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Chapter 16

Variability for Phenotype, Anthocyanin Indexes, and Flavonoids in Accessions from a Close Relative of Soybean, *Neonotonia wightii* (Wright & Arn. J.A. Lackey) in the U.S. Germplasm Collection for Potential Use as a Health Forage

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1. Introduction

The closely related soybean species, *Neonotonia wightii* Wright & Arn. J.A. Lackey is in the *Fabaceae* family and originates from several tropical countries (NPGS, 2012; Cook *et al*., 2005). The plants produce vines with slender stems (2-3 cm in diameter) consisting of glabrous to densely pubescent trichomes and a strong taproot. The leaves are pinnately trifoliolate with elliptic, ovate, or rhombic ovate, acute to obtuse (1.5-15 cm long, and 1.3-12.5 cm wide), glabrous to densely pubescent leaflets. The stipules are lanceolate (4-6 mm long) and the petiole is 2.5-13 cm long. The inflorescence is axillary with dense or lax racemes which are 2-35 cm long on peduncles (3-12.5 cm long) and comprises 20-150 flowers. Each flower is 4.5-11 mm long with white to mauve-blue standards, however small violet streaks are noticeable on the lower part, and will change to yellow or orange at senescence. The 1.5-4 cm long by 2.5-5 mm wide, glabrous to densely pubescence with grey to reddish brown trichomes on the pods are linear, oblong, straight or slightly curved at the apex and transversely grooved with a weak septa between the seeds. Each pod contains 3-8 oblong with rounded corners, laterally compressed, olive green to reddish brown (occasionally mottled, aril white), 2-4 mm long, 1.5-3 mm wide, and 1-1.5 mm thick seeds (Cook *et al.*, 2005). *Neonotonia wightii* consists of diploid (2n = 22) and tetraploid (2n = 44) genotypes which are self-pollinated (cleistogamous) and low outcrossing (Cook *et al*., 2005).

Neonotonia wightii has several common names including glycine (Australia, Kenya); soja perene (Brazil); soya perenne forrajera, soya forrajera, soya perenne (Colombia, Mexico); soja perenne (French); ausdauernde soja (German); soja-perene (Portuguese); Rhodesian kudzu (Taiwan); fundo-fundo (Tanzania); and thua peelenian soybean (Thai) [Cook *et al*., 2005]. This species also has many synonyms including *Glycine javanica* auct., *G. javanica* L. var. *paniculata* Hauman, *G. albidiflora* De Wild., *G. claessensii* De Wild., *G. javanica* sensu auct., *G. javanica* L. var. *claessensii* (De Wild.) Hauman, *G. javanica* L. var. *longicauda* (Schweinf.) Baker, *G. javanica* L. subsp. m*icrantha* (A. Rich.) F.J. Herm., *G. javanica* L. var. *mearnsii* (De Wild.) Hauman, *G. longicauda* Schweinf., *G. mearnsii* De Wild., *G. micrantha* A. Rich., *G. moniliformis* A. Rich., *G. petitiana* Hermann pro parte, *G. pseudojavanica* Taub., *G. wightii* (Wight & Arn.) Verdc. var. *longicauda* (Schweinf.) Verdc., *G. wightii* (Wight & Arn.) Verdc. subsp. *petitiana* (A. Rich.) Verdc. var. *mearnsii* (De Wild.) Verdc., *G. wightii* (Wight & Arn.) Verdc. subsp. *petitiana* (A. Rich.) Verdc., *G. wightii* (Wight & Arn.) Verdc. subsp. *pseudojavanica* (Taub.) Verdc., *G. wightii* (Wight & Arn.) Verdc. subsp. *wightii*, *Johnia wightii* (Wight & Arn.) Wight & Arn., and *Notonia wightii* Wight & Arn. (Cook *et al*., 2005).

