

Article

Towards the Identification of Candidate Genes for Pollen Morphological Traits in *Rubus* L. Using Association Mapping

Jan Bocianowski ^{1,*}  and Agnieszka Leśniewska-Bocianowska ² 

¹ Department of Mathematical and Statistical Methods, Poznań University of Life Sciences, Wojska Polskiego 28, 60-637 Poznań, Poland

² Department of Pathophysiology of Ageing and Civilization Diseases, Poznań University of Medical Sciences, Święcickiego 4, 60-781 Poznań, Poland; abocianowska@ump.edu.pl

* Correspondence: jan.bocianowski@up.poznan.pl; Tel.: +48-61-8487143

Abstract

Rubus L. species display considerable morphological and genetic variability. Traditional taxonomic methods, which rely primarily on the observation of external characters, are often insufficient to resolve this complexity. Consequently, molecular biology tools are being increasingly employed. This study aimed to identify markers associated with candidate genes responsible for four selected pollen morphological traits—namely, the length of the polar axis, length of the ectoaperture, distance between the apices of two ectocolpi, and equatorial diameter—using association mapping. Based on the available literature, this is the first report of association mapping used to identify candidate genes for pollen morphological traits in *Rubus* L. Sixteen *Rubus* species and the complete set of possible markers (65534) were analyzed. Association mapping enabled the identification of 44 markers that are statistically significantly associated with all four morphological traits under consideration. The ten markers with the highest total LOD value for four pollen morphological traits allowed the distinction of six species: *Rubus bifrons*, *Rubus caesius*, *Rubus idaeus*, *Rubus radula*, *Rubus saxatilis*, and *Rubus scissus*. The results demonstrate that the proposed comprehensive approach of analyzing all possible markers may serve as an effective tool for detecting markers linked to genes controlling important traits, not only in *Rubus* species but potentially in other taxa as well.

Keywords: association mapping; percentage of variation explained; Rosaceae; Mahalanobis distance; correlation



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1. Introduction

The genus *Rubus* L., belonging to the family Rosaceae, comprises hundreds of species, including well-known raspberries and blackberries. These species exhibit remarkable morphological and genetic variability [1,2]. Phenomena such as apomixis (asexual reproduction without fertilization), polyploidy (the presence of multiple sets of chromosomes), and frequent hybridization make traditional taxonomic methods based mainly on observations of external characters insufficient and may lead to erroneous conclusions [3]. Consequently, researchers are increasingly employing molecular biology tools, which enable more precise determination of phylogenetic relationships and allow for the verification of taxonomic classifications [4,5]. Moreover, molecular markers facilitate the selection process in breeding programs [6–8].

One of the areas in which modern research methods, including molecular biology, can be applied is the analysis of floral pollen [9,10]. Morphological characteristics of pollen

grains, such as their shape, size, surface ornamentation, and the thickness of the cell wall (exine), are commonly used in taxonomic and palynological studies [11,12]. However, these traits may be influenced by environmental factors and may not fully reflect the underlying genetic variability within a species [13,14]. Therefore, combining morphological analysis of pollen with molecular approaches provides a much more comprehensive understanding of the variability of a given taxon [15].

Molecular markers, such as Inter Simple Sequence Repeats (ISSRs) and Simple Sequence Repeats (SSRs, also known as microsatellites), enable the assessment of DNA variation among individuals or populations. Molecular markers are independent of environmental influences, exhibit consistency across different plant tissues, and enable precise assessment of allelic frequencies [16]. As such, they constitute a reliable tool for genetic analysis. Molecular markers are indispensable in contemporary genetic research and plant breeding programs, as they facilitate the detection of genetic diversity at the DNA level with high resolution [17]. They offer several significant advantages, including resistance to environmental variability, high reproducibility, the ability to detect fine-scale genetic variation, and suitability for high-throughput analysis. An ideal molecular marker should (i) accurately reflect underlying genetic diversity, (ii) demonstrate high reliability and reproducibility, (iii) provide comprehensive genomic coverage and be representative of the entire genome, (iv) be cost-effective, rapid, and technically straightforward, and (v) be compatible with bioinformatics tools and data analysis platforms [7]. Although the development of a single marker system that fully meets all these criteria remains challenging, various marker types can be strategically selected and combined to address specific research objectives. The application of molecular markers in research on *Rubus* enables not only a more detailed understanding of phylogenetic relationships among species but also an assessment of genetic variability within a single species, a variability that is not always evident at the morphological level, yet is crucial, for example, for biodiversity conservation [14,18]. In the case of the genus *Rubus*, where morphology is often misleading due to frequent hybridization and environmental variation, the use of molecular markers is particularly valuable [13,19].

Studies combining pollen analysis with molecular markers demonstrate that there is a correlation between pollen traits and the genetic profiles of species [20,21]. For example, differences in pollen size and surface ornamentation often correspond to genetic variation detected using SSR markers [22]. In certain cases, morphological characteristics may indicate the hybrid origin of a given individual, which can subsequently be confirmed through DNA analysis [23]. Conversely, molecular analysis can also aid in identifying cases of apomixis, when individuals with identical genotypes exhibit morphological differences resulting from environmental influences [24,25].

The application of molecular markers in pollen research of *Rubus* species not only enhances the understanding of their variability but also facilitates their classification, the conservation of genetic resources, and potentially their utilization in plant breeding [26,27]. The integration of morphological and genetic data is particularly important for taxa that are difficult to distinguish based solely on external traits. Molecular markers are valuable tools for the identification of candidate genes, especially when combined with QTL mapping to validate loci associated with quantitative traits [28]. The combination of these methods has helped to overcome their individual limitations [29]. As an alternative to conventional breeding methods, precise genetic engineering based on genome editing technologies may play a pivotal role in accessing and utilizing genetic resources. There is information in the literature on molecular markers of raspberry pollen traits [30–32], but this is limited to specific marker observations. In this study, we present the possibility of analysis conducted on all theoretically possible markers.

The aim of the present study was to identify markers associated with candidate genes responsible for four selected pollen morphological traits (length of the polar axis, length of the ectoaperture, distance between the apices of two ectocolpi, and equatorial diameter) in *Rubus* L. (Rosaceae) using association mapping based on all theoretically possible markers obtainable in the experiment. The proposed approach, which involves analysis of all possible binary combinations—representing every theoretically attainable molecular marker—facilitates complex and time-consuming analyses. Moreover, it offers the potential to reduce the costs associated with the development of molecular markers. These four specific traits were chosen for study because, according to the literature, they best characterize *Rubus* pollen. To our knowledge, this is the first report of association mapping to identify candidate genes for pollen morphological traits in *Rubus* L.

2. Materials and Methods

2.1. Plant Material

Phenotypic data were obtained from Lechowicz et al. [33]. Sixteen species of *Rubus* L. (Rosaceae) were included in the analysis: *Rubus bifrons* (species no. 1), *Rubus caesius* (no. 2), *Rubus constrictus* (no. 3), *Rubus divaricatus* (no. 4), *Rubus gracilis* (no. 5), *Rubus henrici-egonis* (no. 6), *Rubus idaeus* (no. 7), *Rubus nessensis* (no. 8), *Rubus opacus* (no. 9), *Rubus plicatus* (no. 10), *Rubus praecox* (no. 11), *Rubus radula* (no. 12), *Rubus saxatilis* (no. 13), *Rubus scissus* (no. 14), *Rubus sprengelii* (no. 15), and *Rubus sulcatus* (no. 16) (Table A1). The origin of each species was treated as a replicate. Four quantitative traits characterizing pollen morphology were selected for analysis: length of the polar axis (P), length of the ectoaperture (Le), the distance between the apices of two ectocolpi (d), and equatorial diameter (E). These four specific traits were chosen for study because, according to the literature, they best characterize *Rubus* pollen.

2.2. Genotypic Observations

All theoretically possible markers were treated as genotype observations. This approach corresponds to the scenario in which all possible binary (0–1) patterns are generated through DArTseq technology. For 16 genotypes, the total number of such binary configurations is $2^{16} = 65,536$. The extreme configurations 0000000000000000 and 1111111111111111 represent monomorphic cases. Only configurations corresponding to polymorphic cases were retained for further analysis; hence, the final number of marker observations was $2^{16} - 2 = 65,534$. To facilitate the interpretation of specific markers, binary values were converted to decimal notation. Examples of conversions between binary and decimal representations are provided in Appendix B, Formula (A1), and Table A2.

