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Multivariate analysis on isoflavone content for soybean land races in Sichuan Basin

Wan Yan [1]; Ye Maoying[2]; Yang Wenyu[1]; Liu Weiguo[1]; Yong Taiwen [1]

1 Agronomy College, Sichuan Agricultural University, Ya'an 625014, PR China
2 Mingshan Youth League, MingShan 625014, PR China
Corresponding author Yang Wenyu, Email: wenyu.yang@263.net Tel: 086-0835-2882004
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1 SUMMARY

In this work, multivariate analysis techniques including correlation analysis, hierarchical cluster analysis (HCA), and principal component analysis (PCA) were used to estimate soybean isoflavone genetic divergence of the 56 soybean (Glycine max) landraces from Sichuan province in China. It was found that isoflavones compositions were drastic differences among land races, and strong correlations could be observed between total isoflavone content and other isoflavones compositions. PCA showed that three eigenvalues of cumulative variance proportion 91.719 % were selected for evaluation of local soybean varieties in Sichuan. PC1 indicated total Isoflavone content, genistin, 6'-O-malonylgenistin, 6'-O-malonyldaidzin, daidzin were important traits for classification, while daidzein, glycitein were important in PC2. In the PC3 glycitin and 6'-O-malonylglycitin were important. Based on the results of PCA, HCA showed that all materials could be clustered into five groups, which had obvious features, so it would be helpful for parents materials selection and the isoflavone content of soybean to be improved through breeding by utilizing high daidzein or genistein or other special isoflavone composition land races.

2 INTRODUCTION

Soybean (Glycine max L.) is one of the oldest crops of China, and has been consumed by Asians for a significant amount of time in history (Wang and Murphy, 1994.). Soybean is processed into various products such as soymilk powder, soymilk, tofu, soy sauce, soybean oil and tempeh (Tyug et al, 2010). Soybean was not widely consumed as human food source in the West because of its flavor (Carrão-Panizzi M C and Kitamura K., 1995.). A progressive increase in soybean product consumption has recently been observed in Western countries, which is associated with the presence of isoflavones in soybean seed (Locati et al, 2005). Isoflavone contained in soybeans is also known as a phytoestrogen because of its estrogenic activities with potential protective effects against some hormone related diseases, such as breast, prostate, and colon cancers (Davies et al, 1999; Pool-Zobel et al,2000;Akaza et al,2002; Hikosaka et al, 2004; Shaojung and Knobf,2004; Vastag,2007; Xu et al, 2009and ;Kang et al, 2010). Also other biological activities and therapeutic uses such as decreasing certain cancers, osteoporosis, cardiovascular disease and menopausal symptoms(Dören and Samsioe, 2000; Sacks et al,2006; Atmaca et al,2008;D-F Ma et al, 2008; Taylor et al, 2009and Samsioe). Thus, the contents and compositions of isoflavone in soybeans are important factors that affect human health (Yamabe et al, 2007). Together with the increasing interest in soy isoflavones due to health claims related with the consumption of soybeans, scientists have paid much attention to isoflavones. They are produced by the soybean plant as part of their defense mechanism against insects and diseases

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such as *Phytophthora* (Paul et al,1998 ;Connolly et al,1999; Gang et al,1999; Joy et al, 2006), and also in response to environmental stresses such as drought (Caldwell et al,2005; Gutierrez-Gonzalez et al, 2010) . Isoflavones also play an important role in the growing soybean plant by stimulating nodule formation by nitrogen-fixing *Rhizobium* bacteria (Eva et al, 2005).

Isoflavones belong to a group of compounds that share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring. The structure is generally simplified as C6-C3-C6(**Fig 1**) (Robinson et al, 1995;Liu,1997).

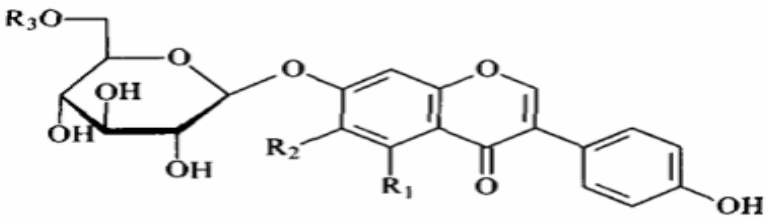

Figure 1: Basic structures of soybean isoflavones Figure

Research has found that naturally occurring soy isoflavones mainly include four groups, they are aglycons (daidzein, genistein, and glycitein), glucosides (daidzin, glycitin, and genistin), malonyl glucosides (6'-O-malonyldaidzin, 6'-O-malonylgenistin, and 6'-O-malonylglycitin), and acetylglucosides (6'-O-acetyldaidzin, 6'-O-acetylgenistin, 6'-O-acetylglycitin) (**Table 1**)(S.H. Kim ,2005; Ha et al ,2009).

