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Analysis of Protein Content and Genetic Diversity in Pea Germplasm in Tibet

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Abstract To grasp protein content and composition of pea resource in Tibet Plateau, 54 pea materials from different eco-geographical environments of Tibet were collected and arranged in this paper. Based on SDS-PAGE, electrophoresis and genetic diversity analysis of water-solubility and salt-solubility proteins from 54 pea materials were conducted, and the relationship between geographical ecological factors(longitude, latitude and altitude) and total protein content was studied. The research results showed that total protein contents of 54 pea materials were between 17.58% and 28.67%, in which water-solubility protein accounted for 86.12% - 91.40%, while salt-solubility protein accounted for 4.76 - 8.29%. Total protein content of Tibet pea showed significantly positive correlation with longitude, certain positive correlation with latitude and certain negative correlation with altitude. SDS-PAGE of water-solubility and salt-solubility proteins from 54 pea materials respectively detected 1588 and 699 protein bands. Based on different mobility ratios, there were 43 kinds of water-solubility protein bands, and diversity index was between 0 and 0.5. Its relative molecular weight was between 24.87 and 149.54 ku, showing the low molecular weight region of 24.71 - 50.41 ku and high molecular weight region of 56.34 - 88.08 ku. There were 24 salt-solubility protein bands based on different mobility ratios, with the diversity index of 0 - 0.5, and relative molecular weight was between 24.85 and 91.24 ku. According to the altitude, 54 pea resources were divided into 4 geographical groups. Gene diversity indexes of each group were respectively 0.23, 0.18, 0.35 and 0.31, and Shannon information indexes were respectively 0.33, 0.41, 0.52 and 0.46. It showed that the variation of pea protein was related to altitude. In clustering analysis, the tested resources were divided into seven classes, showing that water-solubility and salt-solubility proteins could reflect genetic relationship among germplasm resources at certain degree. The research could provide theoretical basis for the development of Tibet pea resources and selection of good parents.

Key words Pea, Protein content, SDS-PAGE, Tibet

1 Introduction

Pisum sativum L belongs to annual or perennial herb of *Pisum Linn* of Leguminosae, has the characteristics of enduring drought, coldness and infertility, is widely distributed[1-2], and is one of important cultivation crops and food materials[3]. *P. sativum* has long planting history in China and is widely distributed in whole country as vegetable, grain and forage[4]. According to the statistics of FAO, dry pea yield of China in 2013 was 1.38 million t, ranked second in the world, which was only next to Canada[5]. *P. sativum* has the characteristics of enjoying coolness and resisting coldness and infertility, is widely distributed in Tibet Plateau, and is often mixedly sown or rotated with highland barley, rape and wheat[6]. In recent years, breeding work of *P. sativum* seriously lags in Tibet, which causes that pea yield and quality decline, and its sowing area gradually declines year by year, and basic research on pea is nearly zero. Therefore, it is urgent to conduct salvage collection, evaluation and genetic analysis of *P. sativum* germplasm resource in Tibet, excavate excellent *P. sativum* germplasm resource and enlarge gene library of *P. sativum* germplasm. Seed storage protein (SSP) indicates seed protein not possessing metabolism and structural functions, which has been applied into four aspects as genetic marker: genetic diversity analysis among materials, identification of genome relationship, preservation of genetic resources, plant domestication and plant improvement breeding related to breeding[7]. Based on SDS-PAGE, seed protein electrophoresis has become a kind of effective tool solving plant classification and evolution problem, identifying variety and mutation, analyzing germplasm characteristics and supplementing the related information of evaluation[8-10]. Prior researches on related crops show that SSP among different ecological groups, varieties and materials is highly polymorphic, and the polymorphism is mainly decided by genetic factors[11-15]. For example, based on the soybean storage protein band, it could quickly identify the variation, mutant and variety of protein subunit[15-19]. Hirata used isozyme and seed protein to analyze genetic diversity of *Glycine max* resource under different habitat conditions of Japan, China and Korea, and their diversity indexes were respectively 0.248, 0.249 and 0.209[20]. Vijayanand used SDS-PAGE for genetic diversity analysis of seed proteins from 722 *P. sativum* samples in 8 eco-geographical areas of Korea, and found that diversity indexes of protein subunits from the tested materials were between 0 and 0.2642, with the mean of 0.1565[8]. SDS-PAGE is used for polymorphism study of glutenin and gliadin in wheat plant. It is found that rich genetic polymorphism exists in whether macromulecule or low molecular weight storage protein site, which could reflect genetic relationship among the tested materials at cer-

