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GENETIC DIVERSITY OF FLAVONOID CONTENT IN LEAF OF HAWTHORN RESOURCES

YUHUI ZHAO, KAI SU, GANG WANG, ZHENDONG LIU, WENXUAN DONG* AND YINSHAN GUO*

College of horticulture, Shenyang Agriculture University, Shenyang, 110866
**Correspondence author's e-mail: wxdong63@126.com, guoyinshan77@126.com*

Abstract

Hawthorn (*Cratageus* spp.) are important medicinal plants. Flavonoids are the main active ingredient in hawthorn. With the help of hawthorn leaf flavonoids efficient detection system, vitexin, rhamnosylvitexin, hyperin, rutin and quercetin of 122 hawthorn resources was precisely measured.The flavonoid contents of 10 hawthorn species were explicited. The comparation of flavonoids revealed the abundant genetic diversity of hawthorn flavones. Large variable coefficient has been observed among 5 flavonoid monomer traits. The coefficients of variation were 44.17%, 132.2%, 157.08%, 113.91% and 31.05 for Vitexin, Rhamnosylvitexin, Hyperoside, Rutin and Quercetin respectively. The sum of these 5 flavonoid monomer contents represented the total flavonoids in hawthorn. The total coefficients of variation was 44.01%. Some high-content-flavone and valuable leaf resources were found. This research could provide accurate date for further production, breeding and the effective use of medicinal resources.

Key words: Hawthorn, Flavone, Genetic diversity.

Introduction

China is abundant in hawthorn resources. Partly estimated about 500 copies of hawthorn resources are in China (Zhao *et al*., 1996). At present, a lot of work has been done on investigation, clearing up and selection for the origin and distribution of hawthorn resources in China. Big fruit, high sugar contents, high Vc contents, storage resistant, good processing quality, dwarf, soft nucleus, peach moth resistant and polyploidy resources has been selected (Li & Zhang, 2000; Li *et al*., 2000). Hawthorn is one of the important medicinal plants. Flavonoids are the main ingredient in hawthorn' physiological activity. But there is no report on the selection of medicinal resources of hawthorn. Previous research showed that the main flavonoids in hawthorn leaves containing mainly include vitexin, rhamnosylvitexin, hyperin, rutin and quercetin (Dong *et al*., 1996; Ding *et al*., 1990; Gong *et al*., 2005; Liu *et al*., 2007; Zhang *et al*., 1999; Chen *et al*., 2006).

The distribution in Chinese hawthorn resources as the object of the research, and measured the flavonoid monomers of 122 copies' flavonoids of hawthorn leaves by the established testing system of HPLC, and we analyzed the diversity of flavonoid contents, so as to compare the difference of the type and contents between different hawthorn flavonoids. We evaluated the quality of hawthorn germplasm resources from the aspect of medicinal active ingredients. Aiming to provide scientific date for further exploitation of hawthorn.

Materials and Methods

Materials: One hundred and twenty-two resources from 9 hawthorn species (*Cratageus* spp.) which are native to China and *Cratageus chlorosarca* Maxim that native to China, Japan and Russia. Materials were provided by National Pomology Germplasm (Shenyang) Hawthorn Garden. Fruits were harvested in Oct. 2012. The materials were as follows: *Cratageus pinnatifida* Bge., *C. maximowiczii* Schneid. var. ninganensis S. Q. Nie et B. J. Jen., *C. kansuensis* Wils., *C. altaica* (Loud.) Lange, *C. songarica* C. Koch, *C. maximowiczii* Schneid., *C. dahurica* Koehne, *C. brettschneideri* Schneid.,*C. chlorosarca* Maxim. and *C. hupehensis* Sarg. Among these, there were 107 cultivars of *C. pinnatifida* Bge.; 5 cultivars of *C. brettschneideri* Schneid.'JifuNO.1', 'JifuNO.2', 'XinghongNO.2', 'Hongroushanlihong'and '555'; 2 resources of *C. hupehensis* Sarg.'Jiatian' and 'Hubei NO.1'; 2 resources of *C. altaica* (Loud.) Lange, one red and one yellow (Table 2).

Hawthorn leaf treatment: Mature, uniform and healthy fruits were collected from the periphery of crown. Then washed the leaves and dried to constant weight at 50°C, and then crushed and filtered through 20 mesh sieve for further use.

