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ARTICLE

Characterization and Quantification of Anthocyanins in Red Kiwifruit (*Actinidia* spp.)

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Red-fleshed fruit occur in a small number of distantly related taxa in different sections of the genus *Actinidia* (kiwifruit). We describe and identify the anthocyanin profile of fruit of several *Actinidia* species. Differences in the relative amounts of cyanidin- and delphinidin-based anthocyanins determine whether the fruit appear red or purple. Cyanidin derivatives have been found in all *Actinidia* species that contain anthocyanins, whereas delphinidin derivatives are limited to two taxa: *A. melanandra* and *A. arguta* var. *purpurea*. The fruit of these not only contain a wider range of anthocyanins, but they also have greater concentrations. Anthocyanins of most *Actinidia* species are usually conjugated with either xylosyl-galactose or galactose, whereas *A. deliciosa* anthocyanins are conjugated with glucose and galactose.

KEYWORDS: ***Actinidia deliciosa***; ***Actinidia chinensis***; ***Actinidia eriantha***; ***Actinidia arguta***; ***Actinidia arguta* var. *purpurea***; ***Actinidia melanandra***; **cyanidin**; **delphinidin**

INTRODUCTION

The genus *Actinidia* Lindl. is widely distributed throughout most of east Asia, with the center of evolution being southwestern China, where there is the most taxa and, hence, the largest variability (*1*). Within the genus, there is great diversity in vine and fruit characteristics, including color of the fruit skin and flesh (*2*). This diversity is used by fruit breeders to produce new kiwifruit that can be differentiated in the marketplace and are thus able to gain increased market share and new customers. Red-fruited kiwifruit appeal strongly to consumers (*3*), and hence, there is much interest in breeding new kiwifruit that have red skins and/or red fruit flesh.

The first step in developing such red-fruited kiwifruit is to characterize the red pigments responsible and determine their distribution within *Actinidia* taxa. Red pigments are found in the hairs of young leaves or shoot tips of many species [e.g., *A. deliciosa* (A. Chev.) C. F. Liang et A. R. Ferguson], in the petioles or stems [e.g., some *A. arguta* (Sieb. et Zucc.) Planch. ex Miq.], and in the petals of species, such as *A. eriantha* Benth. and *A. rubricaulis* var. *coriacea* (Finet et Gagnep.) C. F. Liang, but are less common in the fruit. Thus far, red fruit have been observed only in a small number of *Actinidia* species, sometimes in all the genotypes of a particular taxon, e.g., *A. melanandra* Franch. and *A. arguta* var. *purpurea* (Rehder) C. F. Liang ex Q. Q. Chang (*2*) and sometimes in only a few genotypes within a taxon. Red pigments may occur in all fruit tissues or may be restricted to the skin as a blush, to the fruit pericarp, or to only part of the pericarp, usually the inner pericarp.

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To date, little work has been performed to identify and characterize different anthocyanins in kiwifruit species. Montefiori and colleagues (*4*) tentatively identified and quantified three major and two minor anthocyanins present in various red-fleshed genotypes of *A. chinensis* and *A. deliciosa*. Comeskey and colleagues (*5*) isolated and identified five different anthocyanins from fruit of different *Actinidia* species: cyanidin 3-[2-(xylosyl)galactoside], cyanidin 3-galactoside, cyanidin 3-glucoside, delphinidin 3-[2-(xylosyl)galactoside], and delphinidin 3-galactoside.

In this paper, we describe the identification and quantification of the different anthocyanins present in a wider range of red-fruited genotypes belonging to different *Actinidia* taxa.

MATERIALS AND METHODS

Plant Material. Fruit were collected from the Plant and Food Research (formerly HortResearch) *Actinidia* germplasm collections held at Te Puke (Bay of Plenty, New Zealand) and Riwaka (Nelson Region, New Zealand). The collections consist of about 3500 genotypes, arising from over 300 accessions (*6*), sourced mainly from different provinces of China (**Table 1**). The *Actinidia* taxa with red or purple fruit (*7*−*9*) that were studied are listed in **Table 1**. A few other species are reported as sometimes having red flesh or skin, e.g., *A. henanensis* C. F. Liang found in Henan Province (*8*) and *A. setosa* (Li) C. F. Liang et A. R. Ferguson restricted to Taiwan (Ferguson, personal communication).