2.3. Statistical Methods

The conformity of the empirical distributions of four analyzed morphological pollen traits with the normal distribution was assessed using the Shapiro–Wilk W-test [34]. Homogeneity of variances was evaluated using Bartlett’s test. A multivariate analysis of variance (MANOVA) was carried out to determine the effects of species on all four of the observed traits jointly. Next, one-way analyses of variance (ANOVA) were conducted to assess the effect of species on each trait individually. Arithmetic means and standard deviations were calculated for each trait. Additionally, Fisher’s least significant differences (LSDs) test was estimated at a significance level of $\alpha = 0.05$, and homogeneous groups were identified based on these LSD values. Relationships between the examined traits were evaluated using Pearson correlation coefficients calculated from species mean values. Multivariate methods were also applied for further analysis. Canonical variate analysis was employed to facilitate the multivariate assessment of interspecific similarity by reducing the dimensionality of

the trait space with minimal information loss [35]. This enabled graphical visualization of variation among species based on all observed traits. Mahalanobis distance was used as a measure of multivariate similarity among species [36], and its statistical significance was determined using the critical value D_{α} , referred to as the least significant distance [37].

Association mapping was conducted based on species mean trait values and the generated marker data, using a mixed linear model (MLM) approach. This model incorporated population structure inferred via eigenanalysis and modeled as random effects [38,39]. All statistical analyses and result visualizations were carried out using GenStat 23.1 software [40], specifically employing the QSASSOCIATION procedure. This procedure implements a mixed-model marker–trait association analysis (also known as linkage disequilibrium mapping) for data obtained from a single-trait trial. To control for false-positive associations due to population structure and relatedness, the model included a genetic relatedness correction. The RELATIONSHIPMODEL = eigenanalysis option was used, which identifies major principal components from the marker matrix to account for population stratification [41]. The scores of the significant principal components were incorporated as covariates in the MLM, providing an approximation of the genetic variance–covariance structure via a kinship matrix. The statistical significance of marker–trait associations was evaluated using p -values adjusted for multiple testing using the Benjamini–Hochberg false discovery rate correction method.

3. Results

3.1. Phenotyping

The number of observations per species varied, ranging from two (for *R. constrictus* and *R. opacus*) to five (for *R. bifrons*, *R. caesius*, *R. gracilis*, *R. henrici-egonis*, *R. idaeus*, *R. nessensis*, *R. plicatus*, *R. radula*, *R. saxatilis*, *R. sprengelii*, and *R. sulcatus*) (Table 1). In each case, measurements were made from 30 mature, randomly selected, properly developed pollen grains using light microscopy (LM), measuring a total of 2100 pollen grains.

Table 1. Mean values and standard deviations, values of the F test statistic from the analysis of variance, as well as results of the Shapiro–Wilk W -test for normality and Bartlett test for the length of the polar axis (P), length of the ectoaperture (Le), the distance between apices of two ectocolpi (d), and equatorial diameter (E).

Species	Repl	P ¹		Le ²		d ³		E ⁴					
		Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.				
<i>Rubus bifrons</i>	5	25.43	a	0.771	21.5	a	0.8434	2.656	de	0.2454	21.53	ab	0.502
<i>Rubus caesius</i>	5	24.6	abc	1.789	20.65	abcd	1.638	3.719	a	0.8253	21.65	a	1.892
<i>Rubus constrictus</i>	2	23.68	abcd	0.304	19.91	abcde	0.2758	3.008	bcd	0.3882	20.75	ab	0.806
<i>Rubus divaricatus</i>	4	21.71	e	0.545	18.3	ef	0.6087	2.009	e	0.1177	18.54	cd	1.162
<i>Rubus gracilis</i>	5	24.44	abcd	1.22	20.78	abc	0.972	2.768	cd	0.2767	20.75	ab	1.491
<i>Rubus henrici-egonis</i>	5	22.69	de	1.14	19.16	def	1.0766	2.608	de	0.646	19.67	bc	1.613
<i>Rubus idaeus</i>	5	22.7	de	1.198	19.24	cdef	0.6913	3.487	ab	0.3611	20.46	ab	1.242
<i>Rubus nessensis</i>	5	24.39	abcd	0.951	20.47	abcd	0.6514	2.729	d	0.2812	20.07	abc	0.72
<i>Rubus opacus</i>	2	21.75	e	1.711	18.09	f	0.5162	2.604	de	0.6463	17.74	d	1.223
<i>Rubus plicatus</i>	5	22.94	cde	1.16	19.3	cdef	0.5658	3.119	abcd	0.4014	21.05	ab	1.203
<i>Rubus praecox</i>	4	23.74	abcd	1.364	20.28	abcd	1.2429	2.969	bcd	0.2998	20.88	ab	1.108
<i>Rubus radula</i>	5	25.07	ab	1.213	21.15	ab	1.2769	2.736	d	0.4919	21.5	ab	0.638
<i>Rubus saxatilis</i>	5	23.2	cde	1.025	19.75	bcde	1.1065	3.444	abc	0.1681	20.23	abc	0.655
<i>Rubus scissus</i>	3	23.46	bcde	0.828	19.87	bcde	0.7328	3.234	abcd	0.5522	21.25	ab	0.384
<i>Rubus sprengelii</i>	5	24.17	abcd	0.617	20.56	abcd	0.5339	2.92	bcd	0.2026	20.73	ab	0.472
<i>Rubus sulcatus</i>	5	23.53	bcde	0.93	20.01	abcd	0.8853	2.83	bcd	0.134	20.69	ab	1.346
LSD _{0.05}		1.869			1.61			0.6935			1.914		
F-ANOVA		3.91 ***			3.9 ***			4.49 ***			2.9 **		
Shapiro-Wilk test for normality	W-test p-value	0.987 0.658			0.982 0.41			0.986 0.626			0.969 0.066		
Bartlett test	χ ² p-value	9.51 0.849			12.02 0.677			16.99 0.329			17.7 0.279		

¹ P—length of the polar axis, ² Le—length of the ectoaperture, ³ d—the distance between apices of two ectocolpi, ⁴ E—equatorial diameter; ** $p < 0.01$; *** $p < 0.001$; LSD—Fisher’s least significant difference; a–e—in columns, means followed by the same letters are not significantly different; s.d.—standard deviation.

The empirical distributions of all four pollen morphology traits conformed to the normal distribution (Figure 1, Table 1). Bartlett's test confirmed the homogeneity of variances across all four traits (Table 1).