Sample solution preparation: Referring to the established method (Lu *et al*., 2008). 1.0g of sample was precisely measured, vacuum filtrated and then dissolved in 30 ml 70% ethanol solution. Then extraction of 30 min., at 40°C with the ultrasonic power of 225W. And the liquid was transferred to 50ml volumetric flask through 70% ethanol solution. Filtrated from 0.45μm millipore filter and then got the sample solution.

Hawthorn leaf flavone HPLC conditions: Referring to the established method (Lu *et al*., 2008).
Chromatographic column: Agilent ZORBAX Extend-C18 (250 mm × 4.6 mm, 5μm).

Moving phase: acetonitrile-0.5% phosphoric acid solution gradient elute. (A: acetonitrile; B: 0.5% phosphoric acid solution) (Table 1).

Table 1. Gradient elution program.

Time	A %	B %
0.0 min	18.0	82.0
9.0 min	20.0	80.0
25.0 min	50.0	50.0
33.0 min	100.0	0.0

Testing wavelength: 345nm; Column temperature: 25°C; Flow velocity: 1.0 mL·min-1; Injection volumn: 20μL; Analyzing time: 45 min.

Linear relation: Concentration gradient of reference solutions for vitexin, rhamnosylvitexin, hyperin, rutin and quercetin were prepared according to the earlier stage research of this group. 20μL was injected. Peak area was measured according to the determined chromatographic conditions. Linear regression was done with peak area as the abscissa (X), and sample injection volume as the ordinate (Y).

Calculation of flavone content: Flavonoid monomer content (C) was calculated by standard equation of curve, ω represents the flavone content (%). Calculation fomula is as follows:

$$\omega=[\ C \times V\ /(W\times 10^6)] \times 100$$

C: flavone content calculated by standard curve, (μg);
V: total volume of sample solutions, (mL);
W: weight of dried samples, (g).

Statistic analysis of data: Standard deviation and variable coefficient was calculated by software SPSS.

Results and Analysis

Linear relation: Linear regression was done with peak area as the abscissa (X), and sample injection volume as the ordinate (Y). Linear regression equations were as follows:

Vitexin: Y=0.0132X + 1.6601, r=0.9910
Rhamnosylvitexin: Y=0.0180X + 3.3659, r=0.9789
Hyperin: Y=0.0100X+1.0157, r=0.9986
Rutin: Y=0.0170X+0.9592, r=0.9983
Quercetin: Y=0.0070X+0.4717,r=0.9979

Comparation of leaf flavonoid contents in Chinese Hawthorn: contents of vitexin, rhamnosylvitexin, hyperin, rutin and quercetin were measured and analyzed for the same batch of hawthorn leaves' samples. The sum of five flavonoid monomers' contents represent the total flavonoid content in hawthorn leaf.

Of the 5 flavones tested in 122 resources, rhamnosylvitexin and rutin were the main flavonoid detection. The average of rhamnosylvitexin and rutin contents were 0.3043% and 0.1470%. Lower contents of the other three flavones were detected, being 0.0508%, 0.0309 and 0.0038% for vitexin, hyperin and quercetin respectively. Quercetin content was the lowest (0.0028 0.0093) in all resources, and had the smallest range of variation. Quercetin was detected in only 44 resources. 'Chengoudahong' showed the highest total flavoneoid content (1.0704%). '*C. altaica* Loud. Lange' (yellow fruit) showed the highest conten of rhamnosylvitexin (0.7993%). 'Chuizhishanlihong' showed the highest content of rutin (0.7190%). 'Xiaohuangmianzha' showed the highest content of vitexin (0.3591%). '*C. chlorosarca* Maxim' showed the highest content of hyperin (0.2442%). 'Jiatian' showed the highest content of quecetin(0.0093%). These results could provide evidence for further use of the resources.

We measured flavonoid monomer content in 122 resources covering 10 species, and we found chemical constituents differences in leaves among 10 species. 2 to 4 types of flavonoids were detected in 8 species '*C. maximowiczii* Schneid.', '*C. altaica* (Loud.) Lange.', '*C. kansuensis* Wils.', '*C. brettschneideri* Schneid.', '*C. songarica* C. Koch', '*C. maximowiczii* Schneid. var. ninganensis S. Q. Nie et B. J. Jen.', '*C. dahurica* Koehne' and '*C. chlorosarca* Maxim.'.