Table 1: Side chains of soybean isoflavones

Types	**Isoflavone monomer**	**R_1**	**R_2**	**R_3**
Aglycon	daidzein	H	H	-
	genistein	OH	H	-
	glycitein	H	OCH_3	-
Glucoside	daidzin	H	H	H
	genistin	OH	H	H
	glycitin	H	OCH_3	H
Malonylglucoside	6'-O-malonyldaidzin	H	H	$COCH_2COOH$
	6'-O-malonylgenistin	OH	H	$COCH_2COOH$
	6'-O-malonylglycitin	H	OCH_3	$COCH_2COOH$
Acetylglucoside	6'-O-acetyldaidzin	H	H	$COCH_3$
	6'-O-acetylgenistin	OH	H	$COCH_3$
	6'-O-acetylglycitin	H	OCH_3	$COCH_3$

Several studies have shown that isoflavone concentration and composition are affected by environment, genotype, growth location and the interaction between these factors (Wang and Murphy, 1994). Genetic and agronomic studies showed that the large variation of isoflavone concentration among soybean genotypes and varieties (Wang and Murphy,1994;Aussenac et al,1998; Wang et al ,2000; Philippe et al, 2004; Swanson et al, 2004;and Murphy Devi et al ,2009), and genotype was the most important factor on the

determination of the in individual isoflavone forms and total isoflavone contents (Hocck et al, 2000). Environmental conditions cause significant variations in soybean cultivars (Eldridge AC et al, 1983; Carrão-Panizzi M.C. and Kitamura K., 1995), such as temperatures, the period of seed filling significantly affected the isoflavone content of soybeans. High temperatures decreased the quantity of isoflavones (Eldridge AC, 1982.; Carrão-Panizzi M.C. and Kitamura K., 1995). Sowing date can contribute to seed quality by reducing its isoflavone content, modifying its isoflavone composition (Aussenac et al,1998).Soybeans planted later in the growing season usually have higher seed isoflavone concentrations(Aussenac et al,1998), while early maturating cultivars had low isoflavone content (Kitamura et al,1991). Air temperature and soil moisture status, low temperatures and high soil moisture conditions produced the highest seed isoflavone concentrations with changes in temperature having the larger effect (Lozovaya et al, 2005). Locations would affect the composition of isoflavones as black soybean cultivars cultivated in high altitude possessed significantly higher total isoflavone contents than those grown in low altitude (Ha et al, 2009.). The interaction between environments and genotype would influence the contents of isoflavones, and the effect was significant (Tsukamoto et al, 1995; Macdonald et al, 2005). Fertilizer would influences the concentrations of isoflavones, and research has found that appropriate potassium (K) management could be an effective approach to increase isoflavone concentrations for soybeans produced on low- to medium-K soils (Vyn et al, 2002). Due to wide applications of isoflavones, soybean (Glycine max L.) is in great demand and sometimes in shortage. Landraces are important in keeping the genetic integrity of germplasm during germplasm conservation (LI et al, 2010), and many kinds of soybean landraces are kept in the Sichuan Basin, China. It is, therefore, desirable to determine a reliable and accurate methodology to differentiate the samples collected from Sichuan Basin, China. Although some studies on the compositions of soybean isoflavones using HPLC have been published (i.e., Philippe, 2004), none of them compared the land races germplasm resource of soybean from the Sichuan Basin, China. So the objective of this work is to use HPLC and multivariate analysis combined correlation analysis, hierarchical cluster analysis (HCA), and principal component analysis (PCA) for the identification and selection of soybean landraces with high isoflavone for special usage.

3 METHODOLOGY

3.1 Plant materials. Seed samples of 56 indigenous soybean landraces were directly collected from local farmers in Sichuan Province, China, in 2007. Hilum color and seed weight were determined for each variety. Hilum color among varieties was yellow, green, black, or brown. 100-Seed weight ranged from 12g to 27g (**Table 2**).

Table 2: Soybean landraces Grown in Sichuan Province, China

Sample No.	100-seed Weight/g	Hilum color	Origin	Longitude and latitude	Note
1	12.93	Yellow	Heishui,Aba	102°57'E,32°4'N	wild species
2	23.23	Yellow	Maoxian,Aba	103°48'E,31°36'N	wild species
3	13.29	Yellow	Chongzhou,Chengdu	103°36'E,30°36' N	wild species
4	15.24	Black	Dujiangyan,Chengdu	103°36'E,31°0'N	wild species
5	15.85	Yellow	Qionglai,Chengdu	103°28'E,30°25' N	wild species
6	27.05	Yellow	Xindu,Chengdu Ⅰ	104°8'E,30°49'N	wild species
7	12.56	Black	Xindu,Chengdu Ⅱ	104°8'E,30°49' N	wild species

