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Variation and Heritability Estimates of Anthocyanins and Their Relationship to Antioxidant Activity in a Red Raspberry Factorial Mating Design

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Abstract. **We determined variance components and narrow-sense heritability estimates for total and individual anthocyanin (ACY) content and antioxidant activity (AA) in fruit from 411 genotypes in a red raspberry (*Rubus idaeus* L.) factorial mating design based on 42 full-sib families derived from seven female and six male parents, harvested in 2002 and 2003. Within half-sib family total ACY content ranged from ≈1–60+ mg/100 g fruit in both seasons. The four major ACYs quantified by high-performance liquid chromatography also showed wide ranges each year. Female and male parent contributions to variation in total and individual ACYs were significant ($P \leq 0.001$) in combined year analysis, and together accounted for 29% to 48% of the total variation. A substantial proportion of the female contribution was attributed to the use of a pigment-deficient *R. parvifolius* L. × *R. idaeus* hybrid derivative as a female parent. Female × male interaction was nonsignificant and contributed negligibly to total variance. Year effects accounted for <2.5% of variation in ACYs and were only marginally significant. Year interactions were negligible. Within family variation (among plots and within plot) accounted for ≈50% of the variation in total ACY and 62% to 69% of the variation in individual ACYs. Combined year narrow-sense heritability estimates were high (h^2 = 0.54–0.90 for individual ACYs, 1.00 for total ACY) among all factorial genotypes, but moderate when the progeny of the *R. parvifolius* derivative were excluded (h^2 = 0.45–0.78 for individual ACYs, 0.74 for total ACY). The latter estimates are applicable to breeding programs in which pigment-deficient genotypes are rarely or never used in breeding. Parental main effects were significant for AA, together accounting for 19% of total variance; female × male interaction was nonsignificant. Year effects were marginally significant and year interactions nonsignificant; together these sources of variation contributed <2% of total variation in AA. The majority of AA variation was found within- and among-plots within family. The phenotypic correlation between AA and total ACY was r = 0.53, and ranged from r = 0.21–0.46 between AA and individual ACYs; genetic correlations between AA and the ACYs were similar to the phenotypic correlations, suggesting predominantly additive genetic effects accounted for the phenotypic correlations. Linear modelling for AA based on individual ACYs and their interactions explained ≈0.53 of AA variation, substantially less than that explained by total phenolic content (R^2 = 0.88). Our results show substantial variation and moderate to high narrow-sense heritability estimates for red raspberry ACYs, but ACY content and profile information are ineffective proxies and predictors for AA in red raspberry fruit.**

Red raspberry fruit color is an important quality character in breeding improved genotypes of this crop, as it contributes greatly to the appearance of the fresh fruit and processed products, and therefore influences the acceptance of new cultivars by producers and processors. The color of red raspberries and other berries depends partly on the concentration of anthocyanins, the polyphenolic compounds that constitute one of the classes of flavonoids. Flavonoids and other phenolic compounds present in the plant foods in our diet are of particular interest to human health researchers because they possess antioxidant activity in vitro. Antioxidants are key components in preventing the oxidation of cellular lipids, proteins and nucleic acids by free radical compounds, and oxidative cellular damage has been hypothesized to be the mechanism by which many degenerative and chronic diseases develop, including stroke, coronary heart disease, and neurodegenerative diseases (Cadet and Brannock, 1998; Esterbauer et al., 1992; Markesbery, 1997; Praticò, 2002; Schwenke, 1998; Witztum, 1994). Thus, increasing our intake of dietary antioxidants, including vitamins E and C and the carotenoids, is thought to be important in preventing these diseases, as well as some joint and eye diseases, and possibly some cancers. The presence of the antioxidant flavonoids and other phenolic compounds in fruits and vegetables has been suggested as one of the links between higher fruit and vegetable consumption and lower risk of stroke, cardiovascular disease, and cancer that is noted in some epidemiologic studies (Commenges et al., 2000; Geleijnse et al., 2002; Hertog et al., 1997; Hirvonen et al., 2001; Knekt et al., 2002).

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Breeding for higher levels of antioxidants in fruits and vegetables is one way in which to increase dietary intake of these compounds. Berries may be good candidates for this breeding goal, as many are reported to possess considerable in vitro antioxidant activity (AA) in comparison to other plant foods (Halvorsen et al., 2002, Kähkönen et al., 1999; Wang et al., 1996), possibly as a consequence of their high concentrations of phenolic acids, anthocyanins (ACYs), flavonols, and other phenolic compounds (Häkkinen et al., 1999; Herrmann, 1989; Kähkönen et al., 2001; Proteggente et al., 2002), as well as vitamin C and other nonphenolic antioxidants. Although the efficacy of a food or food extract as an in vivo antioxidant cannot be directly inferred from its in vitro AA, in vitro measurement remains the most practical and efficient method of estimating AA. In our investigation of AA in a red raspberry factorial mating design (Connor et al., 2005), we found high phenotypic and moderate genotypic correlations between AA and total phenolic content (TPH) (0.93 and 0.59, respectively). Although ACYs contribute to the TPH in red raspberry, the importance of their contribution to AA is uncertain, because other phenolic (e.g., ellagitannins) and nonphenolic (e.g., vitamin C) antioxidants are found in substantial concentrations in red raspberries and may account for much of the AA. Deighton et al. (2000) reported that ACYs accounted for ≈18% of the juice phenolics in cultivar Glen Lyon. Using 18 accessions representing 12 *Rubus* L. species, they obtained phenotypic correlations $r = 0.38$ between total ACY content and AA by the TEAC assay and $r = 0.59$ between total ACY content and AA by the FRAP assay. Wang and Lin (2000) and González et al. (2003) reported phenotypic correlations between ACY content and AA that were higher ($r = 0.96$ and 0.85, respectively), but were based on only four cultivars in each study.