For each genotype studied, 15−20 fruit from a single vine were collected. Fruit were assumed to be mature once the seed had darkened. The fruit were held at room temperature until fully ripe, and their firmness was below 1.5 kg/cm^2. Equatorial slices were taken from the fruit, and these therefore included skin, flesh, and core. Each slice was cut into many small pieces. From the pooled pieces for each genotype, three replicates were collected of 1.5−2 g each and immediately frozen in liquid nitrogen. These samples were used to compare total anthocyanins in fruit of

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Table 1. Red-Fruited Kiwifruit, Natural Distribution, and Provenance of Genotypes Studied

taxon	red color	published location of red kiwifruit	provenance of genotypes studied
A. arguta	occasional genotypes have red skin (*8*, *9*)		not recorded
A. arguta var. *purpurea*	purple skin and flesh in ripe fruit	predominantly in southwestern China (*7*)	Wolong (Sichuan)
A. chinensis (red-fleshed genotypes of which have sometimes been classified as *A. chinensis* var. *rufopulpa*)	red color usually limited to the inner pericarp of the fruit but sometimes also in outer pericarp	from a limited area in China: Jiangxi, Zhejiang, Hubei, and Henan (*8*)	Xixia (Henan) and Guilin (Guangxi)
A. deliciosa (red fleshed variants of which have sometimes been classified as *A. deliciosa* var. *coloris*)	red pigments are limited to the inner pericarp	from a very limited area: Dongshangfeng in Hunan, China (*8*)	Dengjia (Chongqinq) and Dongshangfeng (Hunan)
A. melanandra	ripe fruit have red skin and flesh	distributed widely throughout southern China (*2*, *8*)	Wushan and Dengjia (Chongqinq)
A. eriantha	there were no previous records of red pigments in fruit of *A. eriantha*		Guilin (Guangxi)