23 resources among 107 *C. pinnatifida* Bge. contain all of the 5 flavonoids. The 23 *C. pinnatifida* Bge. were tedahuang mianzha, liangshanhong, xiuli, tongliaohong, haitangshanzha, xuzhoudahuo, fushunshangzhuanbai, yanranghong, baiquan7903, zifeng, yu8003, dawuleng, tianxiangyu, yu8002, huangbaoyu NO.1, jinxiandahong, yinyeling NO.2, niejiayu NO.1, yinyeling NO.9, guajiayu NO.1, xifen NO.4, xiajinxing and anshanzirou. 'jiatian' (*C. hupehensis* Sarg.) also contained all the 5 flavonoids.

In "Chinese pharmacopoeia", it is defined that the hyperin content in hawthorn leaves should not be less than 0.05% of the dry weight. Among the 122 resources, 13 were higher than 0.05%. The content of rhamnosylvitexin should not be less than 0.351%. And among the 122 resources, 29 were higher than 0.351%. The content of quercetin was lower in 10 species and had a narrow variation range.

Genetic diversity analysis of flavone: Among the 122 resources, 101 contained rhamnosylvitexin; 92 contained rutin; 111 contained vitexin; 110 contained hyperin; 44 contained quercetin (Table 3).Variation frequency of the character is the quantitative reflection of genetic diversity. The higher the variation frequency is, the more genetic diversity there exists among different cultivars, the more space to select excellent resource for us. It can be seen from Table 2 that, there is large coefficient of variation in all these 5 flavonoid monomers, being 44.17%, 132.2%, 157.08%, 113.91% and 31.05 for Vitexin, Rhamnosylvitexin, Hyperoside, Rutin and Quercetin respectively. The total flavonoid contents (the sum of 5 monomers) reached 44.01%. These means there is abundant genetic diversity in hawthorn flavonoid content. The results of this research could provide evidence for the further effective use of these resources.

The frequency distribution figure (Fig. 1) depicted that the content of rhamnosylvitexin between 0.25-0.30% had the largest frequency distribution and took 24% of the 101 resources, and the content lower than 0.25% took 30.6% of the 101 resources, the content higher than 0.3% took 46%. This means rhamnosylvitexin is a main and widely existed flavonoids in hawthorn. The content of rutin between 0.0186-0.068% had the largest frequency distribution and took 59% of the 92 resources; while the resources whose content was higher than 0.068% took 41%. This is another important flavonoids in hawthorn resources. The content of vitexin between 0.0062-0.025 had the largest distribution frequency, and took 68% of 111 resources. The content of hyperin between 0.0069-0.0166 had the largest distribution frequency, and took 84% of 110 resources. The content of quercetin between 0.0030-0.004 had the largest distribution frequency, and took 26% of 44 resources. The content of quercetin is lowest among the 5 flavones. Quercetin could be detected in only few hawthorn resources, and the content is very low (Table 2).

Table 2. Quantitative analysis results of flavonoids in samples (%, n=3).