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tain degree[21-23]. In clustering analysis, the tested materials are divided into 6 classes, and clustering result could reflect genetic relationship among the tested materials at certain degree[23]. Shi Jianbin *et al.* used SDS-PAGE technique for electrophoresis analysis of albumin from 101 *Vicia faba* L. germplasm resources. Albumin subunit had rich variation among the tested materials and stronger polymorphism. The clustering chart generated by genetic similarity coefficient could reflect the genetic relationship among the tested materials[24]. Song Xiaomin *et al.* used SDS-PAGE for electrophoresis analysis of water-solubility and salt-solubility proteins from seeds of 20 kinds of *V. faba* L. in its main production region of China[25]. Wang Yanping *et al.* selected 57 ecotypes of *G. max* germplasm resources from Shanxi as the materials, and used SDS-PAGE gradient electrophoresis technique to separate each main subunit of 11S globulin and 7S conglycinin. It was found that relative content of the same subunit among different ecotypes of *G. max* germplasm resources had larger difference, and 4 specific *G. max* germplasm resources of natural variation were found[26]. Electrophoresis analysis of seed protein band has been successfully applied in classification and evolutionary relationship between related crops and its close relatives to analyze its genetic homology at molecular level[7]. But so far there is not report on genetic diversity of Tibet *P. sativum* germplasm resource by related DNA molecular marker techniques, such as SSR, ISSR, PAPD and SNP, which is not favorable for further understanding genetic background of *P. sativum* germplasm in Tibet Plateau. In this paper, taking traditional and wild species of Tibet *P. sativum* as the materials, SDS-PAGE technique was used to analyze protein polymorphism of *P. sativum* seed to explore genetic diversity and relationship of Tibet *P. sativum* germplasm resource from protein level. Combining the contents of crude, water-solubility and salt-solubility proteins in seed, *P. sativum* parent materials with high protein content, large genetic difference and far relative relationship were selected, which could provide theoretic basis for the development of Tibet *P. sativum* resource and selection of good parents.

2 Materials and methods

2.1 Materials The tested materials were 51 Tibet *P. sativum* (traditional and wild species) samples, 2 *P. sativum* samples from Kangle County of Gansu and 1 *P. sativum* from Litang County of Ganzi, Sichuan (Table 1). They were collected from each county of Tibet, Kangle of Gansu and Ganzi of Sichuan in 2015, and local altitude, longitude and latitude were recorded. They were sown in the farm of Xizang Agriculture and Animal Husbandry College on March 20, 2016(26°52′ – 30°40′ N, 92°09′ – 98° 47′ E, altitude of 2900 m), and conventional field management was used. During mature period, 5 pods were collected singly from each kind of material, and they were conserved by classification and then used for subsequent test after natural drying.