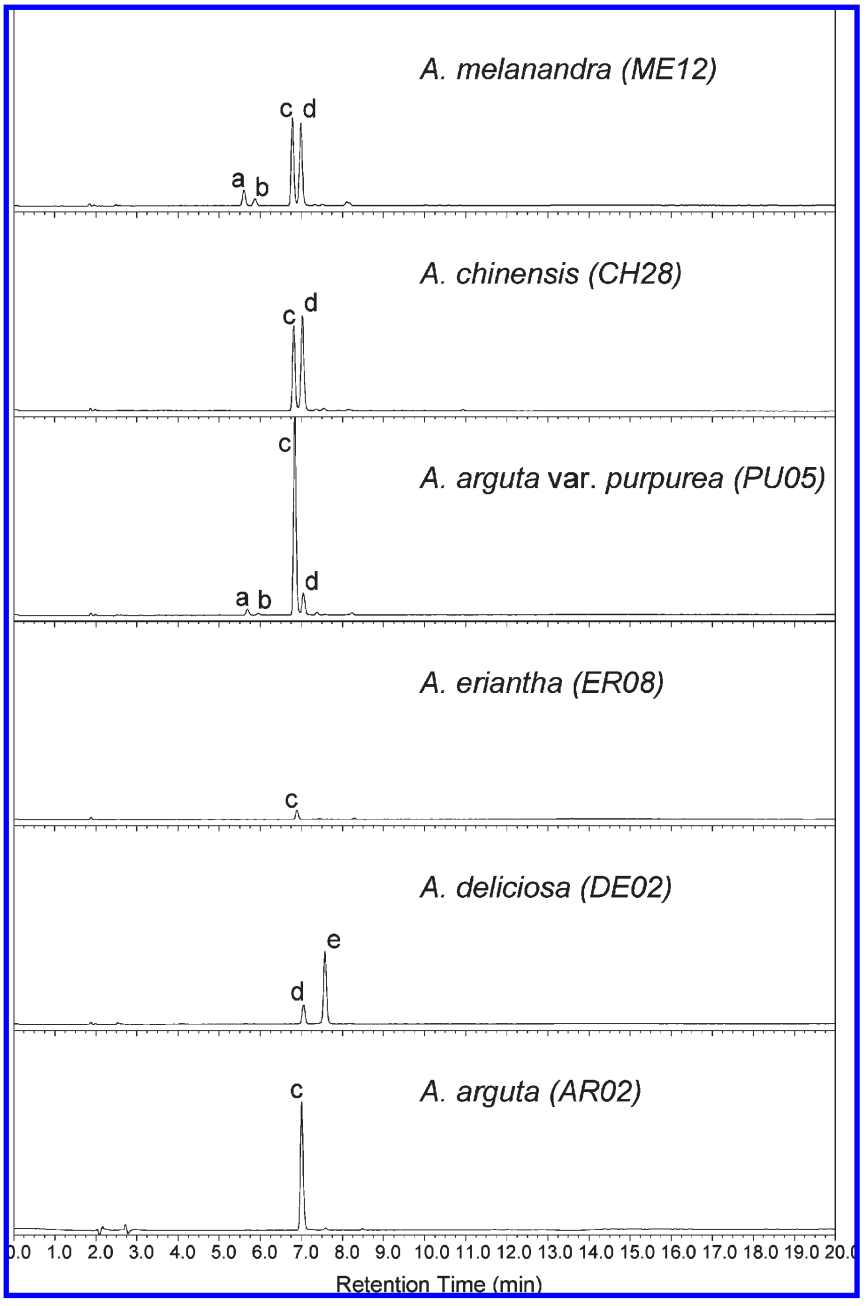

Figure 1. Representative chromatogram traces (530 nm) showing the anthocyanin composition of fruit of six *Actinidia* taxa. Peaks are labeled as follows: (a) delphinidin 3-(xylosyl)galactoside, (b) delphinidin 3-galactoside, (c) cyanidin 3-(xylosyl)galactoside, (d) cyanidin 3-galactoside, and (e) cyanidin 3-glucoside.

Table 2. Anthocyanin Contents of Fruit of Different *Actinidia* Taxa[a]

		anthocyanin					
		cyanidin			delphinidin		
taxa	genotype	Cy-xyl.gal.[b]	Cy-gal.[c]	Cy-glu.[d]	Dp-xyl.gal.[e]	Dp-gal.[f]	total
A. chinensis	CH 15	3.94 ± 0.38	1.60 ± 0.15				5.55 ± 0.52 cd
	CH 16		5.95 ± 0.68				5.95 ± 0.68 cd
	CH 17	4.48 ± 0.43					4.48 ± 0.43 d
	CH 24	11.23 ± 2.14	0.60 ± 0.60				11.83 ± 2.69 bc
	CH 28	46.46 ± 10.09	52.08 ± 13.70				98.55 ± 23.79 a
	CH 29	33.17 ± 3.55	14.76 ± 2.34	1.98 ± 0.36			49.91 ± 6.17 a
	CH 31	10.79 ± 0.69	3.52 ± 0.29				14.31 ± 0.95 b
	CH 47	54.07 ± 5.82	34.86 ± 3.22				88.93 ± 8.78 a
	CH 48	43.76 ± 2.68	38.57 ± 1.40				82.33 ± 4.06 a
	CH 49	38.01 ± 3.69	5.88 ± 0.41				43.90 ± 4.10 a
	CH 51	14.20 ± 2.12	4.11 ± 0.88				18.30 ± 2.99 b
	CH 77	41.88 ± 8.25	20.02 ± 3.95				61.91 ± 12.04 a
A. deliciosa	'Hort16A'						0.00 e
	DE 02		3.64 ± 1.09	14.73 ± 4.08			18.37 ± 5.18 a
	DE 06		0.35 ± 0.35				0.35 ± 0.35 c
	DE 11		1.13 ± 0.34	4.70 ± 1.12			5.82 ± 1.46 b
	DE 18		3.41 ± 0.75	4.20 ± 0.48			7.61 ± 1.22 ab
	DE 26		0.27 ± 0.27	2.94 ± 0.41			3.20 ± 0.64 b
A. eriantha	'Hayward'						0.00 c
	ER 08	3.58 ± 1.12					3.58 ± 1.12
A. melanandra	ME 03	118.11 ± 0.85	0.60 ± 0.60		0.33 ± 0.33		119.05 ± 0.71 b
	ME 04	104.5 ± 4.393	24.69 ± 2.01		3.82 ± 0.22		133.04 ± 3.33 b
	ME 08	88.70 ± 2.66	86.29 ± 6.02		16.38 ± 1.18	7.14 ± 0.84	198.50 ± 10.59 a
	ME 12	88.80 ± 10.19	9.40 ± 1.34		1.97 ± 0.26		100.18 ± 11.78 b
	ME 13	43.67 ± 2.96	5.45 ± 0.71		1.24 ± 0.22		50.35 ± 3.73 c
A. arguta var. *purpurea*	PU 03	126.69 ± 16.48	32.43 ± 7.62		2.06 ± 0.45		161.18 ± 24.31 a
	PU 05	178.1 ± 17.811	19.82 ± 1.22		6.15 ± 0.79	2.00 ± 0.27	206.09 ± 19.09 a
	PU 33	174.1 ± 37.570	6.39 ± 1.68		4.16 ± 1.12		184.65 ± 40.31 a
A. arguta	AR 01	2.61 ± 0.19					2.61 ± 0.19 b
	AR 02	5.09 ± 0.61					5.09 ± 0.61 a
	AR 03	6.75 ± 0.12					6.75 ± 0.12 a
	AR 10	3.03 ± 0.24					3.03 ± 0.24 b

