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Characterization of polyphenolic metabolites in grape hybrids

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Summary

Cultivated and experimental grape hybrids represent an important part of the *Vitis* germplasm for grape improvement. In this study, we characterized the composition and content of polyphenolic compounds in the berries of 48 hybrid grapes for two consecutive years. A total of 48 polyphenolic compounds, including 28 anthocyanins, 6 flavanols, 6 flavonols, 2 hydroxybenzoic acids and 6 hydroxycinnamic derivatives, were identified via HPLC-MS and quantified by HPLC-DAD. The content of total polyphenols as well as individual polyphenolic compounds varied significantly among grape hybrids. A number of grape hybrids with high content of total polyphenols and various individual groups of polyphenolic compounds were identified. Principal component analyses identified several polyphenolic compounds, significantly influencing the content variation of total polyphenols and individual groups of polyphenols. Plot analyses on the basis of PC1 and PC2 values provided some interesting insights into the genetic relationships among these grape hybrids. This work is an important addition to our ongoing effort in developing a comprehensive database of nutrition- and health-related secondary metabolites in the *Vitis* germplasm for future grape improvement.

K e y w o r d s : grapes, *Vitis*, hybrids, polyphenols, secondary metabolites.

Introduction

Grapes are rich in polyphenolic compounds which are beneficial to human nutrition and health. Polyphenolic compounds in grapes can be classified into flavonoids and nonflavonoids on the basis of their primary chemical structures of hydroxybenzes. Flavonoids mainly consist of anthocyanins, flavanols and flavonols, whereas nonflavonoids include hydroxycinnamic and hydroxybenzoic acids (Adams 2006). These polyphenolic compounds affect appearance and quality of grape berries and processed products. Anthocyanins are responsible for the red color of skin in red grapes and often taken as important indicators of grape fruit quality at harvest; flavanols are the basic building blocks of grape tannins, which have significant impact on wine taste and mouthfull (Hollman *et al.* 2000); flavonols are present in grapes and wine as glycosides cofactors for color enhancement (Roggero *et al.* 1997); and nonflavonoids, hydroxycinnamate and hydroxybenzoic acid play critical roles in developing the bitterness and astringency properties of wine (Monagas *et al.* 2006, Suarez *et al.* 2007). In addition to their importance in determining the appearance and quality of grape berries, these polyphenolic compounds were also found to possess antioxidant activities and other health benefits.

Different grape varieties can have very different profiles of polyphenolic compounds, therefore different quality, in their berries. Combining desirable polyphenolic profiles from different breeding material into improved varieties through breeding is an important means for enhancing fruit quality and health benefits of grapes and grape products. To provide support of germplasm for such breeding effort, we recently characterized the composition and content of several dozens of polyphenolic compounds in representative accessions of *Vitis vinifera*, the most widely cultivated grape species, and wild grape species preserved in the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) *Vitis* germplasm repositories in Davis, California and Geneva, New York and identified many accessions of *V. vinifera* and wild grape species with unique composition profiles and high content of various polyphenolic compounds (Liang *et al.* 2011, 2012).

Combining traits, such as polyphenolic profiles, among various varieties of the cultivated species *V. vinifera* is straightforward through conventional crosses and selection. Realizing such trait exchanges among *Vitis* species, including *V. vinifera*, is also possible since, in most cases, there are no hybridization barriers to such inter-specific genetic introgression in the *Vitis* genus. In fact, many *V. vinifera*-wild grape interspecific hybrids have been produced and some of them are widely cultivated for commercial purposes (Luo and Zhang 1990). *Vitis* hybrids represent an important part of *Vitis* germplasm and characterizing the biochemical profiles of these hybrids can provide important insight into how these compounds are inherited and manifested in hybrid background, which in turn can provide a better prediction of the outcomes when these phytochemical traits are introduced from wild grapes into cultivated ones. In this study, we examined the polyphenolic profiles of 48 *Vitis* hybrids accessions preserved in the USDA-ARS *Vitis* germplasm repository in Geneva, NY. Efforts in char-

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acterizing polyphenolic profiles in *Vitis* hybrids, *V. vinifera* - wild grape hybrids in particular, have been previously reported, but the previous studies were mainly focused on anthocyanins in a small number of hybrids (FLAMINI and TOMASI 2000, LIANG *et al.* 2008, POUDEL *et al.* 2008, 2009, NIXDORF and HERMOSIN-GUTIERREZ 2010, DE ROSSO *et al.* 2011). Our current study covered 48 hybrids with diverse genetic background and characterized the profiles of all the major polyphenolic compounds including anthocyanins. This work is a continuation of our effort in establishing a comprehensive database of phytochemicals in the world-wide *Vitis* germplasm for grape improvement.

Material and Methods

Plant material: Forty-eight accessions of *Vitis* hybrids preserved in the USDA-ARS *Vitis* germplasm repository in Geneva, New York were characterized in this study (Tab. 1). Pedigree information for 39 of these hybrid accessions was available at the USDA-ARS GRIN website http://www.ars-grin.gov/. For each accession, two grapevines were available for sampling. All the vines received standard fertilization, irrigation, pruning, and insect and disease control. Berry samples of individual vines were harvested upon their ripening, determined on the basis of seed color change, in two consecutive years of 2008 and 2009.

About 100 grams of representative berries were collected from each individual grapevine. The number of berries was counted and the berry weight was recorded for each sample before being frozen and stored at -80 °C for further processing. The frozen berries were then crashed using a mortar and pestle. After removing all the seeds, flesh and peel tissues were ground in an IKA A11 mill (IKA Works, Inc, NC, USA) while frozen. Then 0.5 g powdery samples were weighed for analysis.