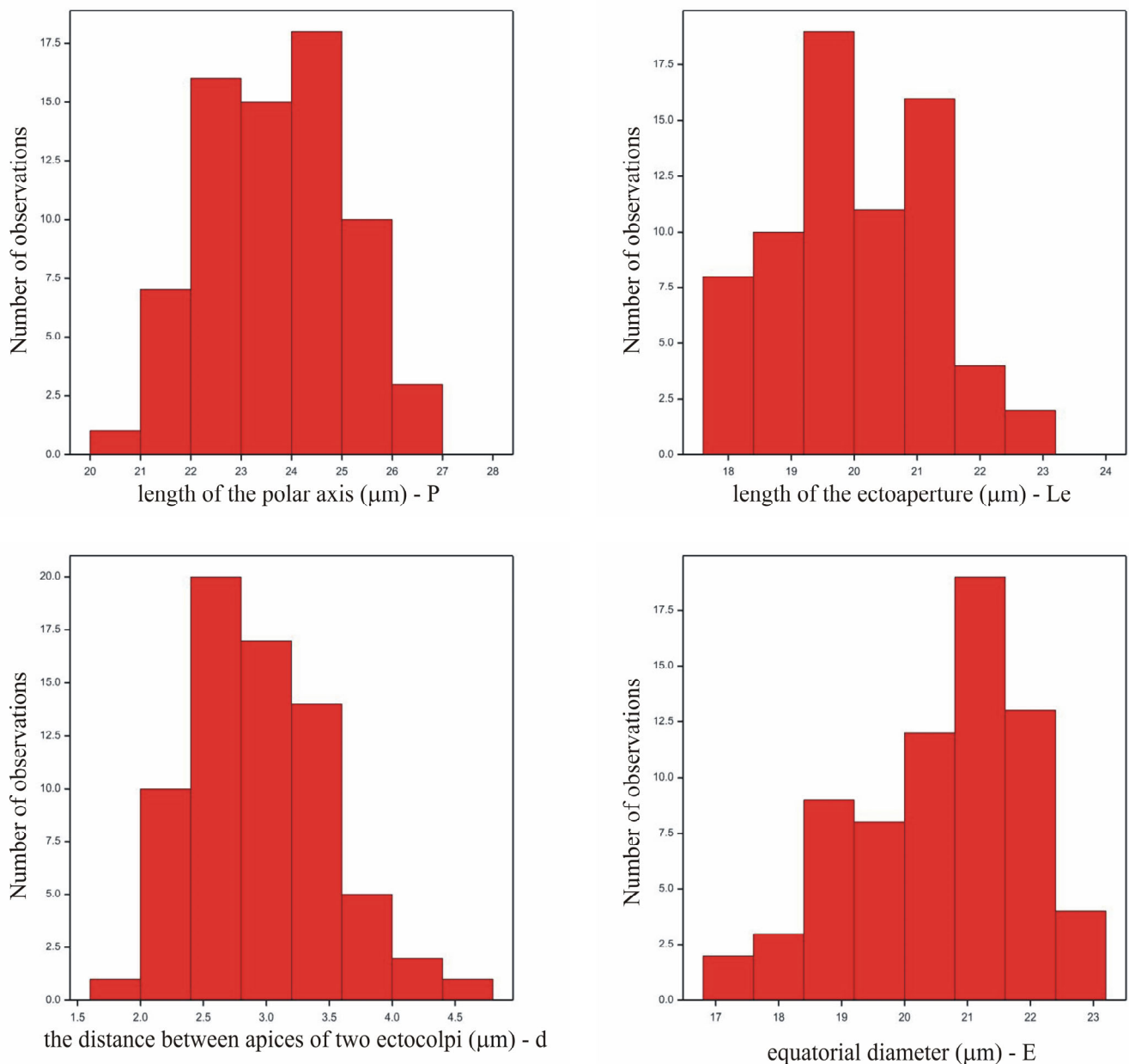


Figure 1. The empirical distribution of observations of the four traits.

The results of the multivariate analysis of variance (MANOVA) indicated that the species differed significantly when all four pollen morphology traits were considered jointly (Wilks' $\lambda = 0.0973$; $F_{60,201} = 2.74$; $p < 0.0001$). The results of the univariate analyses of variance (ANOVA) further confirmed that the main effect of species was significant for each of the four traits individually (Table 1). The mean length of the polar axis ranged from 21.71 μm in *R. divaricatus* to 25.43 μm in *R. bifrons*, with an overall mean of 23.59 μm (Table 1). Based on this trait, species were grouped into five statistically homogeneous clusters. The coefficient of variation (CV) for the mean polar axis length among species was 4.57%, representing the lowest variability among the traits analyzed. The mean length of the ectoaperture varied from 18.09 μm in *R. opacus* to 21.50 μm in *R. bifrons*, with an overall mean of 19.94 μm (Table 1). Species formed six homogeneous groups based on this

trait. The coefficient of variation for ectoaperture length was 4.77%. The mean distance between the apices of two ectocolpi ranged from 2.009 μm in *R. divaricatus* to 3.719 μm in *R. caesius*, with an overall mean of 2.93 μm (Table 1). Five homogeneous species groups were identified based on this trait. The coefficient of variation, 4.57%, was the highest among all four traits examined. The equatorial diameter ranged from 17.74 μm in *R. opacus* to 21.65 μm in *R. caesius*, with an overall mean of 20.47 μm (Table 1). Based on equatorial diameter, four homogeneous species groups were distinguished. The coefficient of variation for this trait was 5.201%.

An almost perfect correlation was observed between the length of the polar axis (P) and the length of the ectoaperture (Le), with a Pearson correlation coefficient of 0.99 (Figure 2). Statistically significant correlations were also found between P and equatorial diameter (E) ($r = 0.80$), Le and E ($r = 0.83$), as well as between E and the distance between the apices of two ectocolpi (d) ($r = 0.55$) (Figure 2). Only two correlation coefficients were not statistically significant: those between P and d ($r = 0.22$) and between Le and d ($r = 0.23$) (Figure 2).

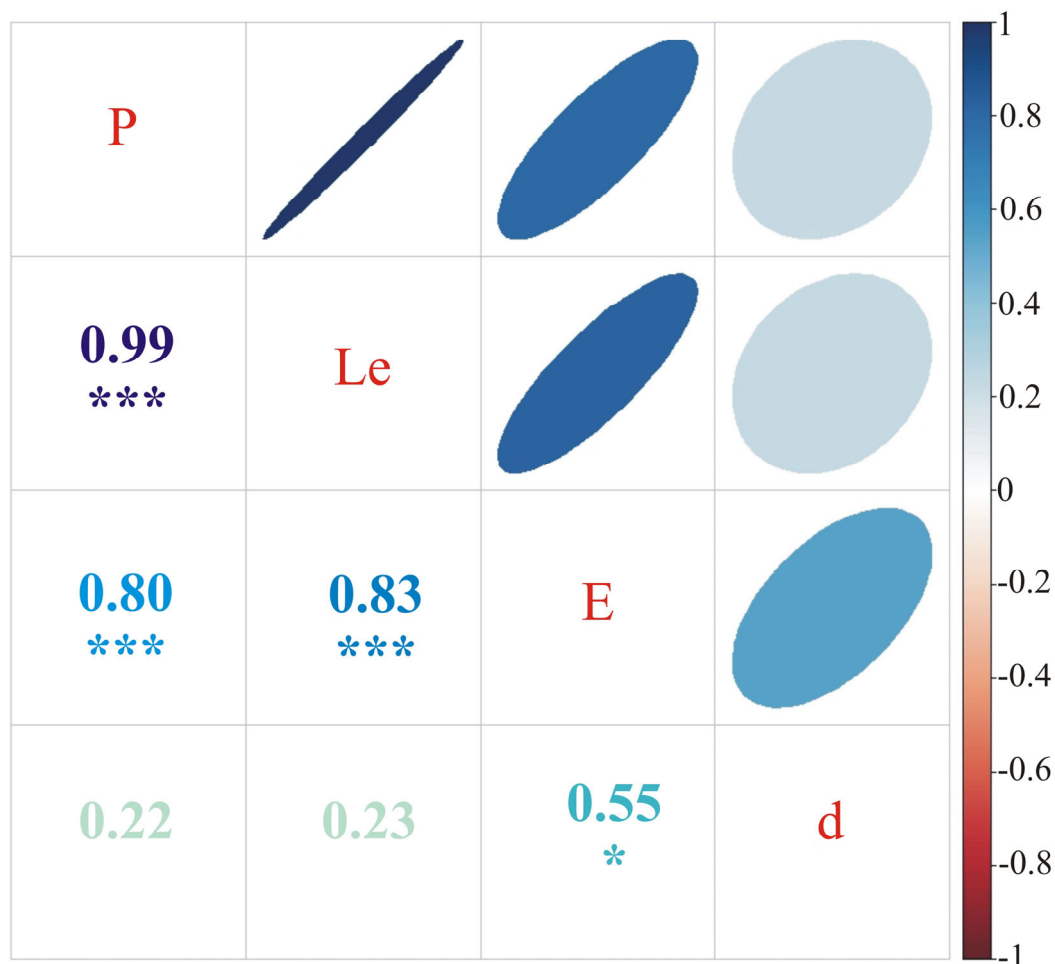


Figure 2. Heatmap showing the correlation coefficients between all pairs of observed traits. P—length of the polar axis, Le—length of the ectoaperture, d—the distance between apices of two ectocolpi, E—equatorial diameter; * $p < 0.05$; *** $p < 0.001$.

Given that individual traits form distinct homogeneous groups and contribute unequally to the overall multivariate variability among the studied species, multivariate analyses were conducted. The results of the canonical variate analysis (CVA), illustrating the distribution of the 16 *Rubus* species in the space defined by the first two canonical

variates, are presented in Figure 3. In this plot, the coordinates of each point represent the species' scores on the first and second canonical variates, respectively. The first two canonical variates together accounted for 82.32% of the total interspecific variability (Figure 3). The distance between the apices of two ectocolpi exhibited the strongest significant positive linear association with the first canonical variate ($r = 0.7013$, $p = 0.0025$), while the length of the polar axis showed a significant negative association ($r = -0.5131$, $p = 0.0421$). The second canonical variate was significantly negatively correlated with all four analyzed traits: length of the polar axis ($r = -0.8147$, $p < 0.001$), length of the ectoaperture ($r = -0.8328$, $p < 0.001$), the distance between the apices of two ectocolpi ($r = -0.7175$, $p = 0.0018$), and equatorial diameter ($r = -0.8481$, $p < 0.001$).