Table 1 Basic information of 54 *P. sativum* germplasm resources

Material number	Collection site	Longitude	Latitude	Altitude//m
1	Tongmu Village, Zhamu Town, Bomi County	95.89	29.79	2874
2	Peng'an Village, Baige Township, Biru County	94.26	31.22	3861
3	San Village, Gongka Town, Mozhu Gongka County	91.82	29.79	3879
4	Yaoziai of Shuangzhai, Suji Village, Kangle County, Gansu Province	103.34	35.20	2000
5	Tarong Village, Nimu Township, Nimu County	90.15	29.44	3841
6	Guoxika Village, Luolin Township, Jiazha County	92.56	29.00	3446
7	Jiadui Village, Cheren Township, Jiangzi County	89.77	28.84	4189
8	Baiguo Village, Chejiu Township, Linzhi County	94.77	29.97	2638
9	Waru Village, Nimu Township, Bianba County	94.37	31.23	3724
10	Dier Village, Chaba Township, Renbu County	89.95	29.17	3972
11	Changga Village, Jiama Township, Qiongjie County	91.65	29.00	3822
12	Boxue Village, Guyu Township, Chayu County	97.20	29.31	3591
13	Nanmulin Town, Nanmulin County	89.09	29.66	3991
14	Guqing Village, Guyu Township, Chayu County	97.21	29.16	3428
15	Changga Village, Jiama Township, Qiongjie County	91.65	29.00	3822
16	Keri Village, Bangdui Township, Dazi County	91.00	30.00	3729
17	Langda Village, Eluo Town, Changdu County	96.97	31.22	3326
18	Jiangda Village, Jiawa Township, Litang County	100.42	29.75	3625
19	Gongrenbu Village, Chaba Township, Renbu County	89.90	29.20	3956
20	Yigongdanka Team, Gang Township, Bomi County	95.69	29.93	2703
21	Chana Village, Kangma Village, Kangma County	89.67	28.62	4264
22	Bairong Village, Changzhu Town, Naidong County	91.75	29.16	3633
23	Jiajiao Village, Dongga Village, Shigatse City	88.89	29.37	3854
24	Shenjiashan of Shuangzhai, Suji Town, Kangle County, Gansu Province	103.34	35.20	2000
25	Chunpi Village, Xiasima Town, Yadong County	88.91	26.47	2918

(to be continued)

(continued)

Material number	Collection site	Longitude	Latitude	Altitude//m
26	Lie Village, Jindong Township, Lang County	93.34	28.98	3112
27	Yonglang Village, Biru Township, Biru County	93.66	31.49	3920
28	Laduo Village, Majia Township, Shajia County	87.85	28.72	4325
29	Xiangga Village, numa Township, Nanmulin County	89.64	29.39	3895
30	Wumo Village, Chaba Village, Renbu County	89.92	29.19	3972
31	Kangbuqi Village, Xiashui Township, Qiongjie County	91.72	29.12	3674
32	Kangbuqi Village, Xiashui Township, Qiongjie County	91.72	29.12	3674
33	Gangma Village, Jiangtang Town, Gongga County	90.62	29.27	3619
34	Xiajiang Village, Xiajiang Township, Qusong County	92.22	29.04	4010
35	Qiangnian Village, Jiru Township, Zhalang County	91.30	29.06	3959
36	Buma Village, Kaga Township, Angren County	87.43	29.21	4313
37	Chuxi Village, Xiegeer Town, Dingri County	87.21	28.59	4266
38	Dier Village, Naisha Township, Jiangzi County	89.38	29.06	3935
39	Xiangruo Village, Jiru Township, Zhalang County	91.31	29.15	3720
40	Zhari Village, Zhari Township, Luozha County	90.69	28.37	4204
41	Chawu Village, Chawu Township, Lazi County	87.58	29.10	4063
42	Cunba Village, Ridang Town, Longzi County	92.30	28.49	4104
43	Panjiu Village, Zhuangzi Township, Jiangzi County	89.48	29.00	3975
44	Disi Village, Langjiexue Township, Gongga County	91.10	29.16	3671
45	Puxia Village, Changguo Township, Gongga County	91.17	29.42	3693
46	Xianglie Village, Jindong Township, Lang County	93.34	28.98	3112
47	Boluo Village, Guyu Township, Chayu County	97.39	29.99	2895
48	Balang Village, Rong Township, Sangri County	92.00	29.23	3582
49	Guju Village, Shengge Township, Luozha County	91.03	28.16	3570
50	Mingtong Village, Dingqing Town, Dingqing County	95.64	31.39	3808
51	Zai Village, Kangsha Township, Luolong County	96.13	30.77	3866
52	Dunba Village, Liemai Township, Longzi County	92.73	28.35	3480
53	Tongba Village, Rong Township, Sangri County	92.14	29.28	3583
54	Jidui Village, Luozha Town, Luozha County	90.77	28.38	4037