Analysis of polyphenols: High performance liquid chromatography/quadrupole-time of flight mass spectrometer (HPLC/Q-TOF MS/MS) (Micromass Q-TOF micro, Waters, USA) was employed for identifying polyphenols. The system was equipped with a Waters Alliance 2695 HPLC Pump, Waters Alliance 2695 Autosampler and Waters 996 photodiode array detector which were coupled directly to the sprayer needle where ions were generated by electrospray ionization (ESI) in both positive and negative ionization modes. A reverse-phase C18 column Inertsil ODS-3 (5 μm particle sizes, 250 mm × 4.6 mm I.D.) from GL Sciences (Japan) and a C18 Nova Pack guard column (Waters, USA) were used for the analysis. The mobile phase consisted of water-formic acid (90:10) as solvent A, and acetonitrile-formic acid (90:10) as solvent B. The gradient profile began at 95 % A, to 85 % A at 25 min, 73 % A at 53 min, then A went back to 95 % at 57 min, and was kept for 5 min. The flow rate was 1.0 mL·min^{-1} and the column temperature was set at 30 °C. The injection volume was 20 μL. Polyphenolic compounds were detected at 280, 320, 360 and 520 nm on the diode array detector, and at the same time, spectrum scans were made from 210 nm to 600 nm. For MS analyses, nitrogen was used as drying and nebulizing gas and nebulizer pressure was 380 Pa. Gas flow was set at 10 L min^{-1} and temperature was 350 °C. The capillary voltage was 3,000 V. Mass spectra of anthocyanins and other polyphenolic compounds were recorded in both positive and negative ionization modes between *m*/*z* 100 and 1000, respectively.

The same HPLC protocol was used on an Agilent 1100 HPLC system (Aglient Technologies, CA, USA) fitted with a Agilent 1100 diode array detector and autosampler for quantifying polyphenolic compounds for all samples. The concentration of individual polyphenolic compounds was quantified based on peak area and standard curves derived from corresponding authentic polyphenolic compounds as described in LIANG *et al.* (2012). Standards for 28 polyphenolic compounds were commercially available and obtained from Sigma-Aldrich (St. Louis, MO, USA), Extrasynthese (Genay Cedex, France) or AApin Chemicals (Abingdon, Oxon, UK). For the polyphenols for which commercial standards are not available, we quantified those compounds using internal standards with similar absorbance wavelength.

Data analysis: Data analysis was carried out using SPSS 13.0 (SPSS, USA). Accession means over years and plants were used in principal component analysis. The boxplot was developed by using Sigmaplot 10.0 for Windows (SPSS, USA).

Results and Discussion

Identification of polyphenolic compounds: Polyphenolic compounds were identified on the basis of retention time, molecular ions, fragment ions and UV-Vis spectra absorbance maxima generated from MS and HPLC profiles. Forty-eight polyphenolic compounds were identified for most hybrids, including 28 anthocyanins, 6 flavanols, 6 flavonols, 2 hydroxybenzoic acids and 6 hydroxycinnamic derivatives. The anthocyanins detected in this study consisted of mono- and di-glucoside derivatives of 5 anthocyanidins: delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv). Other forms of derivatives, including 6-*O*-acetyl, 6-*O*-coumaryl and cyanidin 3-*O*-(6-*O*-caffeoyl)-glucoside, were also detected. Six flavanols, mainly catechin and its derivatives, and 6 flavonols, mostly in the form of flavonoids glycoside, were also identified in this study. Two hydroxybenzoic acids, gallic and vanillic acids, and six hydroxycinnamate derivatives, caftaric, coutaric, chlorogenic, caffeic and ferulic acids and resveratrol, were identified in this study. These polyphenolic compounds were also previously detected in *V. vinifera* and wild grape species (LIANG *et al.* 2011, 2012).

Total polyphenols: The total content of polyphenols was calculated as the sum of the content of individual polyphenolic compounds detected. The mean total content of polyphenols varied significantly among hybrid accessions ranging from 0.301 to 23.588 mg·g^{-1} FW (Tab. 2) with a mean of 4.407 mg·g^{-1} FW. The hybrid Castel 188-15 (PI 588343), which was a hybrid of *V. monticola* x *V. rupestris,* had the highest total content of polyphenols

Table 1

Sources, names and pedigrees of the 48 grape hybrids investigated in this study

Plant introduction number (PI)	Group designation	Cultivar name	Pedigree
175494	Seibel[b]	Cascade (Seibel 13.053)	Seibel 7042 x Seibel 5049. *V. aestivalis, V. cinerea, V. labrusca, V. lincecumii, V. riparia, V. rupestris, V. vinifera*
187207	Seibel	Seibel 7052	Seibel 5163 x Siebel 880. *V. cinerea, V. labrusca, V. lincecumii, V. riparia, V. rupestris, V. vinifera*
187208	Seibel	Seibel 7162	Seibel 5455 x Seibel 5163
200684[a]	Seibel	Vidal Blanc (Vidal 256)	Ugni blanc (syn. Trebbiano or St. Emilion) x Seibel 4986
588295[a]	Seibel	Ill 281-1	Seibel 5813 x Seyve Villard 12-375
597138[a]	Seibel	Seyve-Villard 12.303	Seibel 6468 x Seibel 6905
597141	Seibel	Seibel 5163	Seibel 2510 (Alicante Ganzin x Piquepoul) x Gaillard 2 -- *V. labrusca, V. riparia, V. rupestris, V. vinifera, V. lincecumii*
597175	Seibel	Seibel 6339	Seibel 867 x Seibel 2524 -- *V. cinerea, V. labrusca, V. lincecumii, V. riparia, V. rupestris, V. vinifera*
597184[a]	Seibel	Seibel 5409	Seibel 867 (Seibel 2003 x Noah) x Seibel 452 (Alicante Ganzin x Seibel 4) - *V. aestivalis, V. cinerea, V. labrusca, V. lincecumii, V. riparia, V. rupestris, V. vinifera*
597190	Seibel	Seibel 9280	
597198	Seibel	Seibel A	
181647	LV	Bertille Seyve 5563	French hybrid S.6905 x B.S.3445
215418	LV	Kuhlmann 149-3	Millardet 101-14 x Goldriesling
588067	LV	Moored	Fredonia x Athens
588076[a]	LV	Seneca	Lignan Blanc x Ontario
588096	LV	Canadice (NY 45625)	Bath x Himrod
588111[a]	LV	Golden Muscat	Muscat Hamburg x Diamond
588120[a]	LV	Diamond	Concord x Iona
588122	LV	Campbell Early	Moore Early x (Belvidere x Muscat Hamburg)
588124	LV	Keuka	Chasselas Rose x Mills
588166	LV	Salem	Carter x Black Hamburg
588184	LV	Oconee	SC 4710 (Alden x Ellen Scott O.P.) x Niagara
588211	LV	Agawam	Carter x Black Hamburg
597099	LV	Telegraph	Unknown. V. labrusca, V. aestivalis Chance seedling
597100	LV	Glenora (NY 35814)	Ontario x Russian Seedless
597107	LV	Goff	*V. labrusca* x *V. vinifera*
597111[a]	LV	Brocton	Brighton x NY 125 (Winchell x Diamond)
597122	LV	Wayne	Mills x Ontario
597129[a]	LV	Ripley	Winchell x Diamond
597131	LV	Erie	(Goff x Worden) x Worden
597132	LV	Hector	Chasselas Rose x Brocton
597133[a]	LV	Melton	Triumph x NY 4064 ((Winchell x Diamond) x Jefferson)
588082	Riparia	Teleki 5 A	*V. berlandieri* x *V. riparia*
588091	Riparia	Cosmo 10	*V. berlandieri* x *V. riparia*
588118	Riparia	Couderc 1613	Solonis (*riparia-rupestris-candicans*) x Othello (*Labrusca-riparia vinifera*)
588212	Riparia	Azita	Beta x *V. riparia*
279505[a]	Rupestris	Bertille Seyve 2758	Bertille Seyve 822 x Bertille Seyve 872 - *V. aestivalis, V. labrusca, V. riparia, V. rupestris, V. vinifera*
588170	Rupestris	Ill 796-4	Jaeger 70 x Victoria‘s Choice
588342	Rupestris	Ill 547-2	38 (*V. rupestris*) x B9 (*V. cinerea*)
588343	Rupestris	Castel 188-15	*V. monticola* x *V. rupestris*
597143	Rupestris	Couderc Noir (Couderc 7120)	Jaeger 70 (*V. rupestris* x *V. lincecumii*) x Unknown *V. vinifera*
588083[a]	Unknown	FS 4	
588251	Unknown	Rudelin 15	
588712	Unknown	Galea	
597144	Unknown	Joannes Seyve 26.487	
597147	Unknown	Bertille Seyve 6264	
597152[a]	Unknown	Perbos 226	
597255[a]	Unknown	Hendrickson Seedless	