The plant is used as pasture for grazing, hay, and silage (Cook *et al*., 2005). However, Viswanathan *et al*., 2001 indicated that *N. wightii* seeds are used as food by Malayali tribes in Kollihills of the Namakkal District, Tamil Nadu, India. They found several essential amino acids, fatty acids, potassium, magnesium, manganese, and copper in *N. wightii* seeds. In Kenya, *N. wightii* produced abundant organic matter contributing to soil fertility and tolerated defoliation (Macharia *et al*., 2010). A Brazilian study showed that *N. wightii* was one of several legumes with high crude protein, low NDF, and low phenolic concentrations for use as a ruminant feed (Valarini and Possenti, 2006). Mtui *et al*. (2006) found that *N. wightii* should be a component in dairy cow diets because of its high mineral concentration. *Neonotonia wightii* plants contain low levels of tannins and alkaloids as well (Mbugua *et* al., 2008). Tauro *et al*. (2009) found that *N. wightii* could restore productivity to soils in Zimbabwe that have been cultivated continuously and low in nutrient levels. *Neonotonia wightii* has also been found to contribute the greatest green manure effect in the absence of fertilization in Zambia (Steinmaier and Ngoliya, 2001).

Anthocyanins are chemicals responsible for natural plant colors found in leaves, stems, and flowers. An anthocyanin meter with a 520 nm LED has been used to measure the absorbance near the wavelength at which free anthocyanin aglycones, cyanidin and pelargonidin monogluscosides absorb (Macz-Pop et al., 2004). Several studies have shown potential health benefits of anthocyanins in humans. Chokeberry (*Aronia meloncarpa* E.) anthocyanins (cyanidin derivatives) have been shown to be very potent inhibitors of colon cancer cells (Zhao *et al*., 2004). When several anthocyanins including cyanidin-3,5-diglucoside and cyanidin-3-glucoside are ingested, apoptosis effects were observed and may have potential for human hepatitis B-associated hepatoma (Shin *et al*., 2009). Lacombe *et al.* (2010) found that cyanidin-3-glucoside caused disintegration of E. coli outer membranes. Both cyanidin-3-glucoside and pelargonidin-3-glucoside showed potential for prevention of atherosclerosis (Paixao *et al*., 2011). Corn silage containing anthocyanins may have nutritional value as a ruminant feed (Hosoda *et al*., 2011). *Neonotonia wightii*

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has also been found to be rich in crude protein content and amino acids, but low amounts of the sulfur containing amino acid, methionine (Tokita *et al.*, 2006).

Isofalvones have been associated with reducing sheep fertility (Waghorn and McNabb, 2003). The isoflavone, genistein is a secondary metabolite found in many legumes including *N. wightii* (Ingham *et al.*, 1977; Keen *et al.*, 1989). Genistein can cause reduced fertility in sheep, however after 7-10 days of adaptation, sheep rumen microbes degrade genistein and other oestrogenic compounds to non-oestrogenic metabolites. Therefore, the effects of genistein on sheep fertility is short lived (Waghorn and McNabb, 2003). However, genistein has been shown to protect against mammary and prostate cancer by regulating receptors and growth signaling pathways (Lamartiniere *et al.*, 2002).

2. Materials and methods

2.1. Phenotyping

Neonotonia wightii is a photoperiod and frost-sensitive species requiring seed regeneration in a greenhouse. Twenty-two *N. wightii* accessions from countries throughout the world were used in this study (Table 1). There was not enough room remaining in the greenhouse to accommodate the regeneration of 7 additional *N. wightii* accessions, therefore fourteen accessions were planted in 27.5 cm x 27.5 cm plastic pots containing potting soil grown in a greenhouse from August 1, 2010 – April 1, 2011 each year. Three to four seedlings per pot were maintained for plant production. Due to the vigorous growth habit, all *N. wightii* accessions were grown with trellises. The experimental design was a randomized complete block with 4 replications assigned to *N. wightii* accessions. Phenotypic descriptors including branching, foliage, plant height, plant width, and relative maturity were recorded when each accession reached 50% maturity based on visual observation, while seed numbers were counted at the end of the growing season. Branching and foliage were based on a scale of 1-5 where, 1 = > 90%, 2 = 80-89%, 3 = 70-79%, 4 = 60-69%, and 5 = 50-59%, 6 = 40-49%, 7 = 30-39%, 8 = 20-29%, and 9 = 10-19% of each plant producing branches and/or foliage based on visual observations. Relative maturity dates were based on a scale of 5 to 9 where 5 = mid-season and 9 = very late.

