. A.R., T.I-T., T.Ha., J.D., J.J. and D.F. conducted the data analysis. A.R. and T.Ha. drafted the manuscript draft with contributions from J.J., T.I-T., J.D., A.H.S. and T.Hy. S.K., T.Ha., J.D., B.M., M.A. and S.H.H. acquired the funding. T.Ha., T. Hy. and A.H.S. supervised the study. All the authors reviewed and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available within the manuscript and its Supporting Information files. Raw data and the code used for this project are available from the corresponding author on reasonable request.

ORCID

Attiq ur Rehman https://orcid.org/0000-0002-0131-3928
Terhi Iso-Touru https://orcid.org/0000-0001-8258-9047
Marja Rantanen https://orcid.org/0000-0003-3078-3782
Saila Karhu https://orcid.org/0000-0003-4974-6033
Daniel Fischer https://orcid.org/0000-0002-7513-683X
Muath Alsheikh https://orcid.org/0009-0004-8084-0065
Bruno Mezzetti https://orcid.org/0000-0001-9307-812X
Jahn Davik https://orcid.org/0000-0002-3621-6888
Alan H. Schulman https://orcid.org/0000-0002-4126-6177
Timo Hytönen https://orcid.org/0000-0002-5231-4031
Tuuli Haikonen https://orcid.org/0000-0002-9631-9774

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SUPPORTING INFORMATION

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