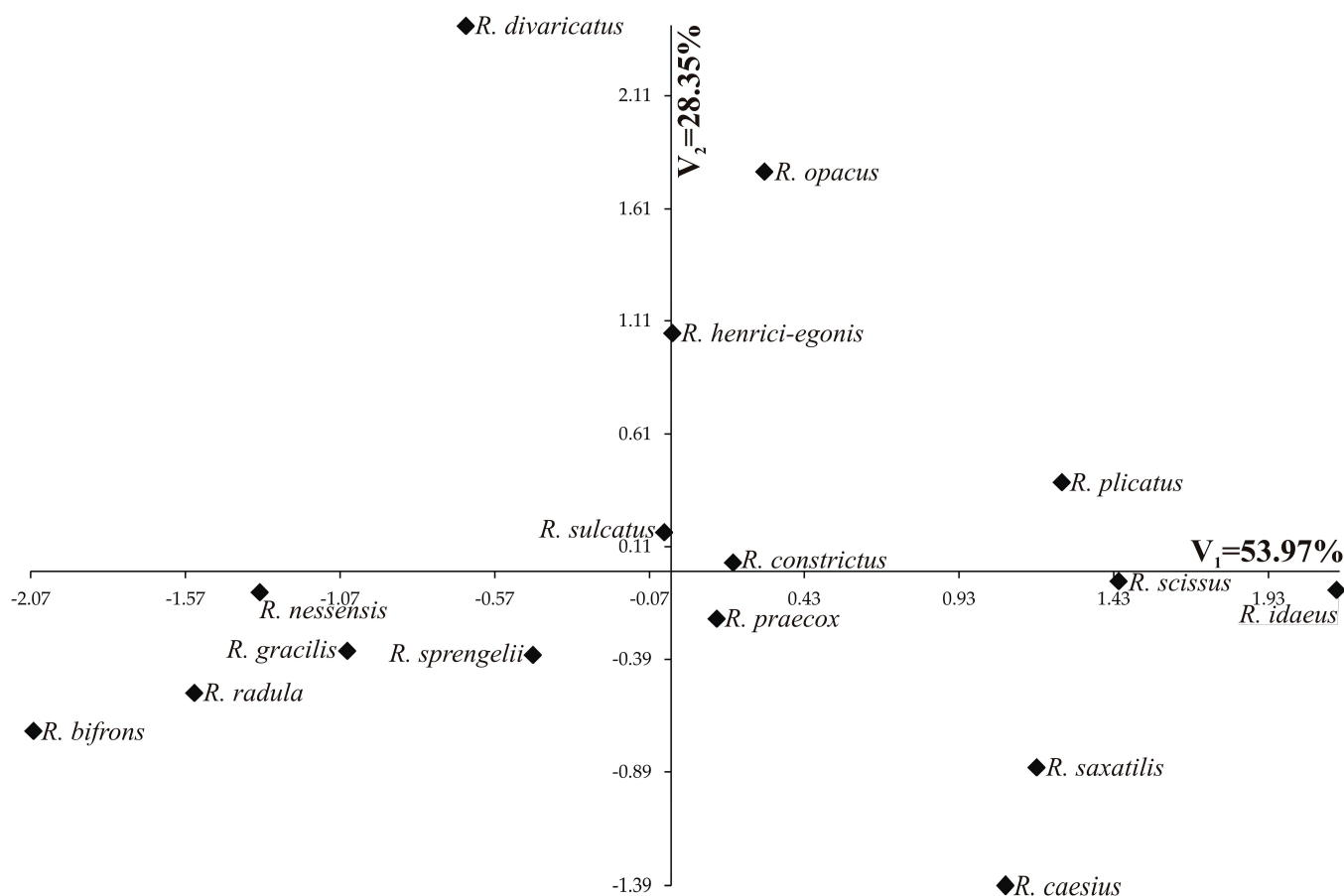


Figure 3. Distribution of 16 *Rubus* species in the space of the first two canonical variables: V_1 and V_2 .

The greatest multivariate divergence, as measured by the Mahalanobis distance, was observed between *R. bifrons* and *R. idaeus* (4.291) (Table S1, Figure 3). In contrast, the highest similarity based on the combined variation of the four traits was found between *R. praecox* and *R. sulcatus*, with a Mahalanobis distance of 0.502 (Table S1, Figure 3).

The distribution of the 16 *Rubus* species within the space defined by the first two canonical variates revealed a clear separation into four distinct groups. The first group comprised four species, *R. bifrons*, *R. gracilis*, *R. nessensis*, and *R. radula*, characterized by the highest values for both the length of the polar axis (P) and the length of the ectoaperture (Le). The second group included *R. divaricatus*, *R. henrici-egonis*, and *R. opacus*, which exhibited the lowest values across all four analyzed traits. The third group consisted of five species, *R. caesius*, *R. idaeus*, *R. plicatus*, *R. saxatilis*, and *R. scissus*, distinguished by the highest values for the distance between the apices of two ectocolpi (d). The fourth group

comprised the remaining four species, *R. constrictus*, *R. praecox*, *R. sprengelii*, and *R. sulcatus*, which displayed intermediate (average) values for all four traits (Figure 3).

3.2. Association Mapping

Association mapping identified 8320 markers that were statistically significant at the 0.05 level with at least one of the four studied traits (Table 2).

Table 2. Characteristics of molecular markers significantly associated with selected morphological traits of *Rubus* L. (Rosaceae) pollen (significant associations selected at $p < 0.05$ with Benjamini–Hochberg multiple testing correction).

Characteristics		P ¹	Le	d	E
The number of significant markers		3346	3340	3342	2882
Effect (absolute value)	Min	1.040	0.916	0.400	1.025
	Max	2.131	1.989	0.980	2.905
The percentage of variation explained	Min	19.4	19.4	19.4	19.4
	Max	63.6	65.1	60.4	70.8
LOD	Min	1.30	1.30	1.30	1.30
	Max	3.88	4.01	3.62	4.57

¹ P—length of the polar axis, Le—length of the ectoaperture, d—the distance between apices of two ectocolpi, E—equatorial diameter.

The length of the polar axis was determined by 3346 markers (Table 2, Figure 4A), and this was the trait for which the largest number of statistically significant markers were detected. The length of the ectoaperture was influenced by 3340 markers (Table 2, Figure 4B), and the distance between the apices of two ectocolpi by 3342 markers (Table 2, Figure 4C). The smallest number of markers (2882) determining the observed traits was for equatorial diameter (Table 2, Figure 4D).

The absolute effects of individual statistically significant markers ranged from 1.040 to 2.131 for the length of the polar axis, from 0.916 to 1.989 for the length of the ectoaperture, from 0.400 to 0.980 for the distance between the apices of two ectocolpi, and from 1.025 to 2.905 for the equatorial diameter (Table 2). The proportion of phenotypic variance explained by each marker varied from 19.4% to 63.6% for the length of the polar axis (markers 5832 and 59703), from 19.4% to 65.0% for the length of the ectoaperture (markers 5824 and 59711), from 19.4% to 60.4% for the distance between apices of two ectocolpi (markers 16972 and 48563), and from 19.4% to 70.8% for equatorial diameter (markers 4224 and 61311) (Table 2). The highest LOD scores ranged from 3.62 (for the distance between apices of two ectocolpi) to 4.57 (for equatorial diameter) (Table 2, Figure 4).

A total of 44 markers were simultaneously statistically significant ($p < 0.05$, LOD > 1.30) for all four morphological traits of *Rubus* L. (Rosaceae) pollen (Table 3). The effects of the individual markers exhibited consistent directions across all traits. The proportion of variation explained by these common significant markers ranged from 19.50% (for markers m [5509] = 0001010110000101 and m [60026] = 1110101001111010) to 46.90% (for markers m [5248] = 0001010010000000 and m [60287] = 1110101101111111) for the length of the polar axis; from 20.40% (for markers m [13443] = 0011010010000011 and m [52092] = 1100101101111100) to 51.80% (for markers m [5248] = 0001010010000000 and m [60287] = 1110101101111111) for the length of the ectoaperture; from 19.40% (for markers m [13761] = 0011010111000001 and m [51774] = 1100101000111110) to 35.70% (for markers m [5504] = 0001010110000000 and m [60031] = 1110101001111111) for the distance between the apices of two ectocolpi; and from 25.00% (for markers m [7361] = 0001110011000001 and m [58174] = 1110001100111110) to 70.80% (for markers m [4224] = 0001000010000000 and m [61311] = 1110111101111111) for the equatorial diameter (Table 3).