2.2. Anthocyanin indexes

An Opti-Sciences CCM-200 chlorophyll content meter was converted to a hand-held anthocyanin meter. The manufacturer replaced the 655 nm light emitting diode (LED) of the CCM with a 520 nm LED to measure the absorbance near the wavelength at which free anthocyanin aglycones, cyanidin and pelargonidin monogluscosides absorb (Macz-Pop et al., 2004). Anthocyanin indexes were determined by inserting each leaflet between the meter and the LED diode, followed by gently pressing the LED directly on to the leaflet and recording from each of three leaflets of 15 *Neonotonia wightii* accessions growing in the greenhouse on 14 February 2008 and 18 March 2009.

Accession (PI)	Origin
156055	Zimbabwe
189613	South Africa
213256	India
213257	India
224976	South Africa
224977	South Africa
224978	South Africa
224979	South Africa
224980 (cultivar-Tropic Verde)	Zimbabwe
224981	Zimbabwe
230324	South Africa
233148	Rhodesia
234874	Congo
235287	Zimbabwe
247677	Congo
258381	Australia
259541	Unknown
259544	South Africa
259545	Brazil
277889	Zimbabwe
314847	South Africa
612241	Taiwan

Table 1. Origin for *N. wightii* accessions used in this study.

2.3. Genistein

As plants matured in the greenhouse, most leaflets were pre-disposed to senescence and only 7 *N. wightii* accessions could be evaluated for genistein variation during 2008. Therefore, preliminary research investigating variability for leaflet weight and genistein content was conducted among these 7 *N. wightii* accessions. Leaflet tissue from each *N. wightii* accession was ground to a fine powder with liquid nitrogen, dried at room temperature, and stored at -20°C until extraction. Approximately 0.15- 0.3 g of dried tissue from each accession was placed into 5 ml test tubes, and their weights were recorded to the nearest 0.001 g. Three ml of extraction solvent consisting of 80% HPLC-grade methanol with 1.2 M HCl was added to each test tube. Each tube with extraction solvent were vortexed and incubated at 80°C for 2 hr with occasional mixing by inversion. An additional 2.0 ml of 5% methanol was added to each test tube, resulting in a final concentration of 50% methanol. A portion of the extract was filtered through a 0.45 μm membrane prior to injection. Analytes were separated and identified by high performance liquid chromatography (HPLC). The stationary phase consisted of a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5μM particle size) (Agilent Technologies) at 40°C with a C18 guard column. The mobile phase consisted of 20% HPLC-grade

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acetonitrile (B) and 80% filtered water with 0.1% formic acid, pH 2.5 (A) at 2.0 ml/min. A gradient flow was used with the following profile: 15% B for 3 min, then 15% to 40% B from 3 min to 20 min. The column was washed with 95% B for 5 min, and then equilibrated for 7 min at 15% B between injections. The injection volume was 10 μl. Flavonoid peaks were monitored at 260 and 370 nm with a diode array detector. The standards for peak identification and quantification consisted of kaempferol, quercetin, myricetin, genistein, and daidzein (Sigma-Aldrich Chemical Co.). Each standard was dissolved in extraction solvent and diluted to the following concentrations: 1, 5, 10, 25, and 50 ng/μl.