Hawthorn species	Name of resources	Rhamnosylvitexin	Rutin	Vitexin	Hyperoside	Quercetin	Total
C. pinnatifida Bge	shen78213	-	0.0528	0.0516	-	0.0031	0.1076
	beijingdenglonghong	-	0.1964	0.1736	0.0245	0.0035	0.3981
	beijingzaosheng	-	0.2506	0.1838	0.0071	0.0046	0.4463
	mengyindajinxing	-	0.1490	0.2575	0.0077	0.0031	0.4174
	mengyinjinxing	-	0.2349	0.2101	0.0074	0.0040	0.4565
	xifenshisheng	-	0.2532	0.1871	0.0106	0.0031	0.4542
	beijingduizhao	-	0.1808	0.2641	0.0139	0.0033	0.4622
	Jiangou No. 2	-	0.2958	0.1925	0.0127	-	0.5011
	zimuhong	-	0.1692	0.1352	0.0079	-	0.3124
	hanfeng	-	0.2639	0.2441	0.0237	0.0031	0.5349
	fenlishanzha	-	-	0.2216	0.0251	0.0032	0.2499
	shandonghongmianzha	-	0.1834	0.1535	0.0085	-	0.3455
	xiaohuangmianzha	-	0.1264	0.3591	-	0.0041	0.4897
	tongtaibaiyesheng	-	0.4045	0.1797	0.0261	0.0039	0.6143
	shuanghong	-	0.3089	0.2548	0.0178	-	0.5817
	chaoxinhong	-	0.2606	0.2174	0.0164	-	0.4945
	wolonggang No. 2	-	0.2834	0.1905	0.0131	0.0037	0.4908
	ganyu No. 2	-	0.1398	0.2659	0.0074	-	0.4132
	magangzaohong	0.0171	0.2625	0.2273	0.0133	-	0.5203
	majiafenrou	0.3015	0.5767	0.0064	0.0079	-	0.8927
	anshanzirou	0.2936	0.0210	0.0090	0.0189	0.0032	0.3458
	jingduan No. 1	0.4855	-	0.0066	0.0194	-	0.5116
	xiajinxing	0.2650	0.0265	0.0084	0.0262	0.0035	0.3298
	xifen No. 4	0.2519	0.0221	0.0123	0.0135	0.0036	0.3034
	donglingqingkou	0.3478	0.0193	0.0142	0.0096	-	0.3910
	jianchangshanzha	0.2901	0.0209	0.0095	0.0102	-	0.3308
	guajiayu No. 1	0.2213	0.0334	0.0062	0.0255	0.0030	0.2895
	yinyeling No. 9	0.2875	0.0311	0.0075	0.0355	0.0033	0.3652
	yinyeling No.1	0.2985	-	0.0121	0.0115	-	0.3222
	xifen No. 1	0.3835	0.0186	0.0091	0.01257	-	0.4238
	piposhi	0.3920	0.0241	0.0311	-	-	0.4473
	liaoyangzirou	0.2635	0.0197	0.0076	0.0119	-	0.3029
	shen 2-4	0.2155	0.0201	0.0086	0.0192	-	0.2634
	jinxianxiaoyeshanzha	0.2438	0.0214	0.0075	0.0262	-	0.2991
	wanqiushanlihong	0.4418	0.0274	0.0079	0.0349	-	0.5122
	xiezishi No. 1	0.2993	0.0221	0.0134	0.0228	-	0.3577
	yiduxiaohuang	0.1969	0.0207	-	0.0263	-	0.2439
	huixiandahongkongqi	0.4234	0.0365	-	0.0404	-	0.5004
	Baili	0.5006	-	0.0066	-	-	0.5073
	linfenbaiyesheng	0.3075	0.0275	0.0065	0.0325	-	0.3741
	huabeixiaoshanzha	0.1164	0.0283	-	0.0143	-	0.1591
	yidutedahuangmianzha	0.6086	0.0474	0.0070	0.0373	0.0031	0.7036
	fengshui No. 1	0.0711	-	0.0065	0.0214	-	0.0991
	niejiayu No. 2	0.4151	-	0.0069	-	-	0.4220
	niejiayu No. 1	0.2725	0.0192	0.0081	0.0142	0.0031	0.3173
	majiadadui	0.2828	-	0.0065	-	-	0.2894
	yinyeling No. 2	0.2415	0.0243	0.0075	0.0251	0.0043	0.3029
	jinxiandahong	0.2581	0.0240	0.0077	0.0267	0.0055	0.3221
	shanchengzi No. 2	0.2853	0.0221	0.0071	0.0271	-	0.3417
	Sishanling	0.3804	-	0.0223	0.0149	-	0.4177
	xihong	0.4252	-	0.0085	-	-	0.4337
	benxi No. 7	0.4552	-	0.0629	-	0.0029	0.5211
	benxi No. 2	0.4025	-	0.0124	0.0102	-	0.4251
	huangbaoyu No.1	0.2886	0.0290	0.0088	0.0492	0.0046	0.3804
	benxi No.4	0.2757	-	0.0069	0.0097	-	0.2925
	shancheng No.1	0.3911	-	0.0200	-	-	0.4112
	niuxintai No.1	0.3278	0.0242	0.0070	0.0322	-	0.3914
	tuguzi No.1	0.3657	0.0237	0.0302	0.0128	-	0.4325
	yu8002	0.2297	0.0272	0.0078	0.0322	0.0029	0.3000
	tianxiangyu	0.3160	0.0433	0.0073	0.0495	0.0048	0.4211
	dawuleng	0.2452	0.0379	0.0071	0.0418	0.0029	0.3351
	tianshui	0.3076	-	0.0076	0.0094	-	0.3247

Table 2. (Cont'd.).