8	23.41	Yellow	Daxian,Dazhou Ⅰ	107°30'E,31°14'N	wild species
9	25.74	Yellow	Daxian,Dazhou Ⅱ	107°30'E,31°14'N	wild species
10	19.89	Yellow	Daxian,Dazhou Ⅲ	107°30'E,31°14'N	wild species
11	19.25	Yellow	Quxian,Dazhou	106°57'E,30°51' N	wild species
12	21.30	Yellow	Guanghan,Deyang	104°15'E,31°0'N	wild species
13	15.50	Yellow	Zhongjiang,Deyang	104°41'E,31°4' N	wild species
14	17.52	Brown	Guangan,Guangan Ⅰ	106°37'E,30°29' N	wild species
15	17.69	Black	Guangan,Guangan Ⅱ	106°37'E,30°29' N	wild species
16	18.27	Yellow	Guangan,Guangan Ⅲ	106°37'E,30°29' N	wild species
17	15.28	Yellow	Yuechi,Guangan Ⅰ	106°26'E,30°33' N	wild species
18	18.69	Yellow	Yuechi,Guangan Ⅱ	106°26'E,30°33' N	wild species
19	22.24	Yellow	Qingchuan,Guangyuan Ⅰ	105°13'E,32°36'N	wild species
20	17.00	Yellow	Qingchuan,Guangyuan Ⅱ	105°13'E,32°36'N	wild species
21	18.95	Yellow	Emeishan,Leshan	103°42'E,29°18'N	wild species
22	18.53	Yellow	Shawan,Leshan	103°44'E',85°0'N	wild species
23	25.38	Yellow	Shizhon,Leshang	103°42'E,29°18'N	wild species
24	17.80	Yellow	Leibo,Liangshan	103°42'E,28°21'N	wild species
25	16.34	Brown	Xichang,Liangshan Ⅰ	102°18'E,27°55'N	wild species
26	19.63	Brown	Xichang,Liangshan Ⅱ	102°18'E,27°55'N	wild species
27	19.92	Yellow	Xichang,Liangshan Ⅲ	102°18'E,27°55'N	wild species
28	22.50	Yellow	Xichang,LiangshanⅣ	102°18'E,27°55'N	wild species
29	18.87	Green	Naxi,Luzhou Ⅰ	105°38'E,28°77 'N	wild species
30	20.32	Yellow	Naxi,Luzhou Ⅱ	105°38'E,28°77 'N	wild species
31	13.88	Yellow	Dongpo,Meishan	103°47'E,30°03 'N	wild species
32	21.23	Yellow	Qingshen,Meishan	103°49'E,29°52'N	wild species
33	26.31	Yellow	Renshou,Meishan	104°09'E,30°0 'N	wild species
34	24.78	Yellow	Jiangyou,Mianyang	104°42'E,31.8 N	wild species
35	14.94	Yellow	Shizhong,Neijiang	105° 3'E,29°36'N	wild species
36	19.26	Black	Weiyuan,Neijiang	104°42'E ,29°34'N	wild species
37	22.16	Yellow	Zizhong,Neijiang	104°51'E ,29°49' N	wild species
38	23.09	Yellow	Langzhong,Nanchong	105°58'E ,31°45'N	wild species
39	25.66	Yellow	Dongqu,Panzhihua	101°7'E,26°6'N	wild species
40	16.71	Yellow	Chuanshan,Suining Ⅰ	105°58'E,30°52'N	wild species
41	19.93	Yellow	Chuanshan,Suining Ⅱ	105°58E,30°52N	wild species
42	18.23	Yellow	Shehong,Suining	105°31E,30°9 N	cultivated species
43	18.87	Yellow	Hanyuan,Yaan	102°40'E,29°24'N	wild species
44	17.03	Yellow	Mingshan,Yaan Ⅰ	103°4'E,30°6'N	wild species
45	20.73	Green	Mingshan,Yaan Ⅱ	103°4'E,30°6'N	wild species
46	22.02	Yellow	Tianquan,Yaan	102°47'E ,30°6' N	wild species
47	14.78	Yellow	Jiangan,Yibin	105°4'E ,28°43'N	wild species
48	16.87	Yellow	Yibin,Yibin	104°34'E,29°46' N	wild species
49	22.50	Yellow	Anyue,Ziyang Ⅰ	105°18'E,30°7'N	wild species
50	13.32	Yellow	Anyue,Ziyang Ⅱ	105°18'E,30°7'N	wild species
51	18.41	Yellow	Anyue,ZiyangⅢ	105°18'E,30°7'N	wild species

52	19.46	Yellow	Anyue,ZiyangⅣ	105°18'E,30°7'N	wild species
53	16.74	Yellow	Lezhi,ziyang	105°02E,30°3'N	wild species
54	16.16	Green	Fushun,Zigong Ⅰ	104°42'E,29°18'N	wild species
55	19.17	Yellow	Fushun,Zigong Ⅱ	104°42'E,29°18'N	cultivated species
56	16.03	Yellow	Rongxian,Zigong	104°24'E,29°24'N	wild species

3.2 Chemicals and Reagents. HPLC grade acetonitrile, glacial acetic acid, and methanol, Sodium Hydrate A.C.S (NaOH) and hydrochloric acid A.C.S. (HCl), were purchased from Fisher Science (NJ, USA). Authentic standard of Isoflavone standards (Daidzin, Genistin, Glycitin) were purchased by Chengdu Mansite Pharmaceutical Co., Ltd (Chengdu, China). The water used for all the solutions and dilutions was prepared with a Millipore water purification system (Bedford, MA, USA). An ultrasonic cleaner (KQ-400KDE, China) was used for extraction. The vacuum concentrator system consisted of a rotary evaporator and a digital bath (EYELA, Japan).

3.3 Standard Solution Preparation. Primary stock standard solutions of the 3 compounds, Daidzin, Genistin, and Glycitin were prepared by dissolving them with acetonitrile, respectively, to get a concentration of 0.2 mg/mL, filtered through a 0.45 mm membrane filter and were stored at 4℃. Working mixed standard solutions were prepared daily by mixing and diluting the stock solutions with acetonitrile. Then the concentrations of daidzin, glycitin and genistin in the mixed standard solutions were 0.004, 0.008, 0.012, 0.016, 0.020, 0.024, 0.028mg·mL^{-1}; 0.0008, 0.0016, 0.0024, 0.0032, 0.0040, 0.0048, 0.0056mg·mL^{-1}; 0.004, 0.008, 0.012, 0.016, 0.020, 0.024, 0.028mg·mL^{-1}. According to the peak area at different concentrations of standard solutions, the regression equation and correlation coefficient was showed in Table **3** which can be used as the quantitative basis to determine the content of isoflavones.