[a]: White grape. [b]: Hybrid groups determined on the basis of pedigree information. Seibel group included Seibel hybrids and those containing Seibel background; LV: hybrids mainly involved *V. vinifera* and/or *V. labrusca*; Riparia: hybrids mainly involved *V. riparia*; Rupestris: hybrids mainly involved *V. rupestris*; Unknown: hybrids with no pedigree information.

(23.588 mg g^{-1} FW). Teleki 5 A (PI 588082), a progeny of *V. berlandieri* x *V. riparia*, had the next highest content of total polyphenols with a mean content of 12.680 mg·g^{-1} FW, followed by Cosmo 10 (PI 588091, 11.954 mg·g^{-1} FW, a progeny of *V. berlandieri* x *V. riparia*), III 547-2 (PI 588342, 11.953 mg g^{-1} FW, a progeny of *V. rupestris* and *V. cinerea*) and Rudelin 15 (PI 588251, 9.714 mg·g^{-1} FW, with unknown pedigree information). They were all distantly higher than the rest of the hybrids. The non-colored hybrids, as expected, had no detectable anthocyanins and lower content of total polyphenols, compared with the colored hybrids.

Table 2

Mean content of hydroxybenzoic acids, flavanols, hydroxycinnamic acdis, flavonols, anthocyanins, and total polyphenols in the 48 grape hybrids