Among other factors, variation in the types and proportions of ACYs in different *Rubus* species may contribute to the discrepancy in correlations among studies. The in vitro AA of individual ACYs depends in part on the specific type of anthocyanidin (e.g., cyanidin or malvidin) that constitutes the basic phenolic structure of an ACY molecule, and the number and types of glycosidic substitutions that are made to that basic structure. The AAs of the six major anthocyanidins are similar in range to those of other flavonoid classes, such as the flavonols and flavanols (Rice-Evans and Miller, 1998). However, in some assay systems, the AAs of some ACYs (i.e., mono- or di-glucosides of the anthocyanidins) are reported to be substantially lower than the AAs of their parent aglycone (Fukumoto and Mazza, 2000; Rice-Evans and Miller, 1998; Tsuda et al., 1994), although not all studies confirm the reduction in AA (Deighton et al., 2002; Wang et al., 1997). This means the contribution of ACYs to AA will vary among and within fruit crops because the total ACY concentration, the types of anthocyanidins synthesized and the degree and type of glycosylation, and the concentration of other antioxidant compounds can all vary among fruits.

The red raspberry factorial mating design we used to investigate AA and TPH let us examine the relationship between individual and total ACYs and AA and to estimate variance components and heritabilities for ACYs. Several genes for raspberry fruit color have been described. These include the *t* gene, which, with epistatic locus *p*, influences ACY production in fruit and spines; the *y* gene, which is dominant for yellow fruit and was identified from *R. phoenicolasius* Maxim., but is expressed in hybrid offspring when backcrossed to *R. idaeus*; the *bl* gene, which derives from *R. occidentalis* L. and is also epistatic to locus *t*, influencing the development of black or purple fruit (summarized by Daubeny, 1996). The *r* gene has also been described, the *R* allele of which is proposed to control production of rhamnose-containing ACYs (rham-ACYs) in red raspberry (Barritt and Torre, 1975a). Two glucose-containing ACYs are produced in all red raspberries, cyanidin 3-*O*-sophoroside (cy-sop) and cyanidin 3-*O*-glucoside (cy-glu). Those red raspberry genotypes that also produce the rham-ACYs cyanidin 3-*O*-(2^G-glucosylrutinoside) (cy-glurut) and cyanidin 3-*O*-rutinoside (cy-rut) are thought to be hetero- or homozygous for the *R* allele. Trace amounts of pelargonidin-based ACYs and cyanidin 3,5-diglucoside have also been reported in some red raspberry genotypes, as noted by Barritt and Torre (1973, 1975b), Mullen et al. (2002), and Torre and Barritt (1977). However, little has been published regarding the heritability of individual or total ACYs in red raspberry. Nestby (1994) reported a broad sense heritability estimate of $H^2 = 0.59$ for fruit color evaluated in one season among 10 offspring from each of 19 three-year-old red raspberry families in two replicates. However, information regarding variation in ACYs was not provided. Furthermore, the relationship between AA and total or individual ACYs has been reported in only a small number of cultivars (González et al., 2003; Liu et al., 2002; Wang and Lin, 2000) but not examined in a mating design.

Materials and Methods

Plants. The design, establishment, and maintenance of the factorial mating design are described in detail by Connor et al. (2005). Briefly, 42 families were derived from 13 red raspberry cultivars and selections in the HortResearch *Rubus* breeding program, seven used as female and six as male parents. Parents were chosen without regard to their AA, TPH, or ACY content. One female parent, an offspring of an open-pollinated *R. parvifolius* x *R. idaeus* hybrid (referred to as "*R. parvifolius* derivative"), was included for its low chilling requirement; its fruit were visually partially pigment deficient, as were the fruit from many of its offspring. Fruit was harvested from two of the four plants in each plot, and the same plants were used for the 2002–03 (2002 season) and 2003–04 (2003 season) harvest seasons (total of 411 plants). Most of the factorial parents were growing in the same orchard in nearby rows and fruit was also harvested from these plants.

Fruit-harvest, extraction, and AA and TPH assays. Detailed description of fruit harvest, extraction, and AA and TPH assays are given in Connor et al. (2005). Briefly, 100 g of fully ripe sound fruit were harvested and kept frozen (–80 °C) until extraction. Extraction was performed using a 20-g sample of drupelets and 80 ethanol : 20 water : 1 glacial acetic acid solvent. Two subsamples per extraction were taken. For AA, the ferric-reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996) was used, as modified by Deighton et al. (2000). A standard curve using ferrous sulfate (0–500 μM) was run with each assay; results are reported as ferrous sulfate equivalents (FE) in $\mu mol \cdot g^{-1}$ fruit. For TPH, a Folin-Ciocalteu reagent-based method (Coseteng and Lee, 1987) was used, modified to allow 90 min for color development. Results are reported as gallic acid equivalents (GAE expressed as mg/100 g fruit). For each assay, single determinations were made on each sub-sample.

Anthocyanin determination. Individual ACYs were determined in the extracts by high-performance liquid chromatography. The HPLC system consisted of a Waters Alliance 2690 HPLC

equipped with a 996 photodiode array detector (Waters Corp., Milford, Mass.). The separation column was a Merck LiChrospher 100 RP-18e (5 μm) 250 × 4.0 mm (Merck, Darmstadt, Germany) protected with a guard column of the same packing. The solvents used were: A) 1.5% H_3PO_4 in water and B) acetonitrile:acetic acid: H_3PO_4:water (24:20:1.5:54.5). The solvent program started at an initial composition of 80% A and 20% B, increasing to 30% A and 70% B at 25 min, and then 10% A and 90% B at 30 min. After a further 5 min the system was reset to the starting composition for the next injection at 40 min. Sample injection volume was 5 μL. Chromatographic data were collected and manipulated using the Waters Millennium Chromatography Manager V4.0. Spectral data were collected (250–600 nm, 2.4 nm resolution) for the entire run and the ACY components were quantified by extracting chromatograms at 530 nm. Total ACY content was calculated as the sum of all ACY components collected between 7 and 30 min. The standards, cyanidin 3-*O*-galactoside, cyanidin 3-*O*-glucoside, and cyanidin 3-*O*-arabinoside, were purchased from Polyphenols Laboratories (Sandes, Norway). The ACY components were quantified using an external calibration and expressed as cyanidin 3-*O*-galactoside equivalents in mg/100 g fruit. Components were identified by comparison with the ACY profile of 'Boysen', which had been previously determined (Cooney et al., 2004).