3.4 Extraction of Isoflavones. Seed powder of soybeans (1g; smaller than 0.25 mm; heated at 40ºC for 48h) was ground in a grinder and extracted with 80% ethanol. T Then the mixture was ultrasonic extracted for 30mins at 4 ºC and then centrifuged at 5000rpm, 4 ºC for 10mins. Then supernatant was transferred into a 50-mL volumetric flask, and 80% ethanol was used to dilute the filtrate to volume, filtered through a 0.45 mm membrane filter before injecting a 15μL sample.

3.5 Chromatographic Conditions. All HPLC analyses were performed using an Agilent 1100 series HPLC chromatograph with a variable wavelength UV-visible detector system, connected to Hypersil Classical Packing Materials and Column-Hypersil ODS C18 (4.0×250mm,5μm), using a linear gradient of A: 0.3% acetic acid and 5% acetonitrile in water and B: 0.3% acetic acid in acetonitrile. The linear gradient was as follows: 0min (90％A) →10min (88％A) →15min (85％A) →20min (80％A) →30min (30％A) →35min (30％A). The injection volume was 15μl, the temperature of column was 4 ºC and the flow rate was 1 mL/min. Spectral data were collected over the run and isoflavone elution was monitored at 254nm.

3.6 Methodology Validation. Injection precision was assessed by repetitive injection of the same sample solution six times in a day. The RSD of relative retention time and relative peak area were 0.19% and 0.95%, respectively.

A sample stability test was determined with one sample at regular intervals of 3 h for 24 h. During this period, the solution was stored at 4 ºC. The RSD of relative retention time and relative peak area were 0.29% and 1.09%, respectively. The similarity of these results indicated that the sample remained stable in 24 h.

Repeatability was determined by analyzing five independently prepared samples of soybean using the same method. The RSD of relative retention time and relative peak area were 0.26% and 1.78%.

The recovery rates were determined by analyzing five independently prepared samples (1g soybean powder of which the content was known and a certain concentrations of standard solutions) using the same method. The recovery rates for daidzin, glycitin, genistin was 95.5%, 97.5%, and 98.3 %. The coefficients of variance (CV) for intraday determinations were all below 2 % (**Table 3**).

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Table 3: The calibration curves of 3 isoflavone compositions standards

Composition	Equation	Correlation coefficient
Daidzin	Y=62057X-20.817	0.9996(**)
Glycitin	Y=95292X-8.0917	0.9994(**)
Genistin	Y=84904X-5.4417	0.9997(**)

3.7 Data collection and Statistical analysis. Data collection was performed using ChemStation software (Agilent). Statistical analyses were performed by Excel 2003 and SPSS 13.0 software package (Chicago, IL, USA) for correlation analysis, principal component analysis (PCA), hierarchical cluster analysis (HCA).

4 RESULTS AND DISCUSSION

4.1 Concentration and composition of isoflavones in soybean seeds. In this study, 8 kinds of isoflavones, including daidzin, glycitin, genistin, 6'-O-malonyldaidzin, 6'-O-malonylglycitin, 6'-O-malonylgenistin, daidzein and glycitein were detected by HPLC analysis form 56 soybean landraces in Sichuan Province. 6'-O-acetyldaidzin, 6'-O-acetylgenistin; 6'-O-acetylglycitin and glycitein were not quantified due to low concentrations in these soybean samples (**Table 4**).

Table 4: The isoflavone content of individual soybean landraces in Sichuan (μg/g)

Sample No.	Daidzin	Glycitin	Genistin	6'-O-malonyldaidzin	6'-O-malonylglycitin	6'-O-malonylgenistin	Daidzein	Glycitein	Total content
1	368.8	57.8	520.1	958.2	98.3	928.4	36.1	26.3	2994.1
2	408.8	64.8	559.9	405.8	71.0	395.3	83.0	89.7	2078.3
3	305.2	84.1	259.1	640.7	152.6	333.3	13.7	10.2	1798.8
4	139.1	24.5	298.1	590.3	52.3	471.3	28.2	37.3	1641.0
5	185.7	55.0	325.6	420.3	86.4	547.9	54.0	115.1	1790.0
6	143.8	37.7	139.9	363.8	62.2	206.2	nd	16.0	969.6
7	225.1	57.1	307.1	447.3	84.4	497.8	22.5	23.3	1664.6
8	508.4	103.4	623.8	971.2	163.6	773.1	22.1	32.5	3198.1
9	251.0	54.1	274.7	535.8	88.1	481.4	nd	18.7	1703.6
10	200.8	18.6	155.8	546.6	nd	279.2	15.2	6.5	1222.7
11	220.9	28.8	231.8	407.5	nd	312.7	46.2	66.6	1314.4
12	487.4	44.7	505.4	1616.2	102.0	1010.6	29.4	30.5	3826.1
13	219.3	53.1	366.0	501.6	101.2	684.9	20.0	34.0	1980.2
14	304.0	57.6	365.1	851.9	120.4	723.8	19.4	17.3	2459.5
15	256.5	60.3	358.2	874.5	135.0	715.9	18.4	19.2	2437.9
16	198.5	46.4	292.7	406.7	65.0	449.4	35.4	56.9	1551.0
17	201.9	62.6	761.2	572.2	97.5	1390.3	nd	41.9	3127.6
18	243.4	59.4	277.8	537.4	104.0	457.1	nd	16.9	1696.2
19	450.4	60.8	538.9	1205.3	85.2	955.9	48.5	33.7	3378.6
20	468.3	66.1	623.5	1612.9	107.9	1281.9	41.5	26.3	4228.4
21	275.6	46.0	292.3	553.6	17.3	412.8	63.2	91.3	1752.1
22	498.5	53.1	627.1	1180.8	86.4	1068.6	26.1	30.7	3571.3