Plant introduction number (PI)	Cultivar name	Anthocyanins		Flavanols		Flavonols		Hydroxybenzoic acids		Hydroxycinnamic derivatives		Total polyphenols	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
175494	Cascade (Seibel 13.053)	2.925	0.310	0.171	0.010	0.031	0.003	0.009	0.003	0.211	0.009	3.345	0.324
187207	Seibel 7052	3.777	1.093	0.206	0.032	0.112	0.013	0.009	0.005	0.251	0.036	4.355	1.178
187208	Seibel 7162	4.218	0.228	0.247	0.014	0.133	0.011	0.009	0.001	0.348	0.031	4.954	0.201
200684[a]	Vidal Blanc (Vidal 256)	0.000	0.000	0.307	0.017	0.040	0.000	0.025	0.003	1.017	0.025	1.389	0.045
588295[a]	Ill 281-1	0.000	0.000	0.047	0.005	0.055	0.001	0.010	0.004	0.189	0.060	0.301	0.070
597138[a]	Seyve-Villard 12.303	0.000	0.000	0.144	0.011	0.219	0.025	0.011	0.002	0.749	0.129	1.123	0.116
597141	Seibel 5163	3.791	0.268	0.627	0.208	0.086	0.005	0.022	0.009	0.884	0.007	5.408	0.039
597175	Seibel 6339	6.190	1.045	0.296	0.052	0.158	0.009	0.015	0.003	1.110	0.085	7.769	1.137
597184[a]	Seibel 5409	0.000	0.000	0.242	0.046	0.082	0.007	0.010	0.001	0.191	0.015	0.524	0.049
597190	Seibel 9280	4.270	0.148	0.240	0.027	0.219	0.002	0.016	0.002	0.265	0.005	5.008	0.169
597198	Seibel A	7.009	0.715	0.334	0.067	0.159	0.039	0.009	0.001	0.346	0.025	7.856	0.798
181647	Bertille Seyve 5563	4.836	0.156	0.429	0.079	0.185	0.007	0.011	0.003	0.685	0.153	6.147	0.323
215418	Kuhlmann 149-3	4.862	0.447	0.251	0.018	0.058	0.003	0.006	0.002	0.322	0.024	5.500	0.429
588067	Moored	0.300	0.003	0.257	0.012	0.091	0.001	0.022	0.002	0.775	0.003	1.443	0.008
588076[a]	Seneca	0.000	0.000	0.690	0.043	0.067	0.003	0.022	0.000	0.415	0.005	1.193	0.041
588096	Canadice (NY 45625)	0.187	0.021	0.194	0.011	0.090	0.004	0.019	0.002	0.507	0.014	0.996	0.044
588111[a]	Golden Muscat	0.000	0.000	0.086	0.010	0.078	0.006	0.004	0.000	0.631	0.041	0.799	0.046
588120[a]	Diamond	0.000	0.000	0.173	0.026	0.075	0.012	0.013	0.001	0.642	0.040	0.903	0.046
588122	Campbell Early	3.398	0.343	0.278	0.052	0.059	0.014	0.019	0.005	0.820	0.050	4.573	0.341
588124	Keuka	0.152	0.015	0.190	0.024	0.069	0.005	0.031	0.003	0.624	0.036	1.065	0.067
588166	Salem	0.344	0.029	0.450	0.096	0.127	0.020	0.022	0.005	0.584	0.039	1.526	0.137
588184	Oconee	3.158	0.438	0.428	0.038	0.125	0.025	0.020	0.004	0.286	0.015	4.016	0.393
588211	Agawam	0.590	0.067	0.318	0.081	0.108	0.006	0.019	0.003	0.758	0.032	1.793	0.154
597099	Telegraph	0.400	0.065	0.846	0.086	0.091	0.004	0.016	0.002	0.657	0.036	2.009	0.168
597100	Glenora (NY 35814)	2.161	0.058	0.184	0.012	0.051	0.005	0.019	0.001	0.346	0.032	2.761	0.059
597107	Goff	1.209	0.112	0.370	0.085	0.098	0.005	0.020	0.006	0.909	0.018	2.605	0.070
597111[a]	Brocton	0.000	0.000	0.076	0.008	0.058	0.011	0.005	0.001	0.477	0.019	0.615	0.013
597122	Wayne	4.725	0.547	0.375	0.032	0.131	0.010	0.020	0.002	1.806	0.082	7.057	0.616
597129[a]	Ripley	0.000	0.000	0.128	0.014	0.150	0.025	0.008	0.001	0.554	0.056	0.838	0.029
597131	Erie	5.172	0.197	0.354	0.004	0.112	0.009	0.021	0.004	0.700	0.005	6.358	0.194
597132	Hector	0.045	0.002	0.322	0.014	0.045	0.004	0.018	0.001	0.897	0.123	1.328	0.138
597133[a]	Melton	0.000	0.000	0.200	0.014	0.122	0.009	0.012	0.003	0.484	0.074	0.816	0.066
588082	Teleki 5 A	11.743	0.121	0.461	0.077	0.099	0.012	0.020	0.004	0.358	0.062	12.680	0.128
588091	Cosmo 10	10.952	0.813	0.723	0.081	0.066	0.007	0.021	0.002	0.192	0.018	11.954	0.859
588118	Couderc 1613	2.742	0.073	0.408	0.045	0.052	0.005	0.024	0.008	0.217	0.018	3.442	0.118
588212	Azita	6.803	0.924	0.310	0.014	0.055	0.006	0.008	0.001	0.253	0.027	7.428	0.961
279505[a]	Bertille Seyve 2758	0.002	0.002	0.185	0.024	0.233	0.021	0.008	0.002	0.732	0.084	1.160	0.118
588170	Ill 796-4	3.044	0.202	0.223	0.019	0.049	0.004	0.018	0.004	1.089	0.084	4.423	0.297
588342	Ill 547-2	10.802	1.052	0.717	0.145	0.148	0.008	0.022	0.005	0.264	0.033	11.953	1.184
588343	Castel 188-15	22.690	0.479	0.554	0.031	0.157	0.020	0.020	0.003	0.168	0.021	23.588	0.474
597143	Couderc Noir (Couderc 7120)	4.186	0.268	0.231	0.018	0.075	0.005	0.020	0.004	0.523	0.023	5.034	0.313
588083[a]	FS 4	0.000	0.000	0.159	0.013	0.091	0.004	0.008	0.001	0.282	0.023	0.540	0.038
588251	Rudelin 15	8.883	0.374	0.391	0.099	0.117	0.016	0.015	0.001	0.309	0.056	9.714	0.427
588712	Galea	8.218	0.465	0.887	0.147	0.105	0.004	0.028	0.007	0.183	0.028	9.421	0.640
597144	Joannes Seyve 26.487	5.842	1.857	0.243	0.043	0.064	0.005	0.008	0.002	0.188	0.008	6.344	1.915
597147	Bertille Seyve 6264	5.514	0.687	0.295	0.041	0.115	0.017	0.009	0.001	0.279	0.012	6.212	0.706
597152[a]	Perbos 226	0.000	0.000	0.171	0.013	0.134	0.020	0.010	0.002	0.280	0.020	0.595	0.025
597255[a]	Hendrickson Seedless	0.000	0.000	0.130	0.008	0.083	0.008	0.014	0.002	0.443	0.025	0.670	0.027

[a]: White grape.

Anthocyanins: Anthocyanins were the main polyphenolic compounds in colored hybrid accessions and accounted for 78.2 % of the total polyphenols in the hybrids (data not shown). The content of total anthocyanins in the 48 hybrid accessions ranged from 0 (non-colored grapes) to 22.690 (colored grapes) mg·g^{-1} FW with a mean content of 3.440 mg·g^{-1} FW (Tab. 2, Fig. 1a). The top 5 accessions with the highest content of anthocyanins were Castel 188-15 (22.690 mg·g^{-1} FW, PI588343), Teleki 5 A (11.743 mg·g^{-1} FW, PI588082), Cosmo 10 (10.952 mg·g^{-1} FW, PI588091), Ill 547-2 (10.802 mg·g^{-1} FW, PI588342), and Rudelin 15 (8.883 mg·g^{-1} FW, PI588251) (Tab. 2). Because anthocyanins are the dominant polyphenolic compounds in colored grapes, these five accessions also had the highest content of total polyphenols as described earlier.