Statistical analyses. Details of the analyses of variance and estimates of variance component distributions, narrow-sense heritabilities, and phenotypic and genotypic correlations are provided in Connor et al. (2005). To summarize, parents, years, replications, rows and plots were treated as random factors. Plot was nested within female × male to calculate analyses of variance (ANOVAs) and variance components distributions. Narrow-sense heritability estimates on an individual plant basis using variance components were based on pooled female and male sources (Hallauer and Miranda, 1981), as degrees of freedom were similar for females and males (six and five, respectively). Linear modelling for AA was performed using stepwise elimination, based on Akaike's information criterion, of terms from a full model that incorporated individual ACYs, parental effects, berry weight, and their first-order interactions (Venables and Ripley, 1994). Linear and quadratic discriminant analyses were used to explore the grouping of factorial genotypes by family, using individual ACYs and their ratios (and their interactions for quadratic analyses) as potential discriminators. Although we investigated AA and ACY, for which the *R. parvifolius* derivative female parent is unusual, it was retained in the analyses since in practice a breeder is interested in a collection of traits. Some parameters (e.g., heritability estimates) were also estimated after excluding this parent. Analyses were performed in S-Plus, version 6.1.2, release 1 (Insightful Corp., Seattle).

Results

Successful harvest for all 411 genotypes occurred in both years; one genotype yielded <20 g sound fruit in 2003, and extraction was adjusted appropriately to account for reduced sample size. Four major ACYs (cy-sop, cy-glurut, cy-glu, and cy-rut) were identified and quantified in the fruit from the factorial and parental plants. Their identities were confirmed by comparison with those identified by LC-MS in the ACY profile of the hybridberry 'Boysen' (Cooney et al., 2004). Together, these accounted for >90% of the ACYs detected. Although several small additional peaks were noted in the chromatograms at 530 nm, the identities of these ACYS were not known or confirmed; they were not considered in the analyses that follow.

The ranges in mean values for total and individual ACYs among female parent half-sib families were similar to those among male parent half-sib families, with the exception of the *R. parvifolius* derivative family, which demonstrated the lowest mean value for total ACY and for each ACY (Table 1). These results reflected the visual impression (see Materials and Methods) that this parent and many of its offspring were partially pigment deficient. Means and ranges for total ACY within half-sib families were similar in 2002 and 2003: the ranges varied from ≈40 mg/100 g fruit ('Chilliwack' half-sib family) to ≈60 mg/100 g fruit ('Sumner' half-sib family). Similarly, for cy-sop, the narrowest range in values was found in the same half-sib family ('Qualicum') in both years, as was the widest range ('Sumner' half-sib family). In contrast, for cy-glu, the half-sib families demonstrating the narrowest and widest ranges in values differed between the 2 years. In general, the changes in range values between years were not marked for cy-glu, cy-glurut, and cy-rut. In contrast to the result for ACYs, the within half-sib family ranges of AA values appeared to change more markedly between years. In 2002, the range in AA varied from 19.7 μmol·g^{-1} fruit ('Meeker' half-sib family) to 37.2 ('Sumner' half-sib family) μmol·g^{-1} fruit, while in 2003, the ranges varied from 19.5 μmol·g^{-1} fruit ('Chilliwack' half-sib family) to 54.1 μmol·g^{-1} fruit (88304ROC6 half-sib family).

Parental effects together accounted for nearly 50% of the variation in total ACY and 29% to 37% of the variation in individual ACYs in combined year analyses of all factorial genotypes (Table 2). The female × male parent interaction variance was <5% of the combined female and male variances, excepting for cy-sop, for which it was 11%, and approached statistical significance ($P = 0.058$). For all variates, both the female and male parent effects were significant, whether using the entire data set, the subset excluding the *R. parvifolius* derivative progeny, or the subset excluding both the *R. parvifolius* derivative progeny and the rham-ACY-negative genotypes.

The female parent contribution to variation was similar for all individual ACYs (19% to 26%), but lower than that for total ACY (36%) (Table 2). The *R. parvifolius* derivative progeny accounted for much of the female parent contribution; exclusion of these genotypes reduced the relative contribution of the female parent at least 2-fold (cy-glurut and cy-rut) and up to 5-fold (cy-glu and total ACY). When all factorial genotypes were considered, females contributed about two to three times more than males to the total variance of total ACY, cy-sop and cy-glu; when the *R parvifolius* derivative progeny was excluded, this situation was reversed (i.e., males contributed two to three times more than females). For cy-rut, female and male contributions were similar, regardless of the data subset; and for cy-glurut, the female parent contribution was always considerably more than that of the male, although the magnitude of this difference was less when *R. parvifolius* derivative progeny was excluded, and was reduced further by the exclusion of the rham-ACY-negative genotypes.

The year effect on ACYs varied around the 5% to 10% significance level (Table 2). However, using all factorial genotypes, year contributed <1% to the ACY (total or individual) variance. Exclusion of the *R. parvifolius* derivative progeny marginally increased the relative contribution of year, and limiting the analyses to rham-ACY-positive genotypes alone increased the contribution still further. However, these increases might be an artefact of the

Table 1. Means (ranges in parentheses) of total and individual anthocyanin (ACY) content (in mg/100 g fruit) and antioxidant activity [AA (in ferrous sulphate equivalents μmol·g^{-1} fruit)] in 2002 and 2003 in red raspberry fruit from factorial parents and their half-sib families.