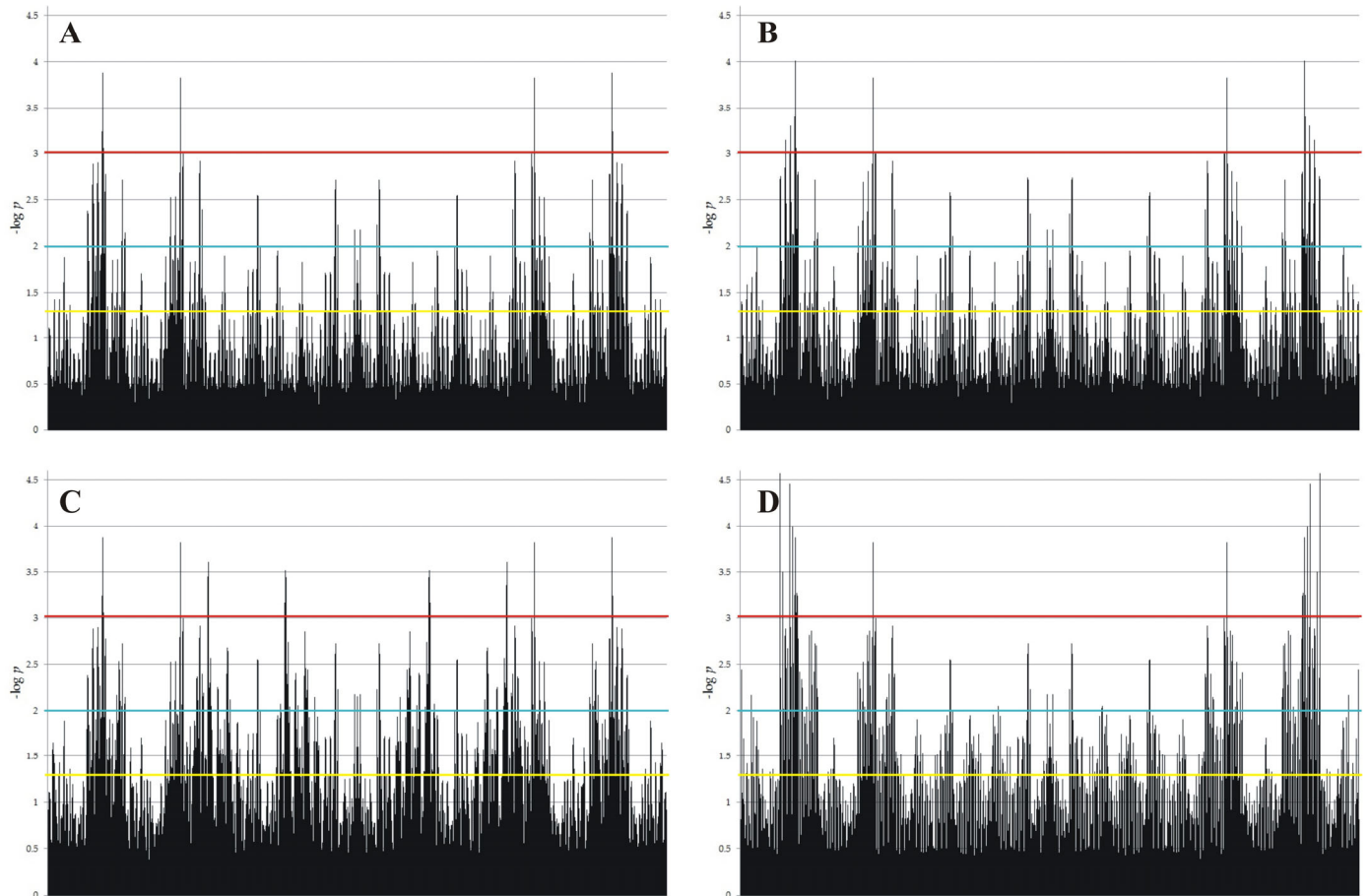


Figure 4. Manhattan plot for: length of the polar axis (A), length of the ectoaperture (B), the distance between apices of two ectocolpi (C), and equatorial diameter (D). The yellow line indicates significance at the 0.05 level (LOD = 1.30), the blue line at the 0.01 level (LOD = 2.0), and the red line at the 0.001 level (LOD = 3.0). The statistical significance of marker–trait associations was evaluated using p -values adjusted for multiple testing using the Benjamini–Hochberg false discovery rate correction method.

Particularly noteworthy is the observation that identical results (apart from the opposite sign of the effect) were obtained for ‘twin’ markers, i.e., markers that have an inverted 0–1 binary pattern. For example, markers $m[13761] = 0011010111000001$ and $m[51774] = 1100101000111110$ yielded identical values for one of the traits, namely the length of the polar axis (P): the proportion of explained variation, p -value, and LOD score were 24.0%, 0.03105, and 1.51, respectively, for both markers (Table 3). However, the effects were -1.136 and 1.136 for $m[13761]$ and $m[51774]$, respectively (Table 3). This indicates that, in subsequent stages of breeding work, it should be sufficient to employ only one of these ‘twin’ markers.

Table 3. Forty-four markers were statistically significant ($p < 0.05$) simultaneously for all four considered morphological traits of *Rubus* L. (Rosaceae) pollen.