Phenotype, anthocyanin index, and isoflavonoid data were subjected to an analysis of variance using SAS (SAS Institute, 2008). Mean separations were conducted using Duncan's multiple range test ($P < 0.05$, $P < 0.01$) and correlations were accomplished using Pearson's correlation in SAS (SAS Institute, 2008). Principal component analysis using PROC PRINCOMP (SAS Institute, 2008) were then used for multivariate analysis of the data. Eigenvalues, the percentage of variances explained by each principal component, and eigenvectors were also determined. Clustering was then performed on the data by entering the similarity matrix into PROC CLUSTER for cluster analysis with the unweighted paired group method using mathematical averages (UPGMA) by specifying the AVERAGE option (SAS Institue, 2008).

3. Results and discussion

3.1. Phenotype

Significant variability for morphological, plant maturity, and seed number characteristics observed among 14 *N. wightii* accessions are reported in Table 2. Only PI 224978 from South Africa produced significantly less branching and foliage production than most of the other accessions. Many plants extended beyond the top of the trellis which allowed us to measure actual plant heights. Plant height ranged from 99.3 to 116.3 cm with both PI 224979 (South Africa) and PI 224981 (Zimbabwe) producing significantly shorter plants (averaging 99.7 cm) than the other accessions. The other twelve accessions averaged 108.4 cm tall. The accessions PI 224976 (S. Africa), PI 224977 (S. Africa), PI 230324 (S. Africa), PI 213257 (India), and PI 224981 produced the significantly narrowest plants averaging 47.6 cm while all other accessions averaged 70.0 cm wide. The accession PI 213256 from India matured the earliest while PI 224976, PI 224977, and PI 213257 averaged maturity at mid season while all others matured late or very late (Fig. 1). The significantly highest seed producing accession was PI 213256 (2214 seeds) followed by the Congonese accession, PI 234874 (725 seeds) and PI 224977 (663 seeds). The significantly lowest seed producers were PI 224979, PI 224980 (cultivar, Zimbabwe), and PI 230324 averaging 70 seeds while all other accessions averaged 307 seeds. Branching significantly correlated with foliage ($r^2 = 0.84^{***}$) and foliage had a significant negative correlation with plant width ($r^2 = -0.28^{*}$). Plant width was significantly correlated with maturity ($r^2 = 0.57^{***}$) and maturity had a significant negative correlation with seed number ($r^2 = -0.39^{**}$). Phenotypic variation for the *N. wightii* accessions can be explained by plant selection leading to the potential development of cultivated varieties or breeding material.

			Plant			Seed
Accession	**Branching**	**Foliage**	**ht. (cm)**	**wd. (cm)**	**Maturity**	**no.**
224978	2.5a	2.3a	106.3abcd	52.5bc	7.5b	201b
224979	1.5b	2.0ab	99.3d	51.8bc	7.5b	89b
156055	1.0b	1.0b	104.0cd	70.3a	9.0a	405b
224976	1.0b	1.5ab	108.8abcd	50.0c	6.0c	304b
224977	1.0b	1.0b	110.0abcd	47.5c	6.0c	663b
213256	1.0b	1.0b	103.3cd	51.7bc	5.0c	2214a
213257	1.0b	1.0b	107.5abcd	47.5c	6.0c	461b
224980	1.0b	1.0b	116.3a	62.3ab	7.5b	78b
224981	1.0b	1.0b	100.0cd	50.5c	7.8ab	131b
230324	1.0b	1.3ab	107.5abcd	42.5c	8.3ab	42b
234874	1.0b	1.0b	105.5bcd	71.0a	9.0a	725b
235287	1.0b	1.0b	106.3abcd	69.5a	9.0a	293b
258381	1.0b	1.0b	115.0ab	71.3a	9.0a	327b
259544	1.0b	1.0b	110.3abc	66.3a	9.0a	336b

Means followed by the same letter are not significantly different.

Table 2. Phenotype and seed reproduction variability.