		Female parents[z]							Male parents[z]					
	Year	Chi.	Hai.	Koh.	Mee.	Qua.	*R.par.*	Tul.	Cit.	F79	Kai.	QH5	ROC	Sum.
Parent values														
Total ACY	2002	55	39	18	21	44	5	32	46	16	21	-----	-----	33
	2003	51	34	17	31	48	6	20	45	23	26	-----	31	33
Cy-sop[y]	2002	24	20	7	12	16	4	22	22	12	13	-----	-----	20
	2003	23	18	7	21	15	4	15	22	16	16	-----	16	21
Cy-glu	2002	7	5	3	5	6	1	5	10	4	5	-----	-----	4
	2003	6	5	2	6	7	1	5	9	6	6	-----	8	4
Cy-glurut	2002	18	9	7	2	16	<1	4	7	0	2	-----	-----	7
	2003	18	8	6	3	17	<1	0	7	0	3	-----	5	6
Cy-rut	2002	5	4	2	1	5	0	2	5	0	1	-----	-----	2
	2003	4	4	2	1	6	<1	0	5	0	1	-----	2	2
AA	2002	42	45	38	49	38	31	36	33	33	32	-----	-----	37
	2003	41	39	35	39	43	33	30	33	32	37	-----	57	35
Half-sib family values														
Total ACY	2002	34	31	25	30	31	12	28	32	24	27	22	30	29
		(19-59)	(16-63)	(<1-52)	(<1-52)	(12-54)	(1-45)	(6-56)	(3-59)	(<1-48)	(1-54)	(1-46)	(2-54)	(1-63)
	2003	35	35	27	33	32	13	30	36	25	28	24	32	31
		(14-52)	(20-61)	(<1-57)	(<1-53)	(15-58)	(1-44)	(15-61)	(5-58)	(<1-49)	(1-47)	(1-47)	(2-61)	(3-61)
Cy-sop	2002	18	18	14	19	14	8	18	17	14	15	11	18	17
		(6-38)	(4-42)	(0-36)	(<1-37)	(5-25)	(<1-39)	(3-46)	(1-36)	(0-39)	(1-37)	(<1-33)	(<1-35)	(1-46)
	2003	19	19	15	21	14	8	19	18	14	16	12	19	19
		(4-35)	(5-38)	(<1-32)	(<1-33)	(6-31)	(<1-31)	(6-48)	(1-38)	(<1-32)	(1-35)	(<1-31)	(1-36)	(1-48)
Cy-glu	2002	6	6	5	7	6	3	5	6	5	5	5	6	5
		(3-15)	(2-15)	(<1-17)	(0-14)	(2-11)	(<1-10)	(1-11)	(2-13)	(0-11)	(<1-14)	(<1-12)	(1-17)	(<1-12)
	2003	6	7	5	7	6	3	6	7	5	5	5	7	5
		(3-12)	(2-18)	(<1-15)	(<1-14)	(2-12)	(<1-10)	(2-11)	(2-15)	(<1-11)	(<1-12)	(1-14)	(1-18)	(1-10)
Cy-glurut	2002	6	4	4	3	7	1	3	5	3	4	4	4	5
		(0-17)	(0-15)	(0-14)	(0-10)	(0-20)	(0-11)	(0-8)	(0-20)	(0-11)	(0-17)	(0-12)	(0-15)	(0-14)
	2003	6	5	4	3	8	1	6	3	5	4	4	5	5
		(0-13)	(0-15)	(0-18)	(0-11)	(0-20)	(0-5)	(0-10)	(0-20)	(0-10)	(0-13)	(0-14)	(0-18)	(0-14)
Cy-rut	2002	3	2	2	1	3	1	1	3	1	2	2	2	2
		(0-8)	(0-10)	(0-7)	(0-4)	(0-9)	(0-2)	(0-4)	(0-10)	(0-9)	(0-7)	(0-7)	(0-9)	(0-6)
	2003	3	3	2	2	3	1	2	4	1	2	2	2	2
		(0-9)	(0-12)	(0-14)	(0-5)	(0-12)	(0-3)	(0-5)	(0-14)	(0-9)	(0-6)	(0-9)	(0-10)	(0-6)
AA	2002	41.9	40.1	37.9	40.3	39.5	33.7	38.6	37.9	38.1	37.2	37.8	41.2	40.2
		(31-61)[x]	(29-58)	(27-49)	(32-52)	(33-54)	(23-47)	(30-51)	(26-55)	(24-52)	(25-54)	(23-56)	(28-51)	(24-61)
	2003	33.9	40.0	36.6	38.7	38.5	34.1	38.3	37.3	36.1	36.7	37.0	40.1	39.2
		(29-49)	(32-79)	(25-71)	(28-49)	(30-49)	(22-53)	(29-50)	(26-71)	(24-52)	(22-49)	(23-48)	(25-79)	(23-56)

[z]Chi. = 'Chilliwack', Hai. = 'Haida', Koh. = 'Kohatu', Mee.= 'Meeker', Qua. = 'Qualicum', *R. par*. = *R. parvifolius* derivative, Tul. = 'Tulameen', Cit. = 'Citadel', F79 = F79, Kai. = 'Kaituna', QH5 = 92387QH5, ROC = 88304ROC6, Sum. = 'Sumner'.

[y]Cy-sop= cyanidin 3-*O*-sophoroside, Cy-glurut = cyanidin 3-*O*-(2^G-glucosylrutinoside), Cy-glu = cyanidin 3-*O*-glucoside, Cy-rut = cyanidin 3-*O*-rutinoside.

[x]Range values rounded to whole numbers for clarity.

Table 2. Combined year variance component distributions (percent) and F-probabilities from the analyses of variance for total and individual anthocyanin contents and antioxidant activity in red raspberry fruit harvested in 2002 and 2003 from all factorial genotypes (n = 411 each year); factorial genotypes excluding *R. parvifolius* derivative progeny (no *R. par* prog.) (n = 355 genotypes each year); and factorial genotypes possessing rhamnose-containing anthocyanins (Rham-ACY only) in either year, but excluding *R. par* prog. (n = 270 in 2002, n = 268 in 2003).

	Relative contribution to total variance			F-probability and d.f. from ANOVA					
				All genotypes		No *R. par* prog.		Rham-ACY only, no *R. par* prog.	
Source	All genotypes	No *R. par* prog.	Rham-ACY only, no *R. par* prog	P	d.f.	P	d.f.	P	d.f.
Total anthocyanins									
Replication	0.2	1.8	1.2	0.035	3	<0.001	3	0.015	3
Female (F)	35.8	7.0	9.1	<0.001	6	<0.001	5	0.001	5
Male (M)	12.4	19.7	21.5	<0.001	5	<0.001	5	<0.001	5
Year (Y)	0.9	1.7	2.5	0.032	1	0.119	1	0.081	1
F x M	2.4	2.8	3.3	0.151	30	0.271	25	0.260	25
F x Y	0.0	0.0	0.0	0.493	6	0.570	5	0.651	5
M x Y	0.0	0.0	0.0	0.218	5	0.515	5	0.701	5
Plot within F x M	11.6	16.4	17.5	<0.001	135	<0.001	116	<0.001	105
F x M x Y	0.0	0.0	0.0	0.132	30	0.080	25	0.015	25
Plot within F x M x Y	0.0	0.0	0.0	1.000	135	1.000	116	1.000	105
Residuals	36.7	50.5	44.9		465		403		253
Total variance	*156.4*	*99.7*	*100.6*						

continued next page

Table 2. Continued.