Marker	P				Le				d				E			
	Effect	Perc ¹	p-Value	LOD	Effect	Perc	p-Value	LOD	Effect	Perc	p-Value	LOD	Effect	Perc	p-Value	LOD
m [4224] = 0001000010000000	−2.131	41.6	0.00416	2.38	−1.989	47.7	0.00184	2.74	−0.71	29.5	0.01742	1.76	−2.658	70.8	0.00003	4.57
m [4225] = 0001000010000001	−1.556	29.1	0.01821	1.74	−1.4	30.7	0.01518	1.82	−0.55	23.4	0.03302	1.48	−1.819	43.7	0.00317	2.50
m [5248] = 0001010010000000	−1.902	46.9	0.00205	2.69	−1.747	51.8	0.00101	3.00	−0.641	34.4	0.01001	2.00	−2.237	69.8	0.00003	4.46
m [5249] = 0001010010000001	−1.566	38.0	0.00653	2.19	−1.397	39.2	0.00559	2.25	−0.553	31.0	0.01477	1.83	−1.745	50.5	0.00123	2.91
m [5250] = 0001010010000010	−1.354	26.6	0.02381	1.62	−1.214	27.9	0.02069	1.68	−0.523	26.9	0.02292	1.64	−1.731	49.5	0.00142	2.85
m [5251] = 0001010010000011	−1.2	23.2	0.03384	1.47	−1.04	22.3	0.03712	1.43	−0.485	26.4	0.02421	1.62	−1.447	38.2	0.00634	2.20
m [5280] = 0001010010100000	−1.496	34.0	0.01041	1.98	−1.306	33.4	0.01123	1.95	−0.507	24.8	0.02854	1.54	−1.679	46.2	0.00227	2.64
m [5281] = 0001010010100001	−1.324	29.8	0.01673	1.78	−1.12	27.0	0.02267	1.64	−0.471	24.5	0.02964	1.53	−1.402	35.4	0.00884	2.05
m [5504] = 0001010110000000	−1.28	23.0	0.03452	1.46	−1.242	29.5	0.01733	1.76	−0.587	35.7	0.00854	2.07	−1.952	64.9	0.00010	4.00
m [5505] = 0001010110000001	−1.136	20.0	0.04664	1.33	−1.064	23.7	0.03214	1.49	−0.541	34.6	0.00981	2.01	−1.64	51.1	0.00111	2.95
m [5509] = 0001010110000101	−1.077	19.5	0.04920	1.31	−0.993	22.2	0.03769	1.42	−0.414	19.5	0.04925	1.31	−1.295	32.5	0.01242	1.91
m [5568] = 0001010111000000	−1.307	28.9	0.01860	1.73	−1.271	36.8	0.00750	2.12	−0.456	22.6	0.03609	1.44	−1.534	43.8	0.00311	2.51
m [5569] = 0001010111000001	−1.215	26.8	0.02322	1.63	−1.147	31.9	0.01323	1.88	−0.444	23.6	0.03246	1.49	−1.348	35.8	0.00848	2.07
m [5601] = 0001010111100001	−1.12	23.1	0.03407	1.47	−1.006	24.4	0.02986	1.52	−0.413	20.7	0.04362	1.36	−1.178	27.3	0.02205	1.66
m [7296] = 0001110010000000	−1.262	22.1	0.03775	1.42	−1.139	23.7	0.03227	1.49	−0.574	33.8	0.01067	1.97	−1.725	49.1	0.00150	2.82
m [7360] = 0001110011000000	−1.291	28.0	0.02049	1.69	−1.181	30.8	0.01500	1.82	−0.445	21.1	0.04197	1.38	−1.335	31.5	0.01391	1.86
m [7361] = 0001110011000001	−1.2	26.0	0.02536	1.60	−1.064	26.5	0.02394	1.62	−0.434	22.2	0.03766	1.42	−1.166	25.0	0.02810	1.55
m [13440] = 0011010010000000	−1.518	35.2	0.00905	2.04	−1.431	41.5	0.00421	2.38	−0.493	23.2	0.03391	1.47	−1.724	49.1	0.00151	2.82
m [13441] = 0011010010000001	−1.343	30.9	0.01488	1.83	−1.229	34.0	0.01046	1.98	−0.459	22.9	0.03477	1.46	−1.441	37.9	0.00662	2.18
m [13442] = 0011010010000010	−1.158	21.1	0.04193	1.38	−1.069	24.0	0.03120	1.51	−0.433	19.6	0.04884	1.31	−1.428	37.1	0.00729	2.14
m [13443] = 0011010010000011	−1.078	19.6	0.04887	1.31	−0.962	20.4	0.04520	1.34	−0.423	20.7	0.04372	1.36	−1.251	29.9	0.01666	1.78
m [13761] = 0011010111000001	−1.136	24.0	0.03105	1.51	−1.101	30.7	0.01525	1.82	−0.403	19.4	0.04998	1.30	−1.212	29.3	0.01769	1.75
m [51774] = 1100101000111110	1.136	24.0	0.03105	1.51	1.101	30.7	0.01525	1.82	0.403	19.4	0.04998	1.30	1.212	29.3	0.01769	1.75
m [52092] = 1100101010111100	1.078	19.6	0.04887	1.31	0.962	20.4	0.04520	1.34	0.423	20.7	0.04372	1.36	1.251	29.9	0.01666	1.78
m [52093] = 1100101010111101	1.158	21.1	0.04193	1.38	1.069	24.0	0.03120	1.51	0.433	19.6	0.04884	1.31	1.428	37.1	0.00729	2.14
m [52094] = 1100101010111110	1.343	30.9	0.01488	1.83	1.229	34.0	0.01046	1.98	0.459	22.9	0.03477	1.46	1.441	37.9	0.00662	2.18
m [52095] = 1100101010111111	1.518	35.2	0.00905	2.04	1.431	41.5	0.00421	2.38	0.493	23.2	0.03391	1.47	1.724	49.1	0.00151	2.82
m [58174] = 1110001100111110	1.2	26.0	0.02536	1.60	1.064	26.5	0.02394	1.62	0.434	22.2	0.03766	1.42	1.166	25.0	0.02810	1.55
m [58175] = 1110001100111111	1.291	28.0	0.02049	1.69	1.181	30.8	0.01500	1.82	0.445	21.1	0.04197	1.38	1.335	31.5	0.01391	1.86
m [58239] = 1110001101111111	1.262	22.1	0.03775	1.42	1.139	23.7	0.03227	1.49	0.574	33.8	0.01067	1.97	1.725	49.1	0.00150	2.82
m [59934] = 1110101000011110	1.12	23.1	0.03407	1.47	1.006	24.4	0.02986	1.52	0.413	20.7	0.04362	1.36	1.178	27.3	0.02205	1.66
m [59966] = 1110101000111110	1.215	26.8	0.02322	1.63	1.147	31.9	0.01323	1.88	0.444	23.6	0.03246	1.49	1.348	35.8	0.00848	2.07
m [59967] = 1110101000111111	1.307	28.9	0.01860	1.73	1.271	36.8	0.00750	2.12	0.456	22.6	0.03609	1.44	1.534	43.8	0.00311	2.51
m [60026] = 1110101001111010	1.077	19.5	0.04920	1.31	0.993	22.2	0.03769	1.42	0.414	19.5	0.04925	1.31	1.295	32.5	0.01242	1.91
m [60030] = 1110101001111110	1.136	20.0	0.04664	1.33	1.064	23.7	0.03214	1.49	0.541	34.6	0.00981	2.01	1.64	51.1	0.00111	2.95
m [60031] = 1110101001111111	1.28	23.0	0.03452	1.46	1.242	29.5	0.01733	1.76	0.587	35.7	0.00854	2.07	1.952	64.9	0.00010	4.00
m [60254] = 1110101010101110	1.324	29.8	0.01673	1.78	1.12	27.0	0.02267	1.64	0.471	24.5	0.02964	1.53	1.402	35.4	0.00884	2.05
m [60255] = 1110101010101111	1.496	34.0	0.01041	1.98	1.306	33.4	0.01123	1.95	0.507	24.8	0.02854	1.54	1.679	46.2	0.00227	2.64
m [60284] = 1110101010111100	1.2	23.2	0.03384	1.47	1.04	22.3	0.03712	1.43	0.485	26.4	0.02421	1.62	1.447	38.2	0.00634	2.20
m [60285] = 1110101010111101	1.354	26.6	0.02381	1.62	1.214	27.9	0.02069	1.68	0.523	26.9	0.02292	1.64	1.731	49.5	0.00142	2.85
m [60286] = 1110101010111110	1.566	38.0	0.00653	2.19	1.397	39.2	0.00559	2.25	0.553	31.0	0.01477	1.83	1.745	50.5	0.00123	2.91
m [60287] = 1110101010111111	1.902	46.9	0.00205	2.69	1.747	51.8	0.00101	3.00	0.641	34.4	0.01001	2.00	2.237	69.8	0.00003	4.46
m [61310] = 11101011101011110	1.556	29.1	0.01821	1.74	1.4	30.7	0.01518	1.82	0.55	23.4	0.03302	1.48	1.819	43.7	0.00317	2.50
m [61311] = 11101011101011111	2.131	41.6	0.00416	2.38	1.989	47.7	0.00184	2.74	0.71	29.5	0.01742	1.76	2.658	70.8	0.00003	4.57

¹ The percentage of variation explained by individual markers.

4. Discussion

Assessing biodiversity without accounting for genetic variability may result in an incomplete evaluation [42]. Analyses based solely on agronomic or morphological traits risk underestimating the true diversity of local populations, as phenotypic plasticity can obscure underlying genetic variation, particularly in environments strongly influenced by climatic factors [43]. In the absence of genetic analyses, it is challenging to determine whether local populations are genuinely distinct or if gene flow contributes to their homogeneity. This has important implications for conservation and valorization efforts. A comprehensive understanding of genetic diversity and intraspecific relationships within plant species is crucial for the development of effective conservation and breeding strategies [44,45]. Molecular markers are indispensable tools for the identification and characterization of plant species and hybrids, as well as for assessing the status of natural populations threatened by disease and genetic contamination [46–48]. In the present study, phenotypic variability was observed for all four morphological traits of *Rubus* pollen under investigation. This variability was evident both at the level of individual traits (Table 1) and in multivariate analyses using Mahalanobis distances (Table S1) and canonical variable analysis (Figure 3). The distribution of the studied *Rubus* species in the space defined by the first two canonical variables is particularly noteworthy, as the information loss resulting from reducing the similarity/dissimilarity structure to two dimensions (a plane) is only 17.68% (Figure 3).

Molecular markers are highly valuable for practical applications, such as the breeding of new cultivars, including raspberries and blackberries. They enable the identification of genetic traits responsible for disease resistance [49], fruit flavor [50], and yield performance [7]. This allows for more targeted and efficient breeding efforts, which is significant not only for scientific research but also for agriculture and the food industry [51,52]. Molecular markers constitute an indispensable tool in studies of *Rubus* L. species [53,54]. They facilitate a deeper understanding of the complex genetic relationships within this plant group, support accurate species identification, and aid in breeding and conservation efforts. Given the challenges associated with classifying plants with such high variability as *Rubus*, modern molecular techniques are not only beneficial but essential.