Among the five classes of anthocyanins, the content of Dp-derivatives ranged from 0 to 8.348 mg·g^{-1} FW and on average accounted for 36 % of the total anthocyanins in the hybrid grapes. Most Dp-derivatives were delphinidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside-5-*O*-glucoside, which together accounted for more than 81.1 % of the total Dp-derivatives (Fig. 1a). The top three accessions with the highest content of Dp-derivatives were Castel 188-15 (PI588343), Cosmo 10 (PI588091) and Teleki 5 A (PI588082). The content of Mv-derivatives (0.982 mg·g^{-1} FW) ranged from 0 to 3.589 mg·g^{-1} FW and, on average,

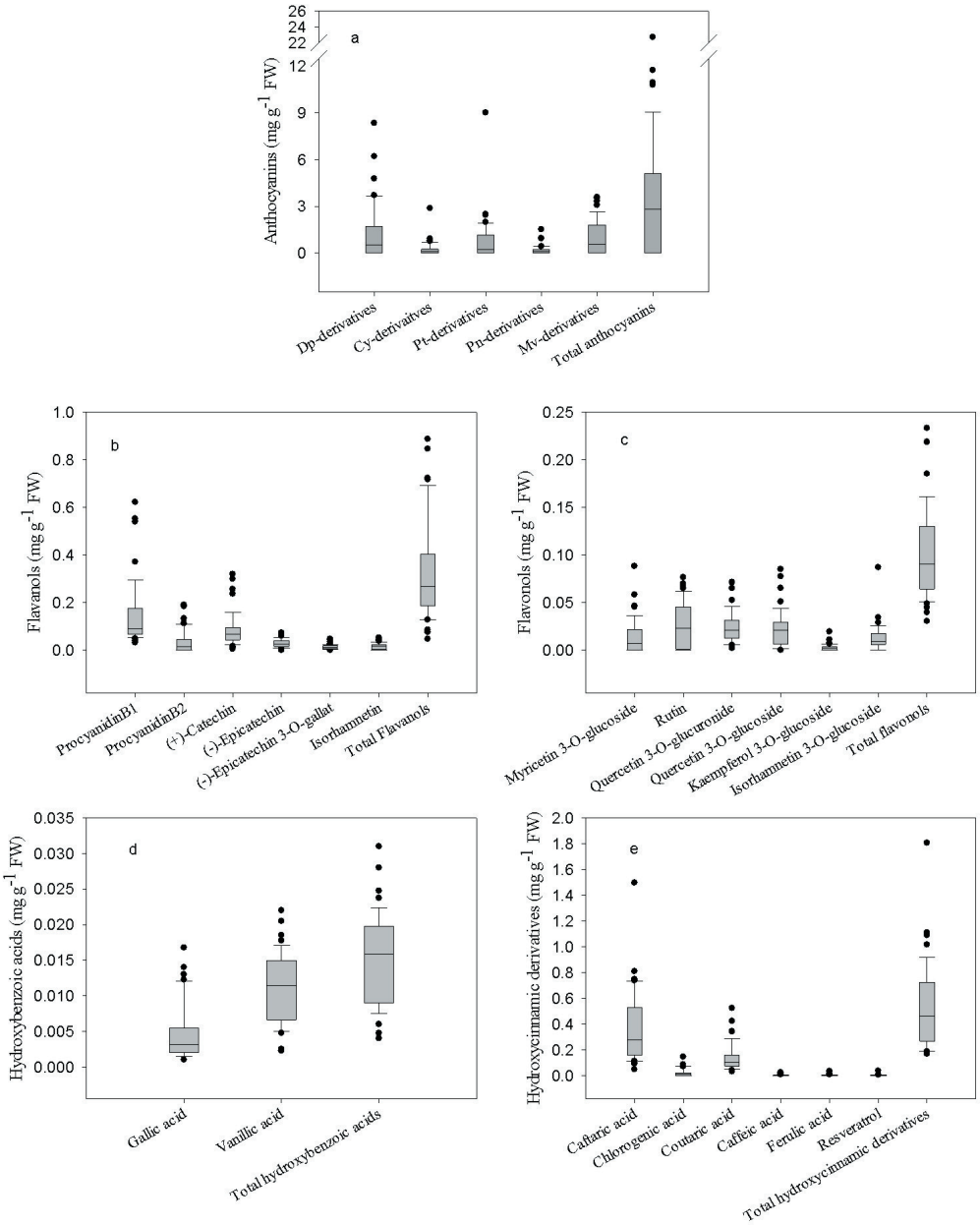

Fig. 1: Content variation of anthocyanins (**a**), flavanols (**b**), flavonols (**c**), hydroxybenzoic acids (**d**), and hydroxycinnamic derivatives (**e**) in 48 grape hybrid accessions. Horizontal lines in the interior of boxes are median values. The height in a box is equal to the inter-quartile distance, indicating the distribution for 50% of the data. Approximately 80 % of the data falls inside the whiskers (the dotted lines extending from the top and bottom of the box). The data outside these whiskers are indicated by black dots. Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pn = peonidin, Mv = malvidin.

accounted for 29 % of the total anthocyanins in the grape hybrids (Fig. 1a, Tab. 2). Malvidin 3-*O*-glucoside-5-*O*-glucoside and malvidin 3-*O*-glucoside were the most abundant compounds and they respectively accounted for 57.7 and 30.1 % of the total Mv-derivatives. The top four accessions with the highest content of Mv-derivatives were Castel 188-15 (PI588343), Rudelin 15 (PI588251), Teleki 5 (PI588082) and III 547-2 (PI588342). The content of Pt-derivatives ranged from 0 to 9.017 mg·g^{-1} FW with a mean of 0.773 mg g^{-1} FW in the 48 hybrid accessions (Fig. 1a). They had a similar variation pattern as observed for the Dp- and Mv-derivatives. The mean content of Cy and Pn-derivatives were 0.243 and 0.185 mg·g^{-1} FW, respectively, and they accounted for 7.1 and 5.4 % of the total anthocyanins (Fig. 1a). The accession of III 547-2 (PI588342) had the highest content of Cy and Pn-derivatives (2.876 and 1.522 mg·g^{-1} FW, respectively). In a previous study, we observed that Mv-derivatives were the most abundant anthocyanin compounds in *V. vinifera*, accounting for more than 68 % of the total anthocyanins (Liang *et al.* 2011, 2012). In contrast to *V. vinifera*, Dp-derivatives were the most abundant anthocyanin compound, as also found in grape hybrids in this study, and accounted for more than 40 % of anthocyanins in the wild species *V. rupestris*, *V. riparia* and *V. labrusca* (Liang *et al.* 2012).