Source	Relative contribution to total variance: All genotypes	No *R. par* prog.	Rham-ACY only, no *R. par* prog	F-probability and d.f. from ANOVA: All genotypes P	d.f.	No *R. par* prog. P	d.f.	Rham-ACY only, no *R. par* prog. P	d.f.
Cyanidin 3-*O*-sophoroside									
Replication	0.0	0.4	0.7	0.829	3	0.201	3	0.299	3
Female (F)	23.0	7.9	12.9	<0.001	6	<0.001	5	<0.001	5
Male (M)	10.5	14.9	13.7	<0.001	5	<0.001	5	<0.001	5
Year (Y)	0.3	0.5	0.8	0.047	1	0.117	1	0.169	1
F x M	3.7	3.4	3.2	0.058	30	0.124	25	0.201	25
F x Y	0.0	0.0	0.0	0.534	6	0.524	5	0.476	5
M x Y	0.0	0.0	0.0	0.552	5	0.720	5	0.695	5
Plot within F x M	11.6	12.3	13.1	<0.001	135	<0.001	116	<0.001	105
F x M x Y	0.0	0.0	0.0	0.191	30	0.168	25	0.030	25
Plot within F x M x Y	0.0	0.0	0.0	1.000	135	1.000	116	1.000	105
Residuals	50.8	60.6	55.6		465		403		253
Total variance	*72.8*	*56.7*	*44.9*						
Cyanidin 3-*O*-glusoside									
Replication	2.0	4.5	3.6	<0.001	3	<0.001	3	<0.001	3
Female (F)	20.6	4.3	9.3	<0.001	6	0.008	5	<0.001	5
Male (M)	9.6	11.5	12.5	<0.001	5	<0.001	5	<0.001	5
Year (Y)	0.5	0.6	1.0	0.069	1	0.103	1	0.113	1
F x M	1.2	1.5	0.0	0.288	30	0.356	25	0.772	25
F x Y	0.0	0.0	0.0	0.724	6	0.700	5	0.863	5
M x Y	0.0	0.0	0.0	0.103	5	0.194	5	0.496	5
Plot within F x M	12.5	15.3	26.4	<0.001	135	<0.001	116	<0.001	105
F x M x Y	0.0	0.0	0.0	0.031	30	0.020	25	0.030	25
Plot within F x M x Y	0.0	0.0	0.0	1.000	135	1.000	116	1.000	105
Residuals	53.6	62.3	47.3		465		403		253
Total variance	*8.2*	*6.9*	*5.2*						
Cyanidin 3-*O*-(2^G-glucosylrutinoside)									
Replication	0.0	0.0	0.0	0.543	3	0.499	3	0.010	3
Female (F)	25.5	16.5	22.4	<0.001	6	<0.001	5	<0.001	5
Male (M)	3.5	3.2	9.1	0.001	5	0.005	5	<0.001	5
Year (Y)	0.2	0.3	1.2	0.055	1	0.048	1	0.106	1
F x M	0.0	0.0	1.5	0.944	30	0.925	25	0.426	25
F x Y	0.0	0.0	0.0	0.312	6	0.490	5	0.516	5
M x Y	0.0	0.0	0.0	0.075	5	0.081	5	0.731	5
Plot within F x M	24.9	28.7	25.9	<0.001	135	<0.001	116	<0.001	105
F x M x Y	0.0	0.0	0.0	0.236	30	0.211	25	0.039	25
Plot within F x M x Y	0.0	0.0	0.0	1.000	135	1.000	116	1.000	105
Residuals	46.0	51.2	39.9		465		403		253
Total variance	*16.7*	*16.7*	*12.4*						
Cyanidin 3-*O*-rutinoside									
Replication	0.1	0.0	0.0	0.229	3	0.213	3	0.006	3
Female (F)	19.1	11.8	16.1	<0.001	6	<0.001	5	<0.001	5
Male (M)	12.2	14.6	17.1	<0.001	5	<0.001	5	<0.001	5
Year (Y)	0.5	0.6	1.5	0.106	1	0.116	1	0.064	1
F x M	0.0	0.0	0.0	0.820	30	0.887	25	0.656	25
F x Y	0.0	0.0	0.0	0.449	6	0.530	5	0.426	5
M x Y	0.3	0.2	0.8	0.006	5	0.010	5	0.161	5
Plot within F x M	25.2	26.9	27.8	<0.001	135	<0.001	116	<0.001	105
F x M x Y	0.0	0.0	0.0	0.175	30	0.208	25	0.003	25
Plot within F x M x Y	0.0	0.0	0.0	1.000	135	1.000	116	1.000	105
Residuals	42.6	45.9	36.6		465		403		253
Total variance	*4.6*	*4.8*	*4.1*						
Antioxidant activity									
Replication	1.6	2.3	2.8	<0.001	3	<0.001	3	<0.001	3
Female (F)	12.2	2.9	5.2	<0.001	6	0.046	5	0.006[y]	5
Male (M)	6.7	7.3	9.2	<0.001	5	<0.001	5	<0.001[y]	5
Year (Y)	1.1	2.1	0.7	0.063[z]	1	0.054	1	0.048[y]	1
F x M	0.0	0.0	0.0	0.806	30	0.751	25	0.940[y]	25
F x Y	0.3	0.0	0.0	0.126	6	0.289	5	0.750	5
M x Y	0.0	0.0	0.0	0.733	5	0.777	5	0.936	5
Plot within F x M	18.5	16.0	18.8	<0.001	135	<0.001	116	<0.001	105
F x M x Y	0.0	0.0	0.0	0.249	30	0.184	25	0.002	25
Plot within F x M x Y	0.0	0.0	0.0	1.000	135	0.999	116	0.968	105
Residuals	59.5	69.4	63.1		465		403		253
Total variance	*38.5*	*32.2*	*35.4*						

[z]The ANOVA F-probability = 0.043 for antioxidant activity by FRAP method, when expressed in Trolox standard equivalents.