[a] Data are for total anthocyanin across all tissues [μg/g of fresh weight ± standard error (SE)]. Analysis of variance (ANOVA) was used to determine differences among genotypes within each species. Different letters represent a significant difference according to Tukey's test (5% level). [b] Cyanidin 3-(xylosyl)galactoside. [c] Cyanidin 3-galactoside. [d] Cyanidin 3-glucoside. [e] Delphinidin 3-(xylosyl)galactoside. [f] Delphinidin 3-galactoside.

different genotypes and species. Additional samples were taken to measure pigment concentrations in separate fruit tissues: skin and pericarp in fruit of *A. arguta*, *A. arguta* var. *purpurea*, and *A. melanandra*; and skin, outer pericarp, and inner pericarp in fruit of *A. deliciosa*, *A. chinensis*, and *A. eriantha*. For these analyses, three replicates of 1.5−2 g were collected in the same way from a pool of 15−20 fruit.

Extraction of Anthocyanins. Fruit samples were lyophilized in a Labconco Centrivap concentrator (Labconco, Kansas City, MO), ground to a powder, and extracted with 5 times volume (v/w) methanol/formic acid (99:1, v/v). The extracts were allowed to stand at room temperature for 4 h and then centrifuged at 3000*g* for 10 min. The supernatant was analyzed by reversed-phase high-performance liquid chromatography (HPLC), as previously described (*5*).

Data Analysis. Analysis of variance (ANOVA) was used to determine differences among genotypes in terms of mean total anthocyanin content. Data for each species were analyzed separately (using Genstat 10.1). To meet the model assumptions, the data were log-transformed, with original zero values having 1 added prior to the transformation. Means were separated posthoc using Tukey's honestly significant difference (HSD) at the 5% level.

Principal Component Analysis (PCA). PCA was used to investigate the relationships between genotypes and the measured attributes. A correlation matrix was used to allow for the differences in the scale of concentrations. The model was fitted in R 2.61 (*10*) using the FactoMiner 1.02 package.

RESULTS AND DISCUSSION

Anthocyanin Profiles. Three cyanidin-based and two delphinidin-based anthocyanins have previously been detected in *Actinidia* fruit (*5*). The chromatographic system used in this study separated all five anthocyanin components. Representative chromatogram traces for the *Actinidia* genotypes studied, showing the variation in anthocyanin content and composition, are shown in **Figure 1**. Analytical results are given in **Table 2**.