Anthocyanin content in *Vitis* hybrids was previously investigated. For example, Liang *et al*. (2008) reported a wide range of anthocyanin content in several *Vitis* hybrids, including those hybrids between *V. vinifera* and *V. labrusca* (0.1 to 97.5 mg·100 g^{-1} FW with a median 6.48 mg·100 g^{-1}

FW) and between *V. berlandieri* and *V. riparia* (more than 200 mg·100 g^{-1} FW). They observed that Mv-derivatives were the most abundant anthocyanin compound, followed by Pn, Cy, Dp and Pt-derivatives. Similarly, DE ROSSO *et al.* (2011) observed that the content and composition of anthocyanins varied widely with the genetic background of hybrids studied: Seibel 8357 (5291 mg·kg^{-1} grape), Burdin 4077 (3372 mg kg^{-1} grape), Bacò 30-12 (2994 mg kg^{-1} grape) and Terzi 100-31 (1880 mg·kg^{-1} grape). While results from these studies were by and large consistent with our current findings, our present work covered a much larger number of hybrids with diverse genetic background.

Flavanols and flavonols: The content of total flavanols in the 48 hybrid accessions ranged from 0.047 to 0.887 mg·g^{-1} FW (Fig. 1b and Tab. 2) with a mean of 0.324 mg·g^{-1} FW. Flavanols accounted for 33.5 % of the total non-anthocyanin polyphenols and comprised of procyanidin B1, B2, (+)-catechin, (-)-epicatechin, (-)-epicatechin 3-*O*-gallate and isorhamnetin (Fig. 1b). Procyanidin B1 accounted for 46 % of the total flavanols and was the most abundant flavanol in all hybrids (Fig. 1b). (+)-Catechin was the second most abundant flavanol (25.6 %), followed by procyanindin B2 (10.5 %). The content of the remaining flavanol compounds all together accounted for no more than 10 % of the total flavanols. We previously reported that flavanols accounted for 36 % and 57.8 % of the total non-anthocyanins polyphenols in *V. vinifera* and wild grape species, respectively (LIANG *et al.* 2011, 2012). Procyanidin B1 was the most abundant flavanol, accounting for 64 % of the total flavanols in *V. vinifera* (LIANG *et al.* 2011) and 34.8 % in wild grape species (LIANG *et al.* 2012). The content of procyanidin B2, on the other hand, accounted for 13.2 % of the flavanols in the wild grape species (LIANG *et al.* 2012), but only about 2 % in *V. vinifera* (LIANG *et al.* 2011).

On average, flavonols accounted for 10.7 % of the total non-anthocyanin polyphenols. The content of flavonols ranged from 0.031 to 0.233 mg·g^{-1} FW in the 48 hybrid accessions with a mean content 0.103 mg·g^{-1} FW (Fig. 1c and Tab. 2). Flavonols mainly comprised of rutin, myricetin 3-*O*-glucoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-glucoside. Rutin was the most abundant flavonol compound and accounted for 25 % of the total flavonols, followed by quercetin 3-*O*-glucuronide and quercetin 3-*O*-glucoside accounting for 23.4 and 21.4 % of the total flavonols, respectively. In wild grape species myricetin 3-*O*-glucoside was most abundant and, on average, accounted for 29 % of the flavonols (LIANG *et al.* 2012). Quercetin 3-*O*-glucuronide and quercetin 3-*O*-glucoside were also abundant, on average accounting for 24.9 and 16.2 % of the total flavonols in wild grapes. In contrast, quercetin 3-*O*-glucuronide was the most abundant flavonol, accounting for 40 % of the total flavonols, and quercetin 3-*O*-glucoside was the second most abundant compound, accounting for 32 % of the total variation of flavonols, in *V. vinifera* (LIANG *et al.* 2011).

Phenolic acids As far as the two hydroxybenzoic acids are concerned, the content of vanillic acid was generally higher than that of gallic acid and ranged from 0.002 to 0.022 mg·g^{-1} FW, with mean content of 0.012 mg·g^{-1} FW, in the 48 hybrid accessions (Fig. 1d and Tab. 2). The content of gallic acid ranged from 0.001 to 0.017 mg·g^{-1} FW (Fig. 1d), with a mean of 0.004 mg·g^{-1} FW. The top three accessions with high hydroxybenzoic acids were 'Keuka' (PI588124), 'Galea' (588712) and 'Vidal Blanc' (Vidal 256) (PI200684) with a mean of 0.031, 0.028 and 0.025 mg·g^{-1} FW, respectively. The content of the hydroxybenzoic acids in these hybrids were largely between those of *V. vinifera* and wild grape species (LIANG *et al.* 2011, 2012).

The variation of the total content of hydroxycinnamate derivatives for the 48 hybrid accessions were presented in Fig. 1e and Tab. 2. The mean content of hydroxycinnamate derivatives was 0.525 mg g^{-1} FW, ranging from 0.168 to 1.806 mg·g^{-1} FW and accounting for 54.3 % of the non-anthocyanin polyphenols in the hybrids. The content of caftaric acid ranged from 0.048 to 1.497 mg·g^{-1} FW and accounted for 69 % of the total hydroxycinnamic derivatives. Coutaric acid was the second most abundant hydroxycinnamic derivative with the content ranging from of 0.032 to 0.523 mg·g^{-1} FW and accounting for 25.9 % of the total hydroxycinnamic derivatives. The rest of the hydroxycinnamic derivatives were relatively low in quantity. Caftaric and coutaric acids were also the most abundant hydroxycinnamic derivatives in wild grape species and *V. vinifera* (LIANG *et al.* 2011, 2012).

Variation patterns revealed by Principal Component Analysis (PCA): On the basis of known pedigree information, the 48 hybrids could be classified into five groups (Tab. 1): 1) Seibel series in which many wild grape species, including *V. aestivalis, V. cinerea, V. labrusca, V. lincecumii, V. riparia, V. rupestris, V. vinifera*, were involved in crosses, 2) hybrids mainly involving *V. vinifera* and/or *V. labrusca,* 3) hybrids mainly involving *V. riparia,* 4) hybrids mainly involving *V. rupestris*, and 5) hybrids with no pedigree information. This proposed classification might not accurately reflect the true genetic relationships among these hybrids, because in some cases multiple grape species were involved in the creation of the hybrids and in some other cases pedigree information were either missing or not accurate. POMAR *et al*. (2005) suggested that profiles of anthocyanins could be used as biomarkers to fingerprint different varieties of *V. vinifera*. To determine whether or not polyphenolic profiles could provide additional information for determining the genetic relationships among the 48 hybrids in this study, we carried out principal component (PC) analyses on the mean content of total polyphenols, 28 anthocyanins, 12 flavones, and 8 phenolic acids, respectively.