2.2 Test methods

2.2.1 The determination of crude protein content from pea. According to the method of GB 50095 – 2010 (N × 6.25), crude protein content from pea was measured.

2.2.2 Separations and content determinations of water-solubility and salt-solubility proteins from pea. Separation and extraction methods of water-solubility and salt-solubility proteins by Song Xiaomin *et al.* [25] were changed properly. 2 – 3 pea seeds were set in precooling mortar and sufficiently ground into powder after removing the peel, and 0.15 g of pea powder was taken in 2 mL of centrifuge tube. According to the proportion of 1∶10, 1.5 mL of distilled water was added for vortex vibration mixing. They were set in 4 ℃ of fridge for extracting for 30 min, and once shaking was conducted per 5 min. It was centrifugated for 20 min at 6500 r/min, and supernatant was transferred to new centrifuge tube, which was water-solubility protein, and it was conserved at 4 ℃. 0.15 g of pea powder was dissolved by 1.5 mL of NaCl (1 mol/L), which was extracted for 30 min in 4 ℃ of fridge. After that, it was centrifugated for 20 min at 6500 r/min, and then supernatant was dialyzed for 24 h in 4 ℃ of fridge, thereby obtaining dry powder. The obtained dry powder was dissolved by 40 μL of NaCl (1 mol/L), which was salt-solubility protein, and it was conserved in 4 ℃ of fridge.

2.2.3 SDS-PAGE electrophoresis of pea protein. 40 μL of water-solubility and salt-solubility proteins were taken respectively. NanoDrop 2000 was used to measure protein content, and 2.5 mg/μL of protein solution was prepared. 12 μL of loading buffer (250 mmol/L, pH 6.8 of Tris-HCl, 10% SDS, 0.5% bromo phenol blue, 50% glycerol, 5% β-mercaptoethanol) was added respectively, and they were boiled for 5 min at 100 ℃. They were centrifugated for 20 s, and sample was added for electrophoresis after cooling. Discontinuous polyacrylamide gel electrophoresis was used, with 5% of concentrated gel concentration and 10% of separation gel concentration. Tris-glycine was taken as electrode buffer solution, and adding amount of each sample was 20 μL, with gel thickness of 1.0 mm. Electrophoresis was conducted under 16 mA of constant flow condition, and it finished after indicator moved to the bottom of gel. Coomassie brilliant blue R-250 solution was used to dye for 90 min, and distilled water was used to wash for 2 times. During decolorization period by decoloring solution (25 mL of absolute ethanol + 50 mL of glacial acetic acid + 475 mL of ddH_2O), 2 – 3 times of decoloring solution was changed until subunit band was clear. After washed by distilled water, it could be taken a photograph.

2.2.4 Statistical analysis of data. SPSS20 was used to conduct correlation analysis between pea protein and longitude, latitude and altitude. Quantity one software was used to automatically read

electrophoresis bands of pea water-solubility and salt-solubility proteins, and supplementary artificial correction was conducted. According to the same position, when there was protein electrophoresis band, it was endowed as 1; when there was not protein electrophoresis band, it was endowed as 0, thereby generating 0, 1 matrix. By NTSYS-pc 2.1 software, clustering of the generated 0, 1 matrix by UP-GMA was conducted to calculate genetic distance and genetic similarity coefficient. Using popgen32 software, allelic variation number, allelic variation frequency, effective number of alleles, Shannon's information index and gene diversity index at one site among different groups were calculated. Via Microsoft Excel, occurrence frequency was counted.