Red Color and Anthocyanin Analysis in Fruit of *Actinidia* Species. Although we had access to a large germplasm collection (*6*), the plants studied are not necessarily representative of a particular taxon. In some *Actinidia* taxa, e.g., *A. chinensis* and *A. deliciosa*, only occasional genotypes had red pigments in their fruit, and we chose those particular individuals for further study. Thus, although 90 accessions of *A. chinensis* were available, only two had fruit that were consistently red. Most genotypes with red fruit came from Xixia (Henan Province, China), while one accession (CH 16) with red fruit came from Guilin (Guangxi Province). Only 2 of the more than 130 accessions from the closely related *A. deliciosa* had any red fruit in the year that we sampled (**Table 1**). In contrast, all genotypes in our germplasm collections of *A. melanandra* and *A. arguta* var. *purpurea* had red fruit every year. We had only a few accessions of these two taxa available in our collection, from which we selected representative genotypes for further analysis (**Table 1**). These fruit had the highest anthocyanin concentrations, nearly twice the highest in any *A. chinensis* fruit (**Table 2**). Anthocyanin production in other *Actinidia* taxa is influenced by environmental factors, and some genotypes, particularly of *A. deliciosa* and *A. chinensis*, do not consistently contain

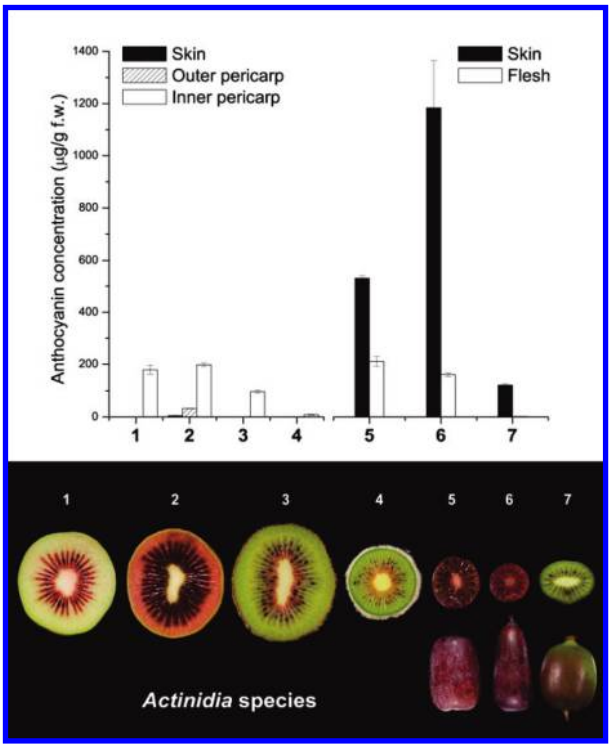

Figure 2. Anthocyanin distribution in fruit tissues of red-fruited genotypes of *A. chinensis* (1 and 2), *A. deliciosa* (3), *A. eriantha* (4), *A. melanandra* (5), *A. arguta* var. *purpurea* (6), and *A. arguta* (7).

anthocyanins each season. We used genotypes that regularly had red fruit, and the data refer to only one fruiting season.

It was not always possible to measure quantitatively flesh color using a chroma meter because the differently colored tissue zones in some species (e.g., *A. chinensis* and *A. deliciosa*) were frequently narrow and irregular. However, it was possible to identify patterns of pigment distribution that allowed *Actinidia* taxa with red fruit to be classified into three main groups.

Group 1, Red Pigments in Pericarp Only. Anthocyanins were detected only in the fruit flesh and not in the skin and were usually present only in the inner pericarp. This pattern was found in fruit of *A. chinensis*, *A. deliciosa*, and *A. eriantha*. Of these, the fruit of *A. chinensis* have the highest anthocyanin concentrations, mainly localized in the inner pericarp of the fruit, in the locules, as previously reported (*4*). Anthocyanin concentrations vary among different genotypes of a taxon (**Table 2**) and sometimes even between individual fruit of the one genotype. While in most of the genotypes tested, anthocyanins were limited to the locules or the inner parts of the locules, as in the commercial cultivars, *A. chinensis* 'Hongyang' (*11*) and 'Chuhong' (*12*) and *A. deliciosa* 'Hongmei' (*13*); in a few genotypes of *A. chinensis*, anthocyanins were also present in the outer pericarp but at lower concentrations, so that the inner pericarp appeared darker red. In such cases, anthocyanins were also detected in the skin, but this was probably due to contamination by outer pericarp tissues (**Figure 2**). Anthocyanins were never detected in the fruit core in these taxa.