23	152.3	32.0	187.1	346.7	97.6	264.2	nd	12.0	1092.0
24	445.7	86.0	806.3	282.5	48.6	326.9	244.8	405.0	2645.8
25	433.0	68.1	620.0	875.9	109.1	761.5	48.1	30.3	2945.9
26	407.5	39.0	653.6	1425.1	80.6	1120.8	39.7	28.8	3795.2
27	156.5	32.1	175.3	530.0	65.7	361.2	nd	8.6	1329.3
28	352.4	53.8	482.8	1329.3	120.2	914.0	nd	17.3	3269.8
29	401.2	68.0	393.0	664.4	112.7	433.4	20.1	22.2	2115.0
30	365.9	33.9	340.7	289.8	nd	219.3	65.9	85.2	1400.5
31	436.5	75.7	558.6	319.0	56.9	349.4	73.7	99.3	1969.2
32	282.5	nd	375.3	886.4	nd	677.3	26.2	32.4	2280.2
33	106.4	40.7	212.0	397.0	111.8	455.5	nd	8.9	1332.3
34	179.5	66.3	248.6	488.7	145.5	511.9	nd	14.7	1655.3
35	275.9	44.5	316.3	650.2	50.9	553.2	20.4	18.3	1929.8
36	501.1	61.4	873.8	1698.4	143.1	1510.2	24.3	46.8	4859.2
37	163.7	27.3	159.8	530.8	nd	355.1	nd	nd	1236.7
38	393.5	59.7	643.5	878.0	75.1	866.2	46.9	56.1	3019.0
39	180.0	17.7	347.8	690.7	45.5	767.3	13.1	25.6	2087.5
40	275.1	39.9	423.2	702.7	67.1	658.9	25.2	27.0	2219.1
41	152.7	34.7	207.9	95.9	nd	103.3	193.6	289.3	1077.5
42	247.8	nd	294.8	794.5	nd	621.7	nd	9.9	1968.7
43	490.5	72.9	686.4	1011.3	96.8	748.6	36.1	34.8	3177.4
44	476.0	55.7	427.0	1127.0	95.2	535.2	88.0	57.1	2861.1
45	311.7	76.5	390.9	610.8	105.2	587.6	41.2	48.3	2172.2
46	294.7	51.8	431.7	490.2	39.8	399.5	65.8	76.3	1849.8
47	339.2	113.6	356.9	426.5	91.0	321.5	51.2	58.5	1758.4
48	261.4	54.7	306.7	681.2	81.7	465.2	nd	14.9	1865.8
49	306.3	nd	378.2	1045.1	nd	733.2	15.8	14.9	2493.6
50	264.0	99.9	317.1	442.9	147.5	313.9	25.2	25.5	1636.2
51	422.2	58.6	655.9	1503.3	96.5	1306.4	18.9	28.2	4089.9
52	470.9	51.5	680.5	1215.9	97.5	1190.0	43.2	38.2	3787.8
53	232.0	nd	339.2	735.3	nd	646.9	23.3	28.2	2004.8
54	462.4	62.1	451.3	909.5	108.9	568.1	22.5	24.0	2608.8
55	178.1	8.3	246.2	650.4	9.6	531.0	13.9	13.7	1651.2
56	331.4	60.7	540.1	886.7	128.0	903.5	19.3	31.7	2901.4
Min	106.4	0.0	139.9	95.9	0.0	103.3	0.0	0.0	969.6
Max	508.4	113.6	873.8	1698.4	163.6	1510.2	244.8	405.0	4859.2
mean	309.1	50.8	414.9	746.7	77.0	633.2	34.5	45.9	2312.0
Range	402.0	113.5	733.8	1602.5	163.6	1406.9	244.8	405.0	3889.6

nd: not detected.

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Drastic differences among landraces were observed for total isoflavone content (from 969.6μg/g to 4859.2μg/g). The total isoflavone content of this study were more or less than in other studies (Eldridge AC et al, 1983; Wang et al, 1994; Carrão-Panizzi et al, 1995; Hocck et al, 2000; S. H. Kim et al, 2005). The five soybean landraces in this study that yielded the highest total isoflavone content were landrace36 (4859.2 μg/g), 20(4228.4 μg/g), 52(4089.9 μg/g), 12(3826.1 μg/g), 26 (3795.2 μg/g). While the lowest five ones were landrace6, 41, 23, 10, 37. The average values for total glucoside, total malonylglucoside, total aglycon and total acetylglucoside of soybean landraces were significantly different (**Fig 2**).