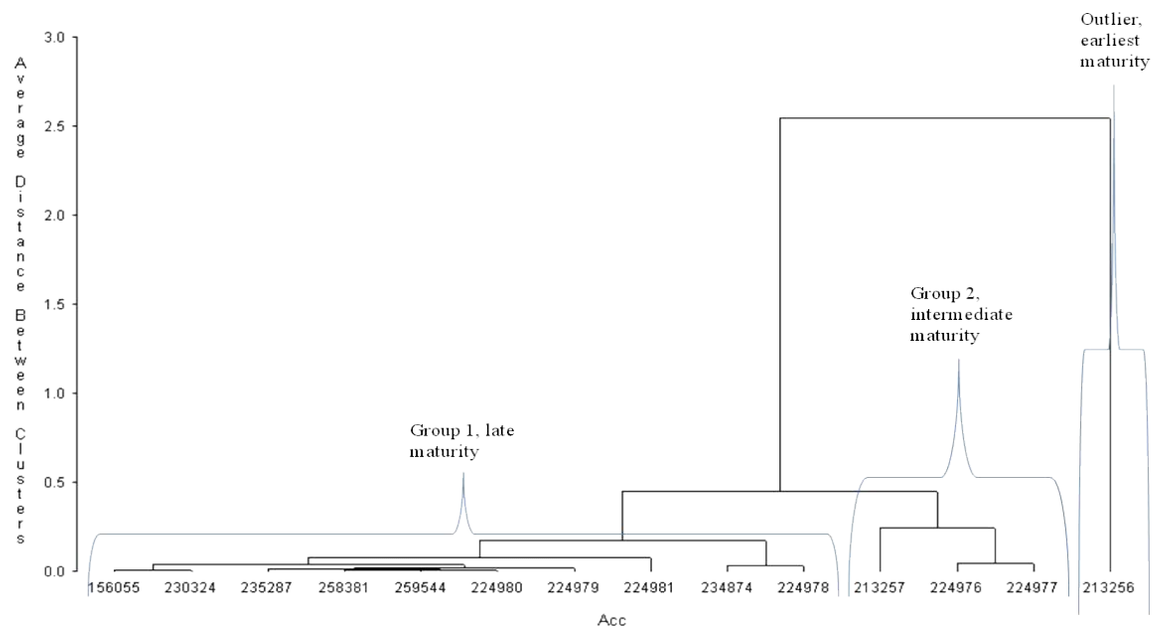

Figure 1. Dendrogram of distance between clusters based on morphological, plant maturity, and seed number differences. Accession numbers are given (Acc). Values on the baseline indicate average phenotypic distances between accessions. Two distinct clusters and one outlier (earliest maturity) for relative plant maturity can be distinguished.

3.2. Anthocyanin index, leaf weight, and genistein

Significant variation for leaf anthocyanin indexes among 15 diverse *N. wightii* accessions were observed also (Table 3). The accession, PI 213257 produced the significantly highest anthocyanin index (7.5) while all other accessions produced an average anthocyanin index of 6.1. This accession also produced significantly higher leaflet weight (0.228 g) than the other 6 accessions (averaging 0.142 g) (Table 4). However, PI 612241 from Taiwan produced significantly more genistein (90.03 μg g^{-1} of leaflet tissue) than all other accessions which averaged 51.3 μg g^{-1} (Table 4). There were no significant correlations among these traits. The flavonoids kaempferol, quercetin, myricetin, and daidzein were minutely or not detected.

Accession (PI)	N	Mean anthocyanin index
213257	4	7.50a
277889	4	7.13ab
314847	4	7.08abc
224976	4	7.00abc
247677	4	6.75abcd
259541	1	6.60abcd
189613	2	6.60abcd
224981	5	6.18abcde
234874	5	6.10bcde
259545	5	5.76cde
235287	5	5.64de
213256	5	5.46de
156055	5	5.44de
233148	5	5.14e
612241	4	5.10e

Means followed by the same letter are not significantly different.