The amount of variation represented by PC1 and PC2 in this study was more than 82 % of the total variation for each of the four categories of polyphenolic compounds analyzed (total polyphenols, anthocyanins, flavones, and phenolic acids). The dominant PC1 and PC2 components (compounds) were summarized in Tab. 3. When the total polyphenols were analyzed, delphinidin 3-*O*-glucoside-5-*O*-glucoside, petunidin 3-*O*-glucoside-5-*O*-glucoside, petunidin 3-*O*-glucoside, and procyanidin B2 were the most dominant PC1 components and peonindin and malvidin 3-*O*-glucoside were the most dominant PC2 components.

Table 3

Dominant PC1 and PC2 components (highlighted in bold) identified in the PC analyses of total polyphenols, anthocyanins, flavones, and phenolic acids

Dominant PC component	Total polyphenols (48 compounds)		Anthocyanins (28 compounds)		Flavones (12 compounds)		Phenolic acid (8 compounds)	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Delphinidin 3-*O*-glucoside-5-*O*-glucoside	**0.926**	-0.34	**0.926**	-0.34				
Petunidin 3-*O*-glucoside-5-*O*-glucoside	**0.943**	-0.301	**0.943**	-0.301				
Petunidin 3-*O*-glucoside	**0.902**	-0.361	**0.902**	-0.36				
Peonidin 3-*O*-glucoside	0.233	**0.626**	0.233	**0.626**				
Malvidin 3-*O*-glucoside	0.533	**0.677**	0.533	**0.678**				
Procyanindin B1	0.076	0.062			**0.994**	-0.105		
Procyanidin B2	**0.925**	0.299			0.169	0.539		
(+)-Catechin	0.259	0.391			0.385	**0.888**		
Caftaric acid	-0.335	0.051					**0.999**	0.044
Coutaric acid	-0.288	-0.148					0.341	**-0.94**

These same compounds, except for procyanidin B2, were also the dominant components when anthocyanins were analyzed. Procyanidin B1 was the dominant component in PC1 and (+)-catechin was the dominant component in PC2 when the 12 flavones were analyzed. Similarly, caftaric acid was the dominant component in PC1 and coutaric acid was the dominant component in PC2 when 8 phenolic acids were considered in PCA analysis.

The scatter plots of PC1 and PC2 were developed for visualizing the inter-relationships of the 48 hybrids (Fig. 2, a-d). Because anthocyanins were the main contributors to the total content of polyphenols in colored hybrids (78.2 %), the distribution patterns of the 48 hybrids in the PC1xPC2 scatter plots of total polyphenols (Fig. 2a) and 28 anthocyanins (Fig. 2b) were very similar. Although drawn on different groups of polyphenols, the four scatter plots of total polyphenols, anthocyanins, flavones and phenolic acids all showed very complex variation patterns among the hybrids and none of the plots had clearly separate group patterns matching with the proposed hybrid groups on the basis of pedigree information. Nevertheless, in the scatter plot of total polyphenols or anthocyanins, the Seibel and LV related hybrids could be recognized as two separate groups. The group of Seibel hybrids was more spread than the LV

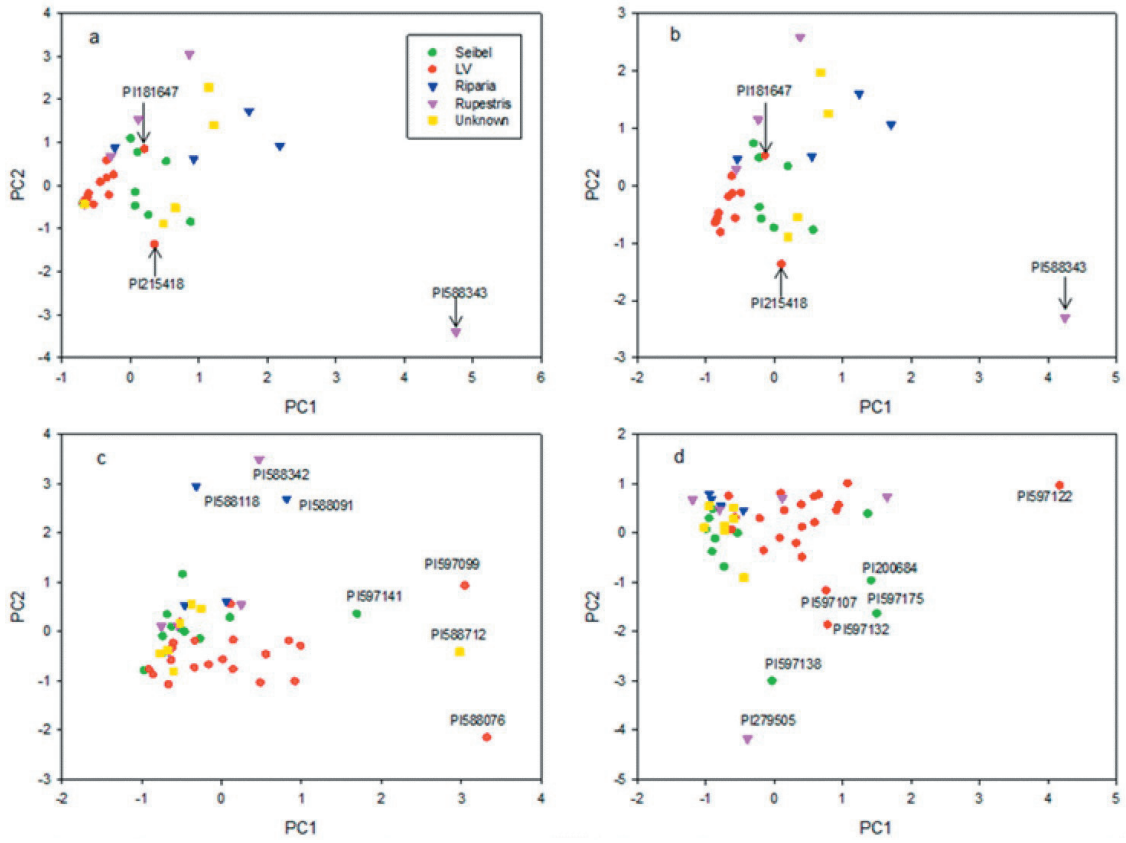

Fig. 2: Distribution pattern of the 48 grape hybrids in the PC1 x PC2 scatter plot of the total polyphenols (**a**), 28 anthocyanins (**b**), 12 flavones (**c**), and 8 phenolic acids (**d**). The identities of the hybrid accessions which were not closely grouped with their respective groups were indicated.