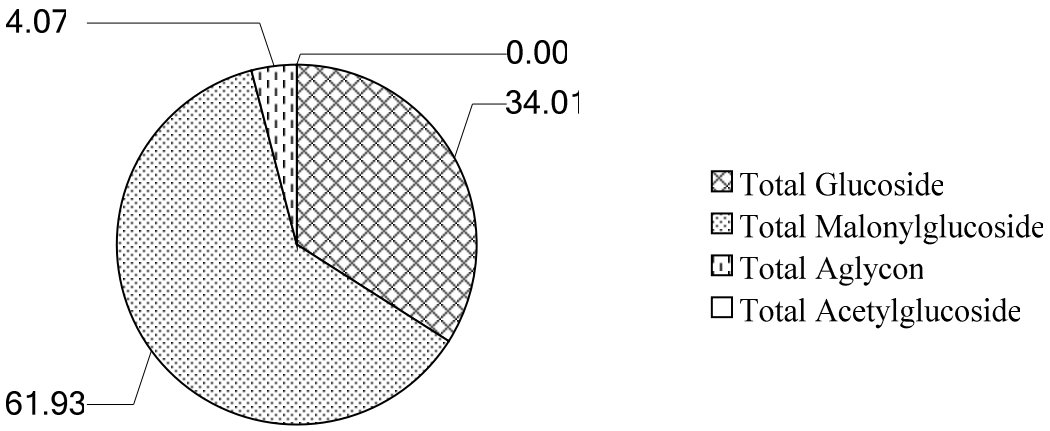

Figure 2: The average ratios of four isoflavone groups of soybean landraces in Sichuan

Malonylglucoside accounted for the vast majority of total isoflavones content, about 62 percent of soybean landraces in Sichuan, and then the glucoside occupied 34%, whereas acetylglucoside (0%) and aglycons (4%) were not detected or were found in very small concentrations. S. H. Kim et al (2005) also found that malonyl conjugates had the highest concentrations, acetyl conjugates and glycitein from aglycon were not detected or were found in trace amounts. Then the differences in the four isoflavone groups were observed in this research between soybean landraces. The five soybean landraces had the highest total malonylglucoside were landrace 36 (3351.7 μg/g), 20 (3002.7 μg/g), 52 (2906.2 μg/g), 12 (2728.8 μg/g), 26 (2626.5 μg/g), which occupied 69.0 %, 71.0%, 71.1%, 71.3% and 69.2 % respectively of their total isoflavones content. It was thought that isoflavones were affected by various genotypic or environmental factors (Wang et al, 1994;Hocck et al, 2000; Philippe et al, 2004; Macdonald, 2005; S. H. Kim et al, 2005; Lozovaya et al, 2005) .

4.2 Correlations analysis of soybean isoflavones content. Strong correlations were observed between total content and daidzin (r=0.795), glycitin (r=0.268), genistin (r=0.875), 6'-O-malonyldaidzin(r=0.902), 6'-O-malonylglycitin (r=0.425), 6'-O-malonylgenistin(r=0.912); It was found that daidzin and glycitin, genistin, 6'-O-malonyldaidzin, 6'-O-malonylglycitin, 6'-O-malonylgenistin, glycitein was strongly associated. The correlations between glycitin and genistin, 6'-O-malonylglycitin were highly correlated. Correlations were found significantly associated between genistin and 6'-O-malonyldaidzin, 6'-O-malonylglycitin, 6'-O-malonylgenistin; 6'-O-malonyldaidzin, 6'-O-malonylglycitin; 6'-O-malonylglycitin and 6'-O-malonylglycitin was strongly correlated with each other. The relationship between daidzein and glycitein was significant (**Table 5**).

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Table 5: Correlation in mainly Isoflavone compositioons of soybean landraces in Sichuan

	Da	Gly	Gen	6'-O-malda	6'-O-malgly	6'-O-malgen	Daid	Glyci
Gly	0.471**							
Gen	0.799**	0.417**						
6'-O-malda	0.660**	0.023	0.600**					
6'-O-malgly	0.349**	0.742**	0.354**	0.331*				
6'-O-malgen	0.516**	0..073	0.746**	0.846**	0.366**			
Daid	0.268*	0.215	0.291*	-0.222	-0.240	-0.225		
Glyci	0.114	0.187	0.239	-0.328*	-0.241	-0.251	0.951**	
Total content	0.795**	0.268*	0.875**	0.902**	0.425**	0.912**	0.029	-0.054

Legend: Da- Daidzin, Gly- Glycitin, Gen – Genistin, 6Omalda-6'-O-malonyldaidzin, 6Omalgly -6'-O-malonylglycitin, 6Omalgen-6'-O-malonylgenistin, Daid-Daidzein, Glyci- Glycitein

4.3

Principal component analysis of soybean isoflavones content. Isoflavones of 56 soybean samples were analyzed by PCA. The aim of this analysis was to see if the constituents can be used to group the different landraces of soybean in Sichuan, and, if so, which constituents were most informative to distinguish the different landraces. The data revealed that 3 principal components having greater than one eigenvalues contributed 91.719 % of the total variation among 56 landraces of soybean (**Table 6**). It was found that principal component 1 (PC1) contributed 49.334%, whereas PC2 and PC3 contributed 26.306% and 16.080%, respectively of the total variation.