[y]Approximate F-ratios and probabilities for these sources in this subset were calculated using an F-test like criterion as suggested by Satterthwaite (1946) and cited by Steel et al. (1997), to avoid negative F-ratios.

decrease in the contribution from the female parents. The contribution of year × parental source interaction to the total variance, although statistically significant for the full data set and for the subset excluding *R. parvifolius*, was negligible.

In combined year analysis, variation among plots within family was highly significant ($P < 0.001$) for total ACY and for all individual ACYs, accounting for slightly more than 10% of the variance for total ACY, cy-sop, and cy-glu, and ≈25% of the variance for cy-glurut and cy-rut. Among-plot and within-plot variances combined accounted for nearly half the total ACY variance and approximately two-thirds of that for individual ACYs in the entire dataset. These proportions tended to be higher for the data subsets, although there were some exceptions (e.g., for cy-glurut and cy-rut in the rham-ACY-positive subset). The combined variances accounted for a lower proportion of total variance than in the full data set (Table 2).

For AA, parental contributions together comprised 19% of total variation (Table 2) with both female and male parental effects being significant ($P < 0.001$). The female × male interaction variance was negligible. The female parent contribution to variation decreased considerably with the exclusion of the *R. parvifolius* progeny, while the male parent contribution changed only slightly. Year was marginally significant in the full factorial ($P = 0.063$ when expressed in ferrous sulfate standard equivalents; $P = 0.043$ when expressed in Trolox standard equivalents) and in each of the subsets ($P = 0.054$ with *R. parvifolius* derivative progeny excluded; $P = 0.048$ with rham-ACY-positive genotypes only). However, its effect on variation in AA was minor, accounting for 1% to 2% of total variance depending on the dataset. The year interactions were negligible.

In combined year analyses of AA, variation among plots within family was highly significant ($P < 0.001$), accounting for 16% to 19% of total variance (Table 2). This source of variation accounted for only 6% of total variation in 2002, and 14% in 2003 (data not shown). Within-plot variances (residuals) accounted for the majority of total AA variance and, when combined with variances among-plots within family, comprised well over three quarters of total variance in AA.

Phenotypic correlations between total ACY and the individual ACYs ranged from moderate ($r = 0.41$ between total ACY and cy-rut) to high ($r = 0.82$ between total ACY and cy-sop) (Table 3). Phenotypic correlations between individual ACYs were generally low to moderate, but a high correlation existed between cy-glurut and cy-rut ($r = 0.85$). Correlations between AA and ACY were low to moderate; the highest was that between AA and total ACY, with $r = 0.53$. The genotypic correlations between total ACY and cy-sop or cy-glu ($r = 0.46$ and 0.44, respectively) were considerably lower than the corresponding phenotypic correlations ($r = 0.82$ and 0.73, respectively), suggesting that there were substantial environmental or nonadditive genetic influences, in addition to additive genetic effects, that impacted the relationship between these variables. Disparities between phenotypic and genotypic correlations among the individual ACYs were also noted. The correlations between (rhamnose-negative) cy-sop and cy-glu (phenotypic and genotypic correlations of $r = 0.64$ and 0.42, respectively) and the rhamnose-positive cy-glurut and cy-rut (phenotypic and genotypic correlations of $r = 0.85$ and 0.47, respectively) demonstrated that non-additive or environmental effects, as well as additive effects, influenced the ACY profile. The genotypic correlations between AA and both the total and individual ACYs were similar to the phenotypic correlations, which in contrast to the above observations, indicate that mostly additive genetic effects contributed to the low-moderate phenotypic correlations. Phenotypic correlations among individual ACY:total ACY ratios and AA were also determined (data not shown). The highest and only significant phenotypic correlation was between cy-glu:total ACY and AA, $r = –0.14$.

The linear models for AA selected using stepwise elimination of effects and interactions contained 20 variates (multiple $R^2 = 0.48$) using data from 2002, 42 variates (multiple $R^2 = 0.64$) using data from 2003, and 49 variates (multiple $R^2 = 0.53$) for combined year data (analyses not shown). In contrast, modelling AA on TPH as sole variate yielded $R^2 = 0.88$, 0.86, and 0.86 in 2002, 2003, and combined years (data not shown). The addition of ACYs to the latter model did not appreciably increase the R^2 value.

Linear and quadratic discriminants were used to group the factorial genotypes by half-sib or full-sib family based on individual ACYs and their ratios as discriminators. The most accurate groupings were achieved with linear combinations. The most effective set of discriminators successfully predicted female half-sib family for 40% of genotypes, male half-sib family for 37% of genotypes, and full-sib family for 19% of genotypes in combined year data.

Heritabilities based on combined year data were high for ACYs, ranging from $h^2 = 0.80$ for cy-glu to $h^2 = 1.00$ for total ACY (Table 4). Individual year heritabilities were moderate, but the estimates were mostly consistent between years (data not shown). Because many *R. parvifolius* derivative offspring were distinguished from the offspring of other parents by their reduced ACY content, estimates were also made excluding the *R. parvifolius* derivative progeny. Combined year heritability estimates thus calculated were lower, but those for total ACY and cy-rut were still high ($h^2 = 0.74$ and 0.78, respectively). Individual year heritabilities were lower as well, ranging from $h^2 = 0.28$–0.72 (data not shown). The combined year heritability estimate for AA based on the full factorial was moderate ($h^2 = 0.54$), considerably lower than found for the ACYs. The difference between individual year estimates was larger than those for the ACYs. Estimates based on data

Table 3. Phenotypic (upper right) and genotypic (lower left) correlations for total and individual anthocyanins (ACY) and antioxidant activity (AA) in red raspberry fruit harvested from factorial mating design in 2002 and 2003, determined on genotype mean basis (n = 411 genotypes each year).

	Total ACY	Cy-sop[z]	Cy-glu	Cy-glurut	Cy-rut	AA
Total ACY	----	0.82	0.73	0.45	0.41	0.53
Cy-sop	0.46	----	0.64	-0.08	-0.14	0.41
Cy-glu	0.44	0.42	----	0.02	0.12	0.41
Cy-glurut	0.34	0.16	0.22	----	0.85	0.24
Cy-rut	0.32	0.13	0.25	0.47	----	0.26
AA	0.43	0.46	0.41	0.25	0.18	----

[z]Cy-sop = cyanidin 3-*O*-sophoroside, Cy-glurut = cyanidin 3-*O*-(2^G-glucosylrutinoside), Cy-glu = cyanidin 3-*O*-glucoside, Cy-rut = cyanidin 3-*O*-rutinoside.