group. Accessions PI181647 and PI215418 in the LV group were distantly located from the remaining group members in the plot. In contrast, the *Riparia* and *Rupestris* related hybrids were much widely scattered with no clear boundaries. One of the *Rupestris* hybrid (PI588343, a hybrid progeny of *V. monticola* x *V. rupestris*) was distantly located far away from the main group. Hybrids with unknown pedigree information were also widely scattered. In the plot of flavones (Fig. 2c), similar patterns were observed. As observed in the plot of total polyphenols or anthocyanins, the Seibel and LV related hybrids were reasonably grouped as two distinct groups, but both were more widely spread. The LV group had two clear outliers: accessions PI588076 and PI597099. In the Seibel group, the accession PI597141 was a clear outlier. Hybrids in the other three hybrid groups were widely spread with no clear group boundaries. In the plot of phenolic acids, hybrids in the *Riparia* group were closely clustered together. In contrast, hybrids from all the other hybrid groups were widely spread and not well clustered into groups within clear boundaries. From these PC-based plot analyses, it appears that the Seibel and LV hybrid groups could be best defined by the content of total polyphenols or anthocyanins and, to a less extent, by that of flavones, while the *Riparia* group was more adequately defined by the content of phenolic acids. There were several hybrids, as indicated in Fig. 2, which were clearly distantly located from the main group of the hybrids.

The PCA analysis results of polyphenolic compounds did not match completely with that of pedigree-based group classification. This is not a surprise because they represent two different sets of information. Nevertheless, the PCA analysis supported the pedigree-based classification results for the Seibel and LV groups, suggesting that some of the groups classified on the basis of pedigree information were informative. The PCA analysis also identified several outlier hybrids which would not be easily recognized solely on the basis of their pedigree information. Indeed, some of these outlier hybrids had very complex pedigrees or no pedigree information. Assigning such hybrids to a group on the basis of pedigree information would be difficult. Furthermore, while supporting the pedigree-based classification for the Seibel and LV groups, the PCA analysis results did not reveal clear group boundaries among the hybrids. This again suggested the complex nature of the genetic background involved in these hybrids. With the recent development of next-generation sequencing technologies, it is possible to gain further insight into the genetic relationships of these hybrids by comparing their profiles of high-density DNA molecular markers.

In summary, tremendous content variation of polyphenolic compounds was observed in the 48 grape hybrids investigated. The polyphenolic profiles of the hybrids showed typical quantitative variation and exhibited intermediate phenotypes between their putative parental species. This work represents the most comprehensive survey of polyphenols in grape hybrids, contributes to the understanding of the inheritance of polyphenolic compounds in interspecific hybrids of *Vitis* species, and enhances the effectiveness of future effort in transferring polyphenolic trait variation from *Vitis* wild species to cultivated grapes.

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Polyphenolic standards and equations

No.	Standard Compounds	WL	Equation	R^2
1	Gallic acid	280	y=0.069x	0.9992
2	Vanillic acid	280	y=0.1003x	0.9994
3	Procyanidin B1	280	y=1.4193x	0.9999
4	Catechin	280	y=0.3526x	0.9998
5	Procyanidin B2	280	y=0.3766x	0.9995
6	Epicatechin	280	y=0.3262x	0.9997
7	Epicatechin gallate	280	y=0.1245x	0.9994
8	Isorhamnetin	280	y=0.2054x	0.9969
9	Caftaric acid	320	y=0.1435x	0.9999
10	Chlorogenic acid	320	y=0.0605x	0.9999
11	Caffeic acid	280	y=0.0389x	0.9999
12	Coutaric acid	320	y=0.1381x	0.9976
13	Ferulic acid	320	y=0.0349x	0.9999
14	Resveratrol	320	y=0.0175x	0.9991
15	Rutin	365	y=0.1044x	0.9995
16	Kaempferol 3-*O*-glucoside	320	y=0.0603x	0.9988
17	Isorhamnetin 3-*O*-glucoside	365	y=0.1412x	0.9991
18	Cyanidin 3-*O*-glucoside-5-*O*-glucoside	525	y=0.0873x	0.9999
19	Delphinidin 3-*O*-glucoside	525	y=0.2183x	0.9938
20	Cyanidin 3-*O*-glucoside	525	y=0.0697x	0.9992
21	Petunidin 3-*O*-glucoside	525	y=0.1105x	0.9979
22	Malvidin 3-*O*-glucoside-5-*O*-glucoside	525	y=0.166x	0.9998
23	Peonidin 3-*O*-glucoside	525	y=0.0888x	0.9998
24	Malvidin 3-*O*-glucoside	525	y=0.1409x	0.9998
25	Delphinidin 3-*O*-(6-*O*-coumaryl)-glucoside	525	y=0.1783x	0.9993
26	Cyanidin 3-*O*-(6-*O*-coumaryl)-glucoside	525	y=0.2909x	0.9952
27	Petunidin 3-*O*-(6-*O*-coumaryl)-glucoside	525	y=0.4017x	0.9900
28	Malvidin 3-*O*-(6-*O*-coumaryl)-glucoside	525	y=0.2948x	0.9996

Note: No standards were available for myricetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-glucoside. They were quantified by using rutin. The anothocyanins that we did not have standards were quantified by using nonacylated anthocyanins. Lowest detection limit was 0.00069 $mg \cdot g^{-1}$.