Group 2, Red Pigments in Both Fruit Flesh and Skin. The fruit have the highest anthocyanin concentrations of any of the *Actinidia* taxa tested. Anthocyanins were present in all parts of the fruit: skin, pericarp, and core. In such fruit (e.g., of *A. arguta* var. *purpurea* and *A. melanandra*), anthocyanins were most concentrated in the skin or the outer pericarp (**Figure 2**).

Group 3, Red Pigmentation as a Skin Blush. In some genotypes of *A. arguta*, a red blush sometimes occurs on exposed fruit surfaces.

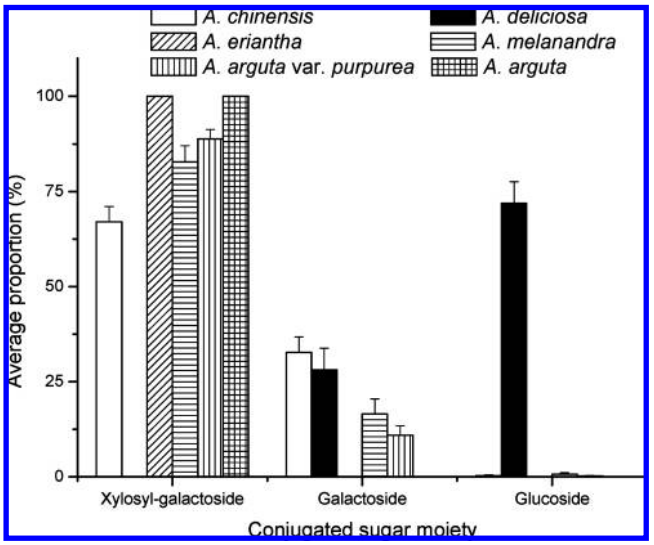

Figure 3. Average proportions of anthocyanin sugar conjugates (percentage of total content ± SE) in different *Actinidia* taxa.

Fruit color is determined by pigment composition and concentration. Anthocyanin chromatic properties are affected by the extent to which the B ring is hydroxylated: increasing hydroxylation shifted the color from orange/pink (pelargonidin) to magenta (cyanidin) to purple/blue (delphinidin) (*14*). Glycosidic substitution or acylation and its position on the aglycone, the vacuolar pH, presence of co-pigments, and cell shape are also important in determining anthocyanin color (*15*, *16*).

The only anthocyanin aglycones thus far detected in *Actinidia* fruit are cyanidin and delphinidin (*5*). The aglycones present determine the color of the fruit, and the presence of a specific aglycone and its derivates seems to be characteristic of the taxon. No single *Actinidia* taxon contained all five anthocyanins (**Table 2**).

Only Cyanidin Present. The fruit (or part of it) appears to be red/magenta. The only anthocyanins identified were different forms of cyanidin, mainly conjugated with the xylosyl-galactoside moiety in fruit of *A. chinensis*, *A. arguta*, and *A. eriantha* or with glucose and galactose in fruit of *A. deliciosa*.

Cyanidin and Delphinidin Both Present. Fruit of *A. melanandra* and *A. arguta* var. *purpurea* appear purple probably because of their high anthocyanin content and the presence of both cyanidin and delphinidin derivatives. Cyanidin xylosyl-galactoside is usually the most abundant anthocyanin present.