3 Results and analyses

3.1 Correlation analysis between geographical ecological factors and protein content of pea The tested *P. sativum* was from plateau region of 103.34 – 87.21° E, 35.20 – 28.16° N, with the altitude of 4325 – 2000 m. The covering range was wide, and it involved complex and diverse terrain and topographic features. Correlation analysis between geographical ecological factors and protein content of pea showed that longitude had significantly positive correlation with crude protein content of pea, while latitude had positive correlation with crude protein, and altitude had negative correlation with crude protein. It illustrated that crude protein of pea had a certain increase from east Tibet to central Tibet to post Tibet area; with latitude increased, crude protein content of pea had an increasing trend; with altitude increased, total protein of pea had slightly declining trend.

3.2 Content analysis of water-solubility and salt-solubility proteins in pea Total protein contents of 54 pea materials in different ecological regions had obvious difference (Table 2). Total protein contents of the tested pea materials changed from 17.58% to 28.67%, in which the material with the highest protein content was from Chayu region of Tibet, while the material with the lowest protein content was from Qiongjie County in central Shannan City of southeast Tibet. Via single-factor variance analysis, it was found that protein contents among 54 *P. sativum* germplasm resources had significant difference. Water-solubility and salt-solubility protein contents had certain difference in 54 pea materials (Table 3), in which water-solubility protein accounted for 86.12% –91.40% of total protein of seed, while salt-solubility protein accounted for 4.76% – 8.29% of total protein of seed, and water-solubility protein was about 10 times of salt-solubility protein. When compared with salt-solubility protein, water-solubility protein had wide application in food and was convenient for eating. Therefore, pea protein had wider application prospect.

Table 2 Contents of total, water-solubility and salt-solubility proteins from 54 *P. sativum* germplasm resources

Material number	Total protein	Water-solubility protein	Salt-solubility protein	Material number	Total protein	Water-solubility protein	Salt-solubility protein
1	20.63 a	88.91	6.96	28	21.67 b	85.63	6.72
2	19.51 a	90.47	5.04	29	20.94 a	88.37	5.98
3	22.59 b	88.66	7.36	30	23.10 b	90.12	7.35
4	24.42 b	91.28	8.21	31	20.88 a	87.63	7.16
5	22.47 b	89.46	7.56	32	19.32 a	87.36	4.82
6	22.00 b	89.60	6.46	33	20.71 a	89.13	5.96
7	25.37 c	92.67	6.27	34	23.04 b	90.16	7.45
8	21.20 a	87.40	7.91	35	20.44 a	88.74	6.96
9	21.93 b	89.87	7.73	36	22.40 b	89.45	7.03
10	21.86 b	87.84	6.36	37	19.01 a	87.32	6.14
11	17.58 a	90.31	5.56	38	23.95 b	90.67	7.49
12	28.67 c	91.20	8.01	39	20.73 a	89.96	7.08
13	23.21 b	87.37	6.11	40	22.30 b	87.63	6.54
14	21.92 b	90.61	7.37	41	18.45 a	86.13	5.18
15	19.19 a	87.49	5.65	42	19.25 a	90.00	4.76
16	20.37 a	88.66	6.24	43	19.54 a	86.79	5.85
17	21.05 a	89.53	7.39	44	22.20 b	90.63	6.63
18	22.86 b	90.01	6.76	45	19.42 a	86.12	5.52
19	24.58 c	91.21	6.37	46	23.51 b	87.15	7.68
20	21.25 a	88.24	5.92	47	22.38 b	89.62	8.52
21	25.71 c	91.22	8.11	48	20.92 a	86.16	7.15
22	23.66 b	90.60	8.29	49	21.31 a	90.43	7.74
23	22.73 b	91.40	5.52	50	23.48 b	88.69	6.83
24	23.60 b	87.80	8.24	51	25.66 c	90.24	6.48
25	21.95 b	89.12	7.83	52	20.84 a	86.42	7.16
26	21.98 b	91.14	5.06	53	20.99 a	87.65	6.52
27	20.56 a	87.60	6.47	54	20.19 a	87.23	6.68

Note: Duncan's multiple range test method was used to analyze, and protein content of each sample was compared with No. 11 sample with the minimum protein content, and different letters at the same row showed significant difference ($P<0.05$, $n=3$).