Table 6: Principal component analysis of eight main Isoflavone compositions

Principal components	PC1	PC2	PC3
eigenvalue	4.440	2.368	1.447
Contribution (%)	49.334	26.306	16.080
Cumulative contribution (%)	49.334	75.640	91.719
Daidzin	0.845	0.272	-0.107
Glycitin	0.517	0.37	0.743
Genistin	0.877	0.306	-0.202
6'-O-malonyldaidzin	0.813	-0.328	-0.365
6'-O-malonylglycitin	0.633	-0.145	0.701
6'-O-malonylgenistin	0.827	-0.287	-0.335
Daidzein	-0.005	0.968	-0.174
Glycitein	-0.092	0.957	-0.135

The isoflavones traits, which contributed more positively to PC1, were genistin (0.877), daidzin (0.845), 6'-O-malonylgenistin (0.827), 6'-O-malonyldaidzin (0.813), while the rest of isoflavones traits under the present study in PC1 showed low eigenvector values. It can be found that pursuit high content of genistin would get low glycitein content landraces and genistin was the eigenvector of PC1.

The eigenvalue of PC2 was 2.368, which contributed 26.306 % of the total variation. The maximum genetic variance to PC2 was contributed by daidzein (0.968), glycitein (0.957), so daidzein was eigenvector of PC2. But 6'-O-malonyldaidzin, 6'-O-malonylglycitin, and 6'-O-malonylgenistin had negative value. The eigenvalue of PC3 was 1.447, glycitin (0.743) and 6'-O-malonylglycitin (0.701) contributed more positively to PC3, as glycitin was the eigenvector. The rest of characters in this component have negative association with low value. It is evident that isoflavones traits contributed positively to first three principal components and hence these could be given considerable importance for the collection suitable soybean varieties under investigation.

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4.4 Hierarchical cluster analysis of soybean isoflavones content. The areas of 8 common peaks (daidzin, glycitin, genistin, 6'-O-malonyldaidzin, 6'-O-malonylglycitin, 6'-O-malonylgenistin, daidzein, glycitein, acetyl genistin, acetyl daidzin, genistein) were calculated (Table 4) ; Hierarchical clustering algorithm based upon principal component analysis used product of the principal component and eigenvector of landraces materials as integrated values for cluster analysis soybean landraces by group average method in Euclidean distance cones. The clustering analysis was operated in SPSS software, and the results are shown in **Fig.3**.