Hawthorn species	Name of resources	Rhamnosylvitexin	Rutin	Vitexin	Hyperoside	Quercetin	Total
	mopan	0.2954	-	-	-	-	0.2954
	8321	0.2303	0.0303	0.0109	0.0457	-	0.3174
	bairangmian	0.3705	0.0188	0.0062	0.0205	-	0.4161
	kaiyuanruanzi	0.5052	0.0223	0.0267	0.0791	-	0.6335
	donglingshanzha	0.3787	0.0206	0.0068	0.0163	-	0.4225
	xinbin No.7	0.1810	0.0262	0.0108	0.0545	-	0.2726
	shen78201	0.2374	-	0.0103	0.0393	-	0.2871
	qiujinxing	0.4926	0.0372	0.0298	0.0088	-	0.5685
	magangdajinxing	0.3453	0.0329	0.0181	0.0260	-	0.4224
	tangchi No. 2	-	-	0.0447	0.0947	0.0041	0.1436
	baiquan7901	0.3016	0.0403	0.0133	0.0187	-	0.3741
	yu8003	0.3615	0.0333	0.0067	0.0486	0.004	0.4547
	xinglongshisheng	0.2940	0.0191	0.0099	0.0121	-	0.3351
	zifeng	0.3244	0.0224	0.0071	0.0227	0.0031	0.3799
	huixiandahong	0.2806	0.0228	0.0259	0.0140	-	0.3433
	baiquan7903	0.1825	0.0259	0.0082	0.0394	0.0034	0.2596
	yanranghong	0.2913	0.0253	0.0159	0.0208	0.0032	0.3568
	fushunshangzhuanbai	0.3744	0.0252	0.0081	0.0183	0.0032	0.4293
	xinbinruanzi	0.3855	0.0321	0.0277	0.0198	-	0.4653
	xuzhoudahuo	0.3052	0.0320	0.0071	0.0551	0.0063	0.4059
	79723	0.2678	0.0271	0.0070	0.0315	-	0.3336
	haitangshanzha	0.4550	0.0212	0.0109	0.0191	0.0031	0.5093
	wanbahong	0.5157	-	0.0078	0.0094	-	0.5330
	luanhong No. 1	0.3148	0.0215	0.0130	0.0112	-	0.3605
	pingyiyinhongzi	0.4464	0.0699	0.0130	0.0311	-	0.5605
	luanhong No. 2	0.3558	0.0205	0.0106	0.0115	-	0.3985
	tongliaohong	0.2985	0.0215	0.0071	0.0292	0.0032	0.3597
	qiuli	0.4250	0.0490	0.0144	0.1061	0.0030	0.5976
	liaoning No. 10	0.2833	0.5236	0.0085	0.0148	-	0.8303
	fushunshanzha	0.2074	0.6845	-	0.0081	-	0.8999
	liangshanhong	0.3260	0.3129	0.0093	0.0074	0.0031	0.6589
	chuizhishanlihong	-	0.7191	-	0.0254	-	0.7445
	duanzhishanlihong	-	0.6907	-	0.0854	-	0.7762
	zhuantaishanzha	0.2162	0.4445	0.0089	0.0068	-	0.6765
	tielingshanzha	0.2547	0.4442	0.0093	-	-	0.7083
	78214	0.3431	0.5740	0.0254	0.0165	-	0.9591
	jiangxian798202	0.0132	-	-	-	-	0.0132
	chengoudahong	0.3917	0.6518	0.0081	0.0187	-	1.0704
	linxianshangkou	0.2448	0.5168	-	0.0201	-	0.7817
	798203	0.3314	0.5130	0.0081	0.0147	-	0.8673
	798201	0.3281	0.4580	-	0.1351	-	0.9213
	shanxitiansheng	0.4098	-	0.0093	0.0133	-	0.4325
	yubeihong	0.5013	-	0.0122	0.0198	-	0.5334
	jiangxianshanzha	0.2766	0.4074	0.0082	0.0295	-	0.7219
	81-2	0.2380	0.3788	-	0.0075	-	0.6244
C.maximowiczii Schneid	*C.maximowiczii* Schneid	0.0135	-	0.0484	0.1437	-	0.2057
C. kansuensis Wils.	*C. kansuensis* Wils.	0.2565	-	0.0280	0.0757	0.0041	0.3644
C.songarica C. Koch	*C. songarica* C. Koch	0.3199	0.0370	0.0074	0.0261	-	0.3906
C.maximowiczii Schneid. var. *ninganensis* S.Q. Nie. et B. J. Jen.	*C.maximowiczii* Schneid. var. ninganensis S. Q. Nie. et B. J. Jen.	0.0167	-	0.0990	0.0698	0.0058	0.1915
C. altaica (Loud.) Lange	red fruit	0.7037	-	0.0206	0.0158	0.0032	0.7434
	yellow fruit	0.7993	-	0.0226	0.1089	0.0028	0.9337
C.Dahurica Koehne.	*C. dahurica* Koehne.	0.0178	-	0.1428	0.0150	-	0.1756
C.brettschneideri Schneid.	555	0.0314	-	0.0953	0.0573	-	0.1841
	jifu No. 1	0.1880	0.0206	0.0290	0.0118	-	0.2495
	jifu No. 3	0.1526	0.0194	0.0211	0.0149	-	0.2081
	xinghong NO. 2	0.2187	-	0.0171	0.0108	0.0036	0.2503
	hongroushanlihong	0.2283	0.0210	0.0241	0.0176	-	0.2912
C. hupehensis Sarg.	hubei No.1	0.2950	0.0199	0.0080	0.0170	-	0.3400
	jiatian	0.1652	0.0324	0.0132	0.1640	0.0092	0.3843
C. chlorosarca Maxim	*C. Chlorosarca* Maxim	0.0466	-	0.1444	0.2442	0.0051	0.4403