Table 4. Combined year narrow sense heritability estimates (SE in parentheses) for total and individual anthocyanin content and antioxidant activity in red raspberry fruit harvested in 2002 and 2003, based on variance component analysis of all factorial mating design genotypes (n = 411 genotypes) and on genotypes excluding R. parvifolius derivative progeny (n = 355 genotypes).

	All genotypes	Excluding *R. parvifolius* derivative progenies
Total anthocyanins	1.00 (0.34)	0.74 (0.37)
Cyanidin 3-*O*-sophoroside	0.83 (0.40)	0.56 (0.29)
Cyanidin 3-*O*-glucoside	0.80 (0.34)	0.45 (0.29)
Cyanidin 3-*O*-(2^G-glucosylrutinoside)	0.90 (0.72)	0.63 (0.37)
Cyanidin 3-*O*-rutinoside	0.91 (0.25)	0.78 (0.39)
Antioxidant activity	0.54 (0.27)	0.29 (0.16)

excluding *R. parvifolius* derivative progeny were much lower for both individual (h^2 = 0.32 and 0.22) and combined years (h^2 = 0.29) (data not shown).

Discussion

Substantial variation in total and individual ACYs was identified in this factorial population, with many half-sib families demonstrating wide ranges for these variables. Both female and male genetic effects were significant for all ACYs, with the female effect generally larger than the male effect. The parental genetic effects contributed nearly 50% of the variation observed in total ACY, and ≈30% of the variation observed in each individual ACY. When the progeny of the pigment-deficient *R. parvifolius* derivative female parent was excluded, however, female effects accounted for a lower proportion of variation, such that parental effects together contributed ≈27% of variation in total ACY and ≈12% to 26% of variation in the individual ACYs. It would be unusual to include a pigment deficient parent in a breeding program for which intense color (high ACY content) was a primary objective, and the variance component analyses excluding the *R. parvifolius* progeny would be more applicable to such breeding programs. On the other hand, the variance component analyses including all data would be more relevant for a program that focused on introducing a trait possessed by a pigment deficient parent (e.g., low chilling in the case of the *R. parvifolius* derivative) whilst maintaining color. In contrast to simple parental effects, the female × male interaction (analogous to specific combining ability in a fixed effects model) made only a minor contribution to total variation in total ACY (2%) or individual ACYs (0% to 4%).

Although year had a statistically significant effect on total ACY and cy-sop, its contribution to overall variation was minimal (≤1%). Similarly, year interactions were only rarely significant for the individual ACYs, including the rham-ACYs for which analyses based on a factorial subset (genotypes that were positive for rham-ACYs) were used to reduce bias from possible major gene effect. For all ACYs, the contributions of year interactions to total variation were negligible. Thus, accurate evaluation and ranking of half-sib or full-sib families for individual ACY content could be achieved with a single year's assessment.

The largest proportion of total variation in individual and total ACYs was within-plot within family, regardless of inclusion of *R. parvifolius* derivative offspring or of rham-ACY offspring. Within-plot variation consisted of both genetic (two full-sibs) and environmental components, as well as experimental error. Within plot environmental variation was limited, since the differences in environmental exposure over the distances between plants were probably small. Although genotype × environment interaction within the plot cannot be excluded, the majority of the within-plot variation was probably genetic. Thus the largest proportion of variation in ACYs was within families rather than among families. However, for all ACYs, 12% to 25% of total variation was found among plots within family. Some of this among plot variation was genetic, since two distinct genotypes were sampled in each plot; but environmental effects also contributed to among plot variance, as plots were located in separate replications. Thus, the "within-family" variation in ACYs we report is likely to be inflated by the environmental variation existing among plots within families.

Parental effects were also significant sources of variation in AA, while year effect and its interactions contributed negligibly to total variation. The female contribution was substantially greater than the male contribution in the full factorial, due to inclusion of *R. parvifolius* derivative progeny. However, female contribution to AA variation was considerably lower than female contribution to variation in any of the ACYs. Within-plot within family variation accounted for the largest proportion of total variance in AA; the among-plot within family variance was also substantial, although environmental influences probably contributed to the among-plot differences. Nevertheless, most variation in AA was found within families rather than among families. The reader is referred to Connor et al. (2005) for a more detailed discussion of variation in AA in this factorial.

Narrow-sense heritability estimates for individual ACYs were moderate to high (h^2 = 0.80-0.91 for combined year data) and for total ACY was very high (h^2 = 1.00) based on the full factorial. However, some of the associated standard errors were large. As with the variance component analyses, more realistic heritabilities might be those based on genotypes excluding *R. parvifolius* derivative progeny, since pigment-deficient parents are unlikely to be included in a breeding program for which increased ACY is a main objective. The heritability estimates calculated on this basis were lower, but still moderate to high for individual ACYs (h^2 = 0.45–0.78 for combined year data) and high for total ACY (h^2 = 0.74). Our combined year heritability estimate for total ACY (excluding *R. parvifolius* derivative progeny) of h^2 = 0.74 was comparable to the broad-sense heritability of H^2 = 0.59 for fruit color reported by Nestby (1994). Other narrow-sense heritabilities to which to compare our estimates were not found in the literature. The higher heritability estimate for total ACY than for the individual ACYs implies that nonadditive genetic variance or environmental variance accounts for a greater proportion of phenotypic variance in the individual ACYs than in the total ACY. It is conceivable that the concentrations of individual ACYs might change markedly and dissimilarly in response to altered environmental conditions, yet the net change in total ACY might be minimal. Some genetic loci contributing to ACY production are common to all individual ACYs, but the loci that differ among ACYs might vary in their environmental responses.