The molecular markers most commonly used in studies of the genus *Rubus* include SSR [18,55–58], SNP [19,59,60], and AFLP markers [61–63]. The application of these markers enables, among other things, the assessment of relatedness among species or individuals [19], the identification of hybrids [55], the confirmation of the taxonomic status of ambiguous systematic units [19,54], and the analysis of genetic diversity within wild and cultivated populations [56].

Numerous studies have demonstrated that molecular markers effectively support species identification and the assessment of intraspecific variability within the genus *Rubus*. Huang et al. [54] elucidated the global phylogenetic relationships within *Rubus*, incorporating an extensive sampling of Chinese endemic species, and accordingly revised the infrageneric classification based on combined morphological and molecular evidence. Sochor and Manning [55] investigated evolutionary patterns and mechanisms in South African blackberries using three types of molecular markers: plastid DNA sequences, nuclear DNA sequences, and nuclear microsatellites. The analysis of SSR markers enabled the assessment of genetic diversity and population structure in wild Korean black raspberry (*Rubus coreanus* Miq.) [56]. AFLP markers were used to construct a genetic linkage map for black raspberry (*Rubus occidentalis*) and red raspberry (*R. idaeus*) based on interspecific progeny [63]. The application of SNP markers has made it possible, among other findings, to confirm the polyphyly of several traditionally circumscribed subgenera, sections, and

subsections within *Rubus*, and to identify 19 well-supported clades that are molecularly, morphologically, and geographically distinct [19]. In addition, SSR markers were used to determine the genetic similarity and to characterize a collection of 21 *R. ellipticus* accessions from different regions of India [18].

When combined with morphological analyses (e.g., pollen studies), molecular markers also make it possible to verify whether external traits are consistent with genetic data, which is invaluable for taxa that are difficult to classify unambiguously [15].

Molecular markers are an indispensable component of contemporary research on the taxonomy, phylogeny, and genetic variability of the genus *Rubus*. By overcoming the limitations of traditional morphology-based methods, they provide robust insights into plant genetic structure. Their application is valuable not only in fundamental science but also in breeding programs, conservation biology, and the identification of morphologically cryptic species. In the context of accelerating environmental change and the increasing homogenization of cultivated germplasm, the judicious use of molecular markers will be pivotal to future *Rubus* research and practice. The results obtained here confirm the utility of molecular markers as an auxiliary tool for classifying and analyzing variability within this genus. They enable the detection of hidden diversity that often remains invisible to morphological assessment. Association mapping pinpointed 44 markers linked to candidate genes that jointly determine the four *Rubus* pollen traits examined. Because many loci occur in “twin” pairs (opposite 0/1 allele configurations), the set of informative markers should be reduced to 22 for subsequent research stages. Importantly, each significant marker showed an allelic effect of the same sign across all four pollen traits. The ten markers with the highest total LOD value for four pollen morphological traits allowed the distinction of six species: *R. bifrons*, *R. caesius*, *R. idaeus*, *R. radula*, *R. saxatilis*, and *R. scissus* (Figure 5). Figure 5 further presents the association mapping scheme for identifying candidate genes for the four considered morphological traits of *Rubus* L. pollen and the most significant results obtained for 16 species. Three statistically significant loci warrant particular attention—m [5248] = 0001010010000000, m [4224] = 0001000010000000, and m [5249] = 0001010010000001 (or, after inversion of the 0/1 coding, m [60287] = 1110101101111111, m [61311] = 1110111101111111, and m [60286] = 1110101101111110)—as they exhibited the highest cumulative LOD values (Table 3). In the face of climate change and ongoing biodiversity loss, these markers may serve as molecular-genetic tools that support the conservation of wild *Rubus* species, many of which inhabit ecologically valuable sites. Association mapping is a tool very often used to analyze marker-trait relationships for different species.

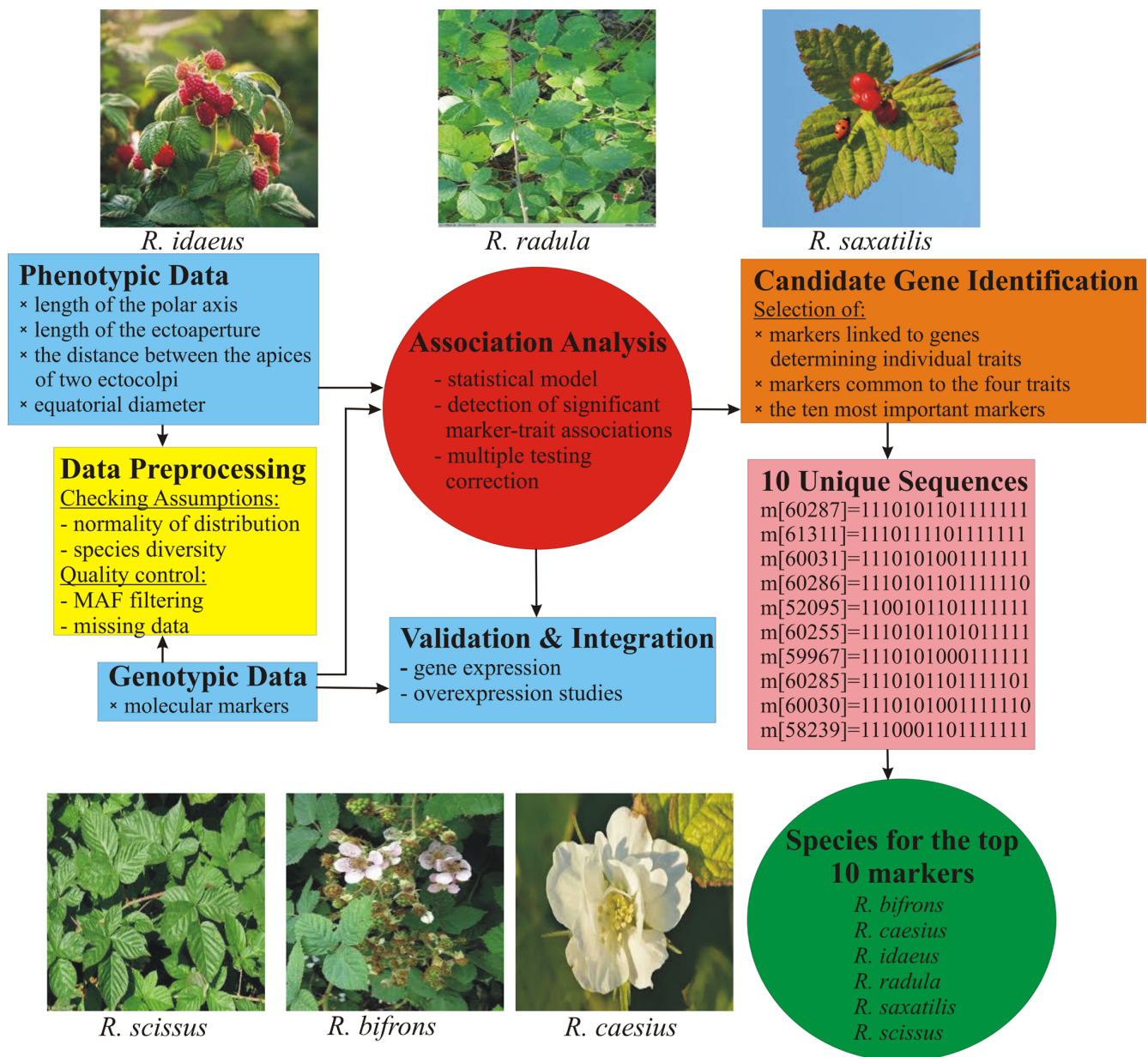


Figure 5. Association mapping scheme for identifying candidate genes for four pollen morphological traits in *Rubus* L., and the most significant results obtained for 16 species.