The notes "-"Not detected

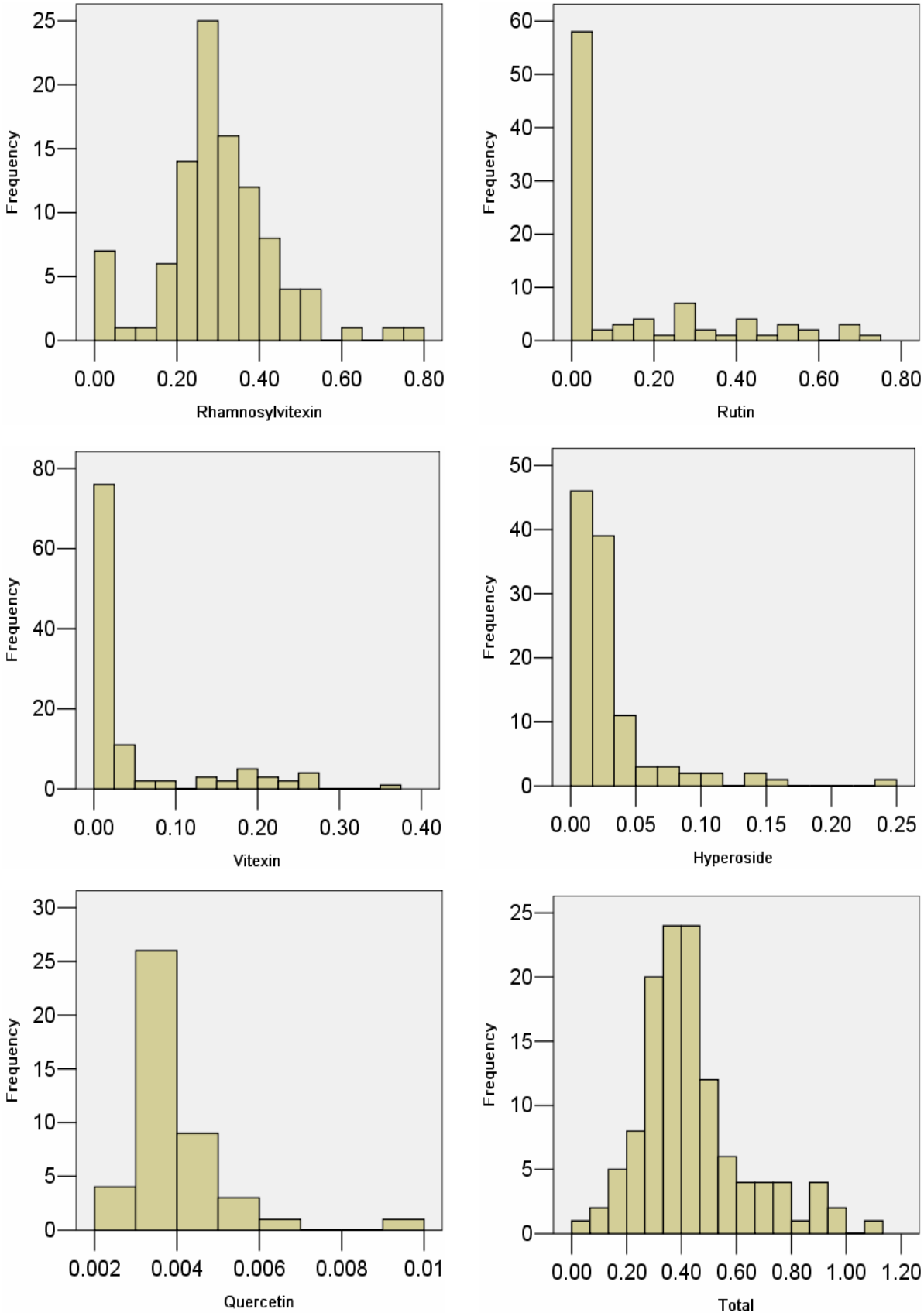

Fig. 1. Frequency distribution of flavone content.

Table 3. Genetic analysis of 5 flavone content in hawthorn resources.

Flavone species	No. of cultivars	Min	Max	Mean SD	C V
Rhamnosylvitexin	101	0.0133	0.7993	0.3043 0.1344	44.17
Rutin	92	0.0186	0.7191	0.1470 0.1943	132.2
Vitexin	111	0.0062	0.3591	0.0508 0.0798	157.08
Hyperoside	110	0.0069	0.2442	0.0309 0.0352	113.91
Quercetin	44	0.0028	0.0093	0.0038 0.0012	31.05
Total	122	0.0133	1.0704	0.4382 0.1929	44.01

Conclusion

Flavone is one of the important medicinal components, which is reported from various kinds of medicinal plants (Cheng, 2004; Sabina, 2009; Kaya, 2012; Jahan, 2013), meanwhile researchers of various countries pay attention to the evaluation and reseach of their native flavonoid resources (Kaya, 2012; Jahan, 2013). Flavonoid content of hawthorn resources which are native to China was precisely measured in the experiment . The content of 10 hawthorn species and common cultivars in *C. pinnatifida* Bge. has been determined. This could provide accurate data for production, breeding and further effective use of these medicinal resources.

Some valuable leaf resources were discovered by comparision of flavonoid ingredient in hawthorn.