The differences in hue observed were due to differences in both anthocyanin concentration (**Figure 2**) and composition. Synthesis of cyanidin requires the enzyme flavonoid 3′ hydroxylase (F3′H) to add a hydroxyl group on position 3′ of the B ring. Synthesis of delphinidin synthesis requires the expression and activity of flavonoid 3′5′ hydroxylase (F3′5′H), a structural enzyme of the flavonoid pathway able to operate at different steps in the anthocyanin biosynthetic pathway: F3′5′H catalyzes the addition of two hydroxyl groups to the B ring, position 3′ and 5′, of either flavanones or dihydroflanonols, eventually leading to the accumulation of delphinidin (*17*, *18*). Both enzymes must be active in fruit of *A. melanandra* and *A. arguta* var. *purpurea*, leading to the production of both cyanidin and delphinidin, whereas in fruit accumulating only cyanidin, F3′H is the only flavonoid hydroxylase active. Regulation of these enzymes is key to determining the relative abundance of cyanidin and delphinidin-based anthocyanins and, hence, the color of the fruit. Identification of the genes for anthocyanin biosynthesis and an understanding of the regulation of the enzymes responsible for cyanidin and delphinidin

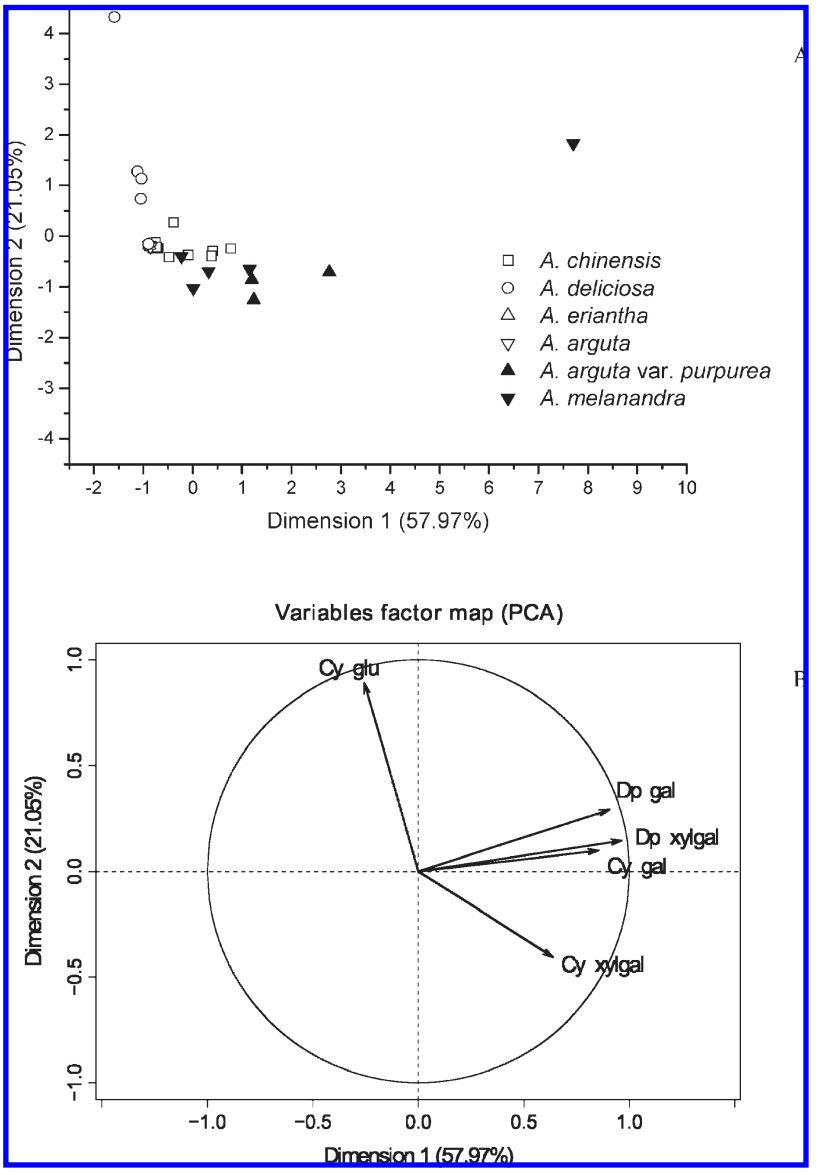

Figure 4. (A) Distribution of red-fruited *Actinidia* species in a two-dimensional PCA plot of the two first principal components. The species are grouped into two groups: with cyanidin and delphinidin (solid simbols) and with cyanidin only (open symbols). (B) Weights of the two principal components.

synthesis could assist breeding programs to develop new types of kiwifruit with different flesh colors.