Table 3. Preliminary leaf anthocyanin index variability among 15 diverse *N. wightii* accessions combined over 2 years (2008 and 2009).

Accession (PI)	N	Leaflet wt. (g)	Genistein (μg g^{-1})
213257	4	0.228a	66.28ab
224976	4	0.175b	80.10ab
314847	4	0.162bc	59.10ab
277889	4	0.160bc	28.90b
247677	4	0.136bcd	37.95ab
189613	2	0.118cd	35.65b
612241	4	0.101d	90.03a

Table 4. Preliminary leaflet weight (g) and genistein variability among 7 *N. wightii* accessions during 2008.

3.3. Principal component analysis

Phenotypic, maturity, and seed number principal component analysis accounted for 44% of the total variation at the first principal component (Table 5). The amount of variation accounted for, cumulatively, by adding principal components 2 through 4 was 75, 88, and 96%, respectively. The first principal component was most correlated with plant width and maturity (Table 6). The second principal component accounted for 31% of the variation and was mostly due to branching and foliage while the third principal component explained 13% of the variation and was composed of primarily plant height. The fourth principal component accounted for 8% of the variation and was most correlated with seed number. Therefore, potential exists to develop cultivars with improved architecture, early or late maturity, and high or low seed yield. Anthocyanin index, leaflet weight, and genistein accounted for 63% of the total variation at the first principal component (Table 7). The cumulative amount of variation for components 2 through 3 was 98 and 100%, respectively. The first and second principal components were mostly correlated with anthocyanin index and genistein, respectively, while the third principal component correlated with both anthocyanin index and leaflet weight (Table 8). Potential exists to develop *N. wightii* cultivars with improved anthocyanin indexes, genistein content, and leaflet weight. Since all traits tested are quantitative, the variability among *N. wightii* accessions is attributed to genetic differences primarily since they were regenerated in a greenhouse.

		Principal	
component	Eigenvalue	% Variability	% Cummulative
1	2.6400	44.00	44.00
2	1.8319	30.53	74.53
3	0.8088	13.48	88.01
4	0.4535	7.56	95.57

Table 5. Eigenvalues and the proportion of total phenotypic, maturity, and seed reproduction variability among 14 *N. wightii* accessions (2010, 2011) as explained by the principal components.

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Principal components	1	2	3	4	5	6
Branching	-0.26	0.58	0.06	0.49	-0.53	-0.22
Foliage	-0.35	0.52	0.23	0.01	0.72	0.15
Plant ht. (cm)	0.33	-0.16	0.88	0.27	-0.03	0.11
Plant width (cm)	0.54	0.06	-0.26	0.46	0.40	-0.49
Maturity	0.50	0.37	-0.24	0.07	-0.10	0.72
Seed no.	-0.37	-0.46	-0.19	0.67	0.11	0.37

Table 6. Eigenvectors, principal components for 6 phenotypic, maturity, and seed traits in 14 *N. wightii* accessions (2010-2011).

		Principal	
component	Eigenvalue	% Variability	% Cummulative
1	1.8958	63.20	63.20
2	1.0540	35.13	98.33
3	0.0501	1.67	100.00

[1]Anthocyanin indexes were based on 15 *N. wightii* accessions.

[2]Leaflet weight and genistein were based on 7 *N. wightii* accessions.

Table 7. Eigenvalues and the proportion of total leaf anthocyanin index[1], leaflet weight (g)[2] (2009) and genistein[2] (2008) variability among *N. wightii* accessions as explained by the principal components.

Principal components	1	2	3
Anthocyanin index[1]	0.71	-0.03	0.69
Leaf wt. (g)[2]	0.62	0.47	-0.61
Genistein[2]	-0.30	0.87	0.36

[1]Anthocyanin index based on 15 *N. wightii* accessions.