'Chengoudahong' held the highest total flavone content (1.0704%), followed by '78214' (0.9591%). In 10 species, the content of total flavone in 2 *C. altaica* Loud. Lange was also high, which is 0.9337% and 0.7434% for yellow fruit type and red fruit type.

C. altaica (Loud.) Lange held the highest rhamnosylvitexin content (0.7993%), followed by 'yidutedahuangmianzha' (0.6086%).

'Chuizhishanlihong' held the highest rutin content (0.7191%), followed by 'Duanzhishanlihong' (0.6907%). The content of rutin was also high in 'Fushunshanzha' (0.6518%) and 'Chengoudahong' (0.6848%).

'Xiaohuangmianzha' held the highest vitexin content (0.3591%), followed by 'Ganyu NO.2' (0.2659%).

C. chlorosarca Maxim. held the highest hyperin content (0.2442%), followed by 'Jiatian' (*C. hupehensis* Sarg.) (0.1640%).

'Jiatian'(*C. hupehensis* Sarg.) held the highest quercetin content (0.0092%), followed by 'Xuzhoudahuo' (0.0063%).

23 resources contained all of the 5 flavones. And 'Yidutedahuangshanzha' held the highest flavone total content (0.7306%). It is a yellow fruit resources.

These hawthorn resources reflect the high advantage, and could be the substitution for abstraction of hawthorn leaf flavone.

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