The narrow sense heritability estimate for AA was moderate based on the full factorial ($h^2 = 0.54$), and low when the *R. parvifolius* derivative progeny was excluded ($h^2 = 0.29$). In both years, the *R. parvifolius* derivative half-sib family demonstrated substantially lower mean content of total and most individual ACYs than the other half-sib families. The *R. parvifolius* derivative progeny also demonstrated lower mean AA content, although the distinction of its mean AA from other half-sib family AA was not as marked as the differences between their respective mean ACY contents. While ACYs, as polyphenolic compounds, contribute to AA, they are only some of many phenolics that contribute to AA in the fruit (Häkkinen et al., 1999; Kähkönen et al., 2001). The genetic contributions of the *R. parvifolius* derivative to ACY-related loci diminished, rather than enhanced, ACY production, but the genetic influences on total AA could have been through both ACY and non-ACY loci. For example, genotypes with lower ACY production could have abnormalities in the flavonoid synthetic pathway at points proximal to that at which ACY production is initiated, affecting production of other compounds with AA. Thus, the influences of the *R. parvifolius* derivative parent on ACY and AA content could be diverse.

Our heritability estimates were not adjusted for parental relatedness. Adjusted heritabilities are more applicable to baseline (or noninbred) populations. The parental coancestries among families in this study ranged from 0.026 to 0.213 (not presented). However, as pointed out by Connor et al. (2005), there may be greater utility for unadjusted heritability estimates than adjusted ones with regard to red raspberry breeding populations, because breeding programs worldwide make use of parents with considerable coancestry.

We found a moderate phenotypic correlation between AA and total ACY ($r = 0.53$), and low to moderate correlations between AA and individual ACYs ($r = 0.24$–0.41). These were markedly lower than the correlation between AA and TPH in this population, $r = 0.93$ (Connor et al., 2005). The method used (Folin-Ciocalteu reagent-based) to determine TPH in that study measures oxidizable compounds but is not entirely specific for phenolics (Singleton et al., 1999). Oxidizable nonphenolic substances (such as vitamin C, which is present in some fruits, including red raspberries) can interfere with the determination, if they are present in the fruit extracts. Thus, the correlation between AA and true TPH might be slightly lower than 0.93. However, the Folin-Ciocalteu-based method is a widely-used and accepted method and is considered reliable, so we consider the higher correlation between AA and TPH compared to AA and ACY to be legitimate. As pointed out earlier, ACYs are polyphenolics and they contribute to both TPH and AA. However, it appears from these data that TPH would be more useful than ACY content as a surrogate or predictor of red raspberry AA if AA could not be directly determined. Furthermore, the most successful linear model for AA that we derived with stepwise analysis based on individual ACYs and interactions yielded an $R^2 = 0.53$, compared to an $R^2 = 0.88$ for a model of AA using only TPH. Thus, total or individual ACYs or ACY profiles do not appear to provide any more information regarding the AA of red raspberry fruit than can be obtained by measuring TPH if AA assays are unavailable.

Although the major focus of our study was the relationship between ACYs and AA, breeding for increased ACY content without regard to AA has long been a major goal for raspberry breeding programs because fruit color influences the acceptability of fresh and processed products. Additionally, it has been suggested that ACYs may have health benefits that are not entirely dependent on their AA (Andriambeloson et al., 1998; Martin et al., 2003; Rossi et al., 2003). It is possible that specific ACY profiles will be perceived as more efficient in providing these benefits, and thus be desirable "targets" for selection. Our study indicates that simultaneous increase (or decrease) of some individual ACYs appears realistic because their genotypic correlations were moderate (e.g., between cy-glu and cy-sop and between cy-glurut and cy-rut). However, simultaneous increases in some ACYs, such as cy-rut and cy-sop, would be difficult to achieve because of their low genotypic correlations; and selective increase of a single ACY while decreasing (or not altering) the concentration of another with which it shares a moderate genetic correlation will be even more difficult.

We used linear and quadratic discriminant analysis to investigate grouping genotypes by ACY profile. If half-sib or full-sib grouping were successful, it would suggest that parental ACY profiles could be used to predict family profiles; this might be advantageous in developing genotypes with "tailored" ACY profiles. Our predictions for half-sib and full-sib family grouping were based on the same data used to derive the linear discriminants, and thus represent the upper limit of predictive accuracy achievable using ACYs as discriminators. Although they were better than expected for random allocation (37% to 40% compared with ≈15% for half-sib families, and 19% compared with 2.4% for full sib families), ACY profiles did not appear to be distinctive or consistent enough to be used alone as family grouping variables. Furthermore, as already noted, although the heritability of total ACY was high, that of individual ACYs was lower. Thus, even if it were possible to demonstrate that a specific ACY profile was optimal for a particular health benefit, it would be extremely difficult to deliberately breed a genotype with that profile.

In calculating the variances of the rham-ACYs cy-glurut and cy-rut, we presumed the presence of the major gene, *R*, which is proposed to determine the ability of a red raspberry genotype to produce rhamnose-containing ACYs (Barritt and Torre, 1975a). Detecting the effects of any minor quantitative genes influencing the levels of these ACYs required minimizing or equalizing the effect of *R*, which we attempted by including only those genotypes with demonstrated rham-ACYs and excluding *R. parvifolius* derivative progeny. Genetic variation was reduced in this subset as compared to the full factorial, but it was sufficient to suggest the presence of minor genes influencing the content of rham-ACYs. Although the significance of several genetic interactions differed between the full factorial and the subset, their contribution to variance did not appreciably alter.

In summary, our 2-year study of a red raspberry factorial mating design demonstrated the importance of female and male parental contributions to total variance of ACY in the fruit, and indicated that the female × male interaction contribution was negligible. Year effects and year interactions accounted for only a small proportion of variation. Most variation was found within families, and a considerable proportion of this was identified among plots within family, suggesting that both environmental influences and genetic effects were important. Combined year narrow sense heritability estimates were high for individual and total ACYs. Phenotypic correlations between total and individual ACYs were moderate to high. Many of the individual ACYs showed substantial differences between phenotypic and genotypic correlation coefficients, suggesting that non-additive and environmental effects contributed to the phenotypic correlation between variates. In addition, we found a moderate phenotypic correlation ($r = 0.53$) and low to moderate genotypic correlation ($r = 0.43$) between

total ACY and AA. This phenotypic correlation is markedly lower than that between TPH and AA, determined previously in this same factorial. The high heritability and considerable variation in ACYs indicate high expected gains in ACY content in red raspberry. However, the study also suggests that ACY content and profiles are relatively poor surrogates of AA in red raspberry fruit; with regard to screening and breeding genotypes for higher AA, direct assays of AA (or of TPH as a proxy for AA) would be more time- and cost-effective.

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