5. Conclusions

Molecular markers play a pivotal role in the study of genetic variability within the genus *Rubus*. Based on the available literature, this is the first report of association mapping used to identify candidate genes for pollen morphological traits in *Rubus* L. The results of this study demonstrated that molecular markers are effective and reliable tools for evaluating morphological traits among *Rubus* genotypes. The ten markers with the highest total LOD value for four pollen morphological traits allowed the distinction of six species: *R. bifrons*, *R. caesius*, *R. idaeus*, *R. radula*, *R. saxatilis*, and *R. scissus*. Notably, six markers $m[5248] = 0001010010000000$, $m[4224] = 0001000010000000$, $m[5249] = 0001010010000001$, $m[60287] = 1110101101111111$, $m[61311] = 1110111101111111$, and $m[60286] = 1110101101111110$ exhibited particularly high cumulative LOD scores, reflecting their strong discriminatory power. In summary, the findings suggest that these markers are highly useful for the characterization of genetic resources, diversity assessment, and inference of phylogenetic relationships within the genus *Rubus*. The approach presented here—based on analyzing all possible binary marker

combinations—can be applied broadly to other species. It offers a means to streamline the otherwise complex and time-consuming process of association mapping, while also reducing the costs associated with developing molecular markers. Moreover, selected molecular markers that significantly determine the observed traits can be used to select genotypes characterized by the most favorable parameters determining the pollen structure.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f16091395/s1>, Table S1: Mahalanobis distances between 16 *Rubus* species were calculated based on four observed traits.

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Data Availability Statement: All phenotypic data on *Rubus* L. (Rosaceae) pollen morphology are included in the manuscript and presented in Table A1 in Appendix A. Data for all possible 65,534 theoretical markers were generated and stored in a binary system according to the method described in Appendix B.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ISSR	Inter Simple Sequence Repeats
SSR	Simple Sequence Repeats
DNA	Deoxyribonucleic acid
QTL	Quantitative Trait Loci
MANOVA	Multivariate analysis of variance
ANOVA	Analysis of variance
LSD	Least significant difference
s.d.	Standard deviation
P	Length of the polar axis
Le	Length of the ectoaperture
d	The distance between the apices of two ectocolpi
E	Equatorial diameter
LOD	The limit of detection
SNP	Single-nucleotide polymorphism
AFLP	Amplified fragment length polymorphism

Appendix A

Table A1. Data on four quantitative traits characterizing pollen morphology were used for analysis [33].

No.	Species	P ¹	Le ²	d ³	E ⁴	No.	Species	P	Le	d	E
1	<i>Rubus bifrons</i>	25.53	21.22	2.360	21.40	9	<i>Rubus opacus</i>	20.54	17.73	2.147	16.88
1	<i>Rubus bifrons</i>	24.84	20.89	2.968	21.54	9	<i>Rubus opacus</i>	22.96	18.46	3.061	18.61
1	<i>Rubus bifrons</i>	26.69	22.98	2.842	22.36	10	<i>Rubus plicatus</i>	23.11	19.75	2.638	21.30
1	<i>Rubus bifrons</i>	24.78	21.09	2.580	21.35	10	<i>Rubus plicatus</i>	21.15	18.36	2.833	18.97
1	<i>Rubus bifrons</i>	25.33	21.31	2.531	21.01	10	<i>Rubus plicatus</i>	24.29	19.74	3.138	21.79

Table A1. Cont.

No.	Species	P ¹	Le ²	d ³	E ⁴	No.	Species	P	Le	d	E
2	<i>Rubus caesius</i>	21.89	18.26	2.347	18.39	10	<i>Rubus plicatus</i>	23.46	19.33	3.333	21.25
2	<i>Rubus caesius</i>	26.15	22.38	3.850	22.92	10	<i>Rubus plicatus</i>	22.70	19.30	3.653	21.95
2	<i>Rubus caesius</i>	25.72	21.75	3.770	22.97	11	<i>Rubus praecox</i>	24.29	20.70	2.811	21.67
2	<i>Rubus caesius</i>	25.58	21.03	4.553	21.77	11	<i>Rubus praecox</i>	22.27	18.87	3.384	19.53
2	<i>Rubus caesius</i>	23.68	19.83	4.075	22.22	11	<i>Rubus praecox</i>	23.04	19.78	2.700	20.44
3	<i>Rubus constrictus</i>	23.89	20.10	3.283	21.32	11	<i>Rubus praecox</i>	25.36	21.77	2.979	21.90
3	<i>Rubus constrictus</i>	23.46	19.71	2.734	20.18	12	<i>Rubus radula</i>	26.76	23.15	2.428	22.14
4	<i>Rubus divaricatus</i>	22.34	19.15	1.834	18.93	12	<i>Rubus radula</i>	23.63	20.09	2.620	20.58
4	<i>Rubus divaricatus</i>	21.01	17.70	2.066	16.81	12	<i>Rubus radula</i>	25.63	21.29	3.153	21.84
4	<i>Rubus divaricatus</i>	21.76	18.17	2.088	19.27	12	<i>Rubus radula</i>	24.27	19.98	3.324	21.11
4	<i>Rubus divaricatus</i>	21.72	18.19	2.047	19.15	12	<i>Rubus radula</i>	25.05	21.25	2.153	21.82
5	<i>Rubus gracilis</i>	25.63	21.65	3.098	22.08	13	<i>Rubus saxatilis</i>	24.48	21.57	3.669	20.31
5	<i>Rubus gracilis</i>	23.70	20.33	2.581	20.70	13	<i>Rubus saxatilis</i>	24.12	20.00	3.421	20.86
5	<i>Rubus gracilis</i>	24.69	21.41	3.000	21.43	13	<i>Rubus saxatilis</i>	22.63	19.24	3.345	20.83
5	<i>Rubus gracilis</i>	22.74	19.29	2.442	18.23	13	<i>Rubus saxatilis</i>	22.22	18.80	3.544	19.77
5	<i>Rubus gracilis</i>	25.46	21.23	2.720	21.30	13	<i>Rubus saxatilis</i>	22.54	19.15	3.239	19.37
6	<i>Rubus henrici-egonis</i>	22.67	18.84	2.266	18.64	14	<i>Rubus scissus</i>	22.73	19.18	2.597	20.94
6	<i>Rubus henrici-egonis</i>	24.42	20.87	3.722	22.53	14	<i>Rubus scissus</i>	23.29	19.80	3.529	21.68
6	<i>Rubus henrici-egonis</i>	22.42	18.91	2.151	19.13	14	<i>Rubus scissus</i>	24.36	20.64	3.576	21.13
6	<i>Rubus henrici-egonis</i>	21.23	17.92	2.288	18.91	15	<i>Rubus sprengelii</i>	24.80	21.15	3.222	21.08
6	<i>Rubus henrici-egonis</i>	22.70	19.26	2.614	19.12	15	<i>Rubus sprengelii</i>	23.50	19.75	2.774	20.72
7	<i>Rubus idaeus</i>	22.16	19.23	3.444	20.13	15	<i>Rubus sprengelii</i>	24.74	20.91	2.707	19.92
7	<i>Rubus idaeus</i>	24.62	20.32	4.053	22.46	15	<i>Rubus sprengelii</i>	24.22	20.52	2.997	21.00
7	<i>Rubus idaeus</i>	22.81	19.15	3.076	19.43	15	<i>Rubus sprengelii</i>	23.58	20.45	2.900	20.93
7	<i>Rubus idaeus</i>	21.39	18.39	3.321	19.51	16	<i>Rubus sulcatus</i>	24.34	20.41	3.011	21.65
7	<i>Rubus idaeus</i>	22.52	19.12	3.542	20.77	16	<i>Rubus sulcatus</i>	22.46	19.31	2.696	18.37
8	<i>Rubus nessensis</i>	23.10	19.60	2.549	19.00	16	<i>Rubus sulcatus</i>	23.10	19.55	2.780	21.33
8	<i>Rubus nessensis</i>	24.00	20.26	3.070	20.11	16	<i>Rubus sulcatus</i>	23.09	19.38	2.927	20.66
8	<i>Rubus nessensis</i>	25.63	21.34	2.597	20.80	16	<i>Rubus sulcatus</i>	24.66	21.38	2.734	21.42
8	<i>Rubus nessensis</i>	24.90	20.82	2.987	20.63						
8	<i>Rubus nessensis</i>	24.31	20.34	2.440	19.79						

¹ P—length of the polar axis, ² Le—length of the ectoaperture, ³ d—the distance between apices of two ectocolpi, ⁴ E—equatorial diameter.

Appendix B

Example of converting a number from binary (1100111₂) to decimal (103₁₀):

= 1 + 1 + 0 + 0 + 1 + 1 + 1 = 64 + 32 + 40 + 0 + 4 + 2 + 1 = (A1)

Table A2. Example of converting a number from decimal (103₁₀) to binary (1100111₂).

Integer from Division by 2	Residual from Division by 2	Direction of Writing Digits in Binary Number Notation
103		
51	1	
25	1	
12	1	
6	0	↑
3	0	
1	1	
0	1	

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