[2]Leaflet weight and genistein based on 7 *N. wightii* accessions.

Table 8. Eigenvectors, principal components for two phytochemical traits and leaf weight in *N. wightii* accessions (2008, 2009)

4. Cluster analysis

Average distance cluster analysis grouped the original 14 *N. wightii* accessions into well defined phenotypes with two distinct relative plant maturity groups and one outlier (Fig. 1). Cluster or group 1 represents 10 late maturing *N. wightii* accessions and group 2 consists of three intermediate or mid-season maturing accessions. The outlier, PI 213256 represents the earliest maturing accession. The *N. wightii* accessions clustered in group 2 showed relatively closer genetic relationships than those in group 1. Using the distance values indicated in Fig. 1, the groupings at any similarity level can be identified. For example, PI 224976 and PI 224977 originate from South Africa with a phenotypic distance index of 0.0473, which indicates their close morphological similarities.

Average distance cluster analysis grouped 7 *N. wightii* accessions into well defined phenotypes with three distinct genistein producing accessions and one outlier (Fig. 2). Group 1 represents 2 intermediate genistein producing accessions while group 2 consists of two high genistein producing accessions. Group 3 is representative of two very high genistein producing accessions and one outlier (PI 277889) accession producing low amounts of genistein. Overall, *N. wightii* accessions showed similar genetic relationships in all groups and one outlier. However, PI 612241 from Taiwan and PI 224976 from South Africa had a phenotypic distance index of 0.3030, which indicates their close morphological similarities.

These results show substantial variability for various phenotypic traits, maturity, seed reproduction, and genistein in these *N. wightii* accessions regenerated in a greenhouse. However, additional studies are warranted for investigating if similar results will occur when *N. wightii* accessions are grown in field conditions over multiple years.

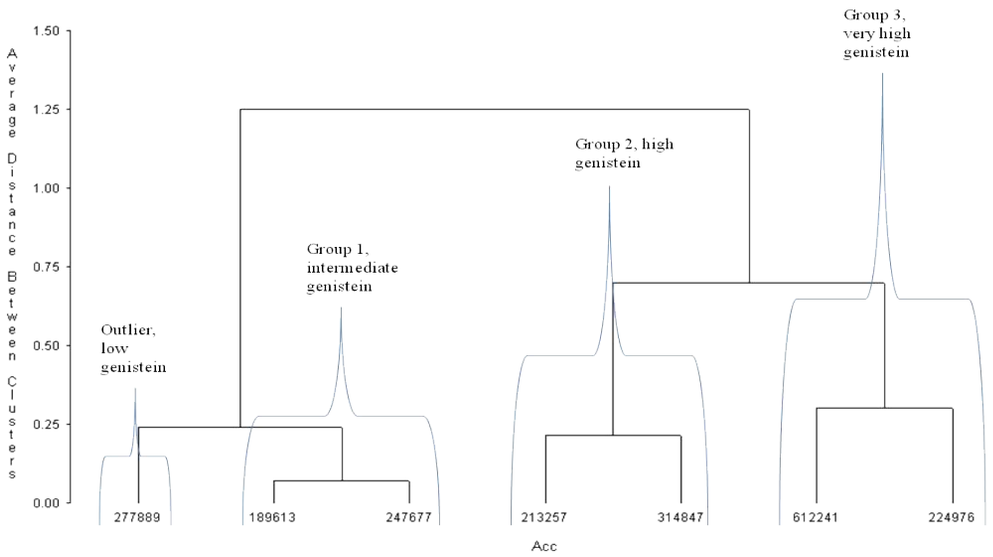

Figure 2. Dendrogram of distance between clusters based on anthocyanin indexes and genistein differences. Accession numbers are given (Acc). Values on the baseline indicate average phytochemical distances between accessions. Three distinct clusters and one outlier (low) for genistein can be distinguished.

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