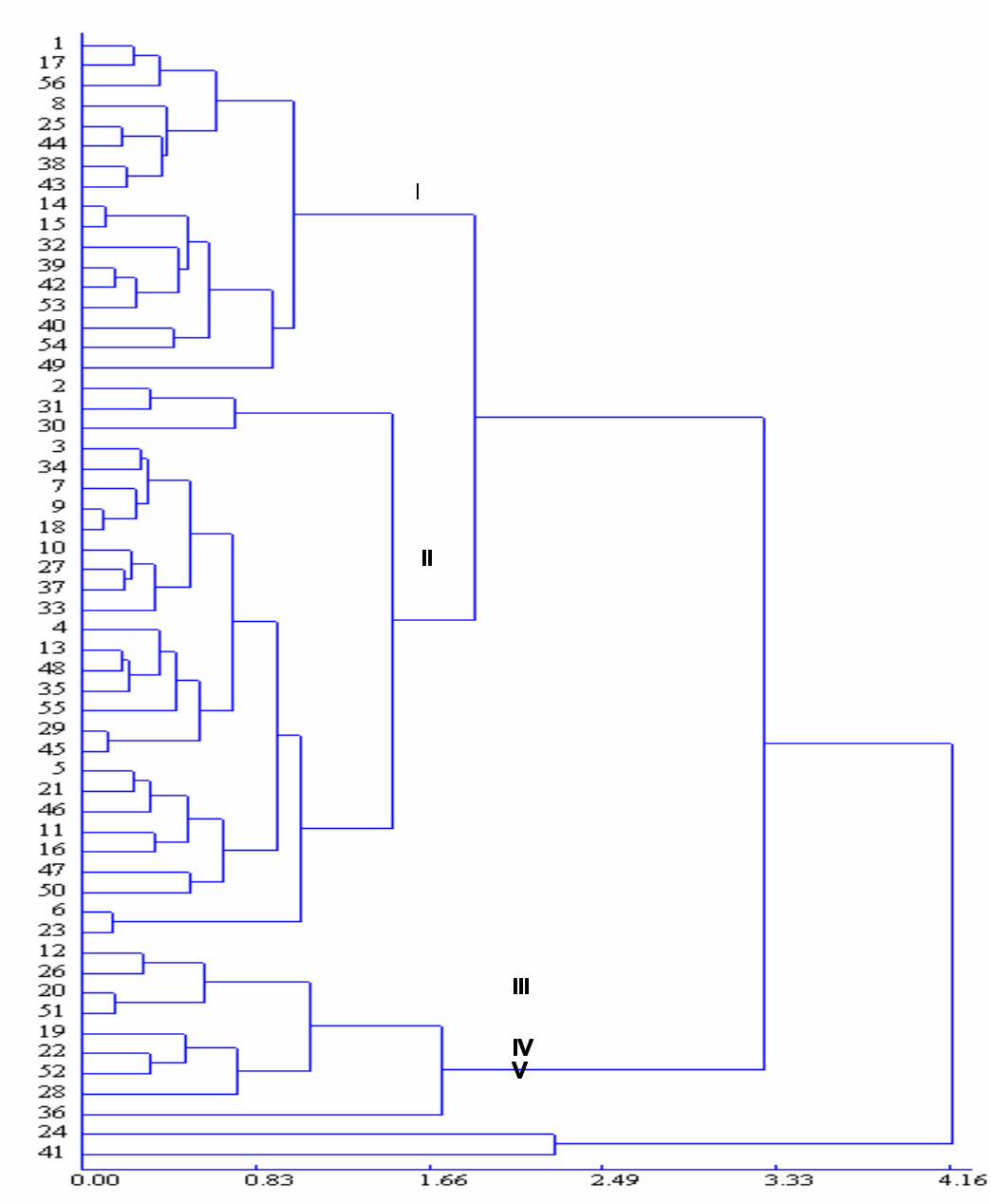

Fig 3: Clustering scheme of 56 soybean landraces

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The dendrogram showed the existence of 5 sub-clusters groups with the similarity values about 1.87. About 17 kinds of materials were included as a separate cluster Ⅰ, which contained one material from Aba, one material from Dazhou, three materials from Guangan, one from Liangshan, one from Meishan, one from Nanchong, one from Panzhihua, two from Suining, two from Yaan, two from Ziyang, and two from Zigong. Category cluster Ⅱ contains 28 kinds of landraces, which were one material from Aba, five materials from Chengdu, three from Dazhou, one from Deyang, two from Guangan, two from Leshan, one from Liangshan, two from Luzhou, two from Meishan, one from Mianyang, two from Neijiang, two from Yaan, two from Yibin, one from Ziyang, one from Zigong. Category Cluster Ⅲ contains 9 kinds of soybean landraces, one from Deyang, two from Guangyuan, one from Leshan, two forms Liangshan, one from Neijiang, two from Ziyang. No. 24 form Leibo, Liangshan and No. 41 from Chuanshan, Suining were clustered into another two clusters Ⅳ and Ⅴ. It could be found that there was no strong association between the clustering results and the geographical distribution. Further analysis shown in table 7 found that, cluster Ⅰ had the lowest content of Glycitein and Daidzein, while contents of 6'-O-malonyldaidzin, 6'-O-malonylgenistin were significantly higher than average. In group cluster Ⅱ the contents of Daidzin, Genistin, 6'-O-malonyldaidzin, 6'-O-malonylgenistin, Daidzein, Glycitein were lower than average except Glycitin. The Daidzin, 6'-O-malonyldaidzin , 6'-O-malonylglycitin, 6'-O-malonylgenistin of Cluster Ⅲ were highest while the Daidzein and Glycitein were below the average. In,cluster Ⅳ the Glycitin Genistin, Glycitein and Daidzein contents were of the highest, while the contents of 6'-O-malonyldaidzin 6'-O-malonylglycitin, 6'-O-malonylgenistin were much lower than average. Group cluster Ⅴ had the lowest content of Daidzin, Glycitin, Genistin, 6'-O-malonyldaidzin,6'-O-malonylglycitin, 6'-O-malonylgenistin, but had a higher content of Daidzein and Glycitein.

Table 7 : Average of Isoflavone compositions in every cluster

	Da	**Gly**	**Gen**	**6'-O-malda**	**6'-O-malglyc**	**6'-O-malgen**	**Daid**	**Glyci**	**Total content**
cluster Ⅰ	338.2	45.8	479.7	868.9	78.9	765.9	27.1	30.0	2634.4
cluster Ⅱ	246.6	52.0	300.8	495.6	71.4	416.1	27.1	38.9	1648.4
clusterⅢ	451.0	54.3	626.8	1420.8	102.2	1150.9	30.2	31.2	3867.4
cluster Ⅳ	445.7	86.0	806.3	282.5	48.6	326.9	244.8	405.0	2645.8
clusterⅤ	152.7	34.7	207.9	95.9	0.0	103.3	193.6	289.3	1077.5
mean	309.1	50.8	414.9	746.7	77.0	633.2	34.5	45.9	2312.0

Legend: Da- Daidzin, Gly- Glycitin, Gen – Genistin, 6Omalda-6'-O-malonyldaidzin, 6Omalgly -6'-O-malonylglycitin, 6Omalgen-6'-O-malonylgenistin, Daid-Daidzein, Glyci- Glycitein

5 CONCLUSION.

In this paper, HPLC analysis method of characteristic bioactive isoflavones of 56 soybean landraces were used and were adopted as multivariate analysis for classification those landraces. The combination of correlation analysis, PCA, and HCA provided a more reliable and comprehensive result and multivariate analysis is a valid system to deal with germplasm collection for soybean landraces. Here found kinds of landraces with high total isoflavone content and 5 different clustering groups of landraces for specific isoflavone compositions. The samples from different habitats provided plentiful variations among specific germplasm resources of soybean, and it could be found that there was no strong association between the clustering results and the geographical distribution.

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