3.3 SDS-PAGE analysis of water-solubility protein of pea

Via SDS-PAGE electrophoresis, water-solubility proteins of 54 pea materials showed the band with different mobility ratios, and the band number was different by the sample (Fig. 1A). 1588 water-solubility protein bands were separated from the tested materials, in which 43 bands had different mobility ratios, and change range of their number was 6 – 43 bands, and average band number of water-solubility protein in each material was 20.4. Among them, band number of water-solubility protein separated from No. 36 pea material was 36, which was the most, while band number of water-solubility protein separated from No. 18 and No. 40 materials was 22, which was the minimum. In 43 bands with different mobility rates, occurrence times of each band in 54 tested materials was 6 – 54, and corresponding occurrence frequency was 11.1% – 100.0%, and diversity index (I) was between 0 and 0.5 (Table 4). No. 3, 4, 5, 7, 21 bands all appeared in the tested materials, and their occurrence frequencies were 100%, and diversity index was 0, which was common band. Occurrence frequency of No. 6 band in 54 pea materials was 11.1%, which was the lowest, and it only appeared in 6 pea materials, and other pea materials lacked it, with lower diversity index. Occurrence frequencies of No. 8, 12, 15, 27, 34, 40 bands in 54 pea materials were all 40 times, but band diversity index was 0.5, which was the maximum, showing that these bands had high variation frequency and strong polymorphism. Molecular mass of each subunit of water-solubility protein from pea was between 24.87 and 149.54 ku, in which electrophoresis band was concentrated in two regions: 24.71 – 50.41 ku of low molecular mass of protein subunit and 56.34 – 88.08 ku of high molecular mass of protein subunit. In 43 protein bands, the bands of proteins with molecular masses of 88.08, 68.25, 62.38, 44.76 and 29.89 ku were constant and obvious. These data showed that Tibet *P. sativum* germplasm had rich genetic difference at water-solubility protein level.

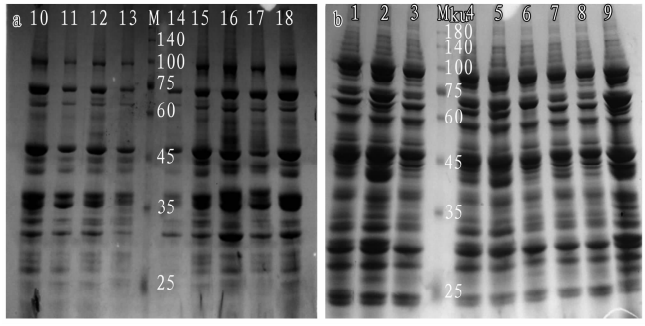

Fig. 1 SDS-PAGE electrophoresis of water-solubility (a) and salt-solubility (b) proteins from 54 pea materials

Table 3 Statistics of SDS-PAGE electrophoresis bands of water-solubility and salt-solubility proteins from 54 pea materials

No.	Water-solubility protein band	Salt-solubility protein band	No.	Water-solubility protein band	Salt-solubility protein band	No.	Water-solubility protein band	Salt-solubility protein band
1	30	12	19	27	14	37	34	16
2	30	11	20	24	13	38	26	17
3	28	14	21	30	10	39	32	9
4	30	15	22	34	12	40	22	15
5	26	15	23	27	11	41	30	12
6	28	9	24	29	12	42	29	15
7	28	14	25	31	17	43	27	16
8	27	16	26	32	10	44	32	6
9	28	10	27	26	12	45	29	8
10	29	15	28	26	14	46	27	14
11	30	12	29	33	14	47	30	14
12	33	15	30	30	18	48	30	14
13	32	12	31	36	13	49	30	12
14	32	7	32	34	11	50	28	10
15	26	17	33	34	9	51	34	13
16	27	16	34	34	13	52	30	17
17	32	14	35	30	9	53	25	13
18	22	16	36	31	12	54	27	14