In addition to the differences in the aglycones, there are also differences in the conjugated sugars. Glycosylation occurs at the 3 position in the *Actinidia* taxa studied (*5*). Xylosyl-galactoside is the most common sugar moiety (**Figure 3**), except in fruit of *A. deliciosa*, in which cyanidin is present mainly as cyanidin 3-glucoside. Fruit of one genotype of *A. chinensis* (CH 16) containing low anthocyanin concentrations differed from all other *A. chinensis* genotypes studied in that only cyanidin 3-galactoside was detected. It may be significant that this genotype came from a different area in China (Guilin), well-separated in origin from the other red-fleshed *A. chinensis* genotypes.

Anthocyanins and Chemotaxonomy of Red-Fruited *Actinidia* Species. The taxonomy of the genus *Actinidia* has been debated with several species being periodically separated and then recombined, e.g., *A. chinensis* and *A. deliciosa* (*2*) or *A. melanandra* and *A. arguta* (*19*).

Anthocyanins and polyphenols in general have often been used in other plants to distinguish species or even cultivars (*14*, *20*−*22*). Because red-fleshed fruit occur in different *Actinidia* taxa and there are differences in the aglycones and sugar moieties, we investigated whether anthocyanin composition could be used to resolve some of the taxonomic questions in *Actinidia*. Although our results show that in the *Actinidia* genotypes studied cyanidin derivatives are always the most abundant anthocyanins in red-fruited species and all of the anthocyanins identified are 3-glycoside derivatives, there are some obvious differences between different taxa. With PCA, it has been possible to separate the plants studied into two main groups according to their anthocyanin composition (**Figure 4A**): those with and those without delphinidin. The first two PCA dimensions (**Figure 4B**) explain 80% of the total variation in the five variables. The first dimension is highly correlated with the delphinidin derivates (galactoside and xylosyl-galactoside) and cyanidin galactoside and to a lesser extent with cyanidin xylosyl-galactoside. The second dimension is highly correlated with cyanidin glucoside. This is illustrated by the vectors in **Figure 4B**. For example, the symbol identifying the *A. deliciosa* in the top left corner on **Figure 4A** had the highest content of cyanidin glucoside (corresponding position in **Figure 4B**).

Similarly data points in the right-hand side had higher scores for those attributes mapping on the right. The overall percentage variation explained changed very little and maintained the same vectorial direction when we removed the two outlier genotypes, *A. melanandra* and *A. deliciosa*, from the analysis.

One group includes *A. arguta* var. *purpurea* and *A. melanandra* (solid symbols in **Figure 4A**), characterized by the presence of both cyanidin and delphinidin. These two taxa are very closely related, and both of them are listed in the same section *Leiocarpae* (Dunn) Li and series *Lamellatae* C. F. Liang. Interestingly, fruit of *A. arguta* seem to belong to a different group, but this could be because the analysis has been limited to anthocyanins only and the small amount and occasional red pigmentation in fruit of *A. arguta* might not be appropriate for this analysis (*19*).

The second group (open symbols in **Figure 4A**), with only cyanidin derivates detected in the fruit, includes *A. arguta* (*Lamellatae*) and *A. chinensis*, *A. deliciosa*, and *A. eriantha* that are usually placed in a quite different section (*Stellatae*) of the genus. The presence of cyanidin glucoside instead of cyanidin xylosyl-galactoside in fruit of *A. deliciosa* seems to distinguish this species from *A. chinensis*. These two species are closely related even if the level at which they should be separated is still being debated (*3*). Further chemotaxonomic analysis would require the study of many more genotypes from different accessions and possibly the use of other components, such as other polyphenols, to determine whether it is really useful in elucidating relationships within *Actinidia*.

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NOTE ADDED AFTER ASAP PUBLICATION

Several errors were reported after the original ASAP posting of July 2, 2009. These have been corrected with the posting of July 13, 2009.

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