3.4 Gel electrophoresis analysis of salt-solubility protein of pea

Via SDS-PAGE electrophoresis, 699 water-solubility protein bands were separated from 54 pea materials, in which there were 24 bands with different mobility ratios, with variation range of 6 – 17 bands, and average band number of each material was 20.4. Among them, band number of salt-solubility protein separated from No. 30 pea material was 18, which was the most, while band number of No. 44 pea material was 6 (Table 3, Table 5). Occurrence frequencies of 24 bands with different mobility ratios in 54 tested materials were between 18.5% and 100.0%, and diversity index was between 0 and 0.5. No. 6 band appeared in all tested materials, and its occurrence frequency was 100%, which was common band; occurrence frequency of No. 17 band was 18.5%, which was the minimum, and it only appeared in 10 pea materials. Diversity index of No. 13 and No. 22 bands was 0.5 and was the maximum, showing that the band had the most rich variation. Molecular mass of each subunit from salt-solubility protein of pea was between 24.85 and 91.24 ku. These data showed that Tibet *P. sa-*

tivum germplasm had rich genetic difference at salt-solubility protein level.

Table 4 Diversity analysis of water-solubility protein bands from 54 pea materials

Protein Band	Relative molecular mass	Occurrence times	Occurrence frequency//%	Gene diversity index (H)	Protein Band	Relative molecular mass	Occurrence times	Occurrence frequency//%	Gene diversity index (H)
1	149.54	44	81.48	0.48	23	44.93	17	31.48	0.30
2	138.95	51	94.44	0.34	24	44.76	43	79.63	0.48
3	125.37	54	100.00	0.00	25	43.49	50	92.59	0.39
4	116.28	54	100.00	0.00	26	42.31	47	87.04	0.43
5	107.76	54	100.00	0.00	27	41.27	41	75.93	0.50
6	96.66	6	11.11	0.11	28	40.34	12	22.22	0.20
7	88.08	54	100.00	0.00	29	38.02	44	81.48	0.49
8	82.56	39	72.22	0.50	30	37.35	37	68.52	0.49
9	82.07	35	64.81	0.48	31	35.67	18	33.33	0.31
10	78.28	33	61.11	0.47	32	34.08	23	42.59	0.36
11	75.36	22	40.74	0.36	33	33.61	45	83.33	0.47
12	69.96	40	74.07	0.50	34	33.15	40	74.07	0.50
13	68.25	31	57.41	0.47	35	32.55	33	61.11	0.47
14	65.68	31	57.41	0.45	36	31.97	15	27.78	0.26
15	62.38	42	77.78	0.50	37	31.12	44	81.48	0.48
16	60.08	30	55.56	0.44	38	29.89	54	100.00	0.00
17	59.55	44	81.48	0.48	39	28.45	52	96.30	0.26
18	57.19	23	42.59	0.37	40	27.19	39	72.22	0.50
19	56.34	8	14.81	0.13	41	25.84	14	25.93	0.24
20	50.41	35	64.81	0.48	42	25.47	52	96.30	0.26
21	49.31	54	100.00	0.00	43	24.71	53	98.15	0.19
22	46.12	31	57.41	0.46					

Table 5 Band analysis of salt-solubility protein

Protein Band	Relative molecular mass	Occurrence times	Occurrence frequency//%	Gene diversity index (H)	Protein Band	Relative molecular mass	Occurrence times	Occurrence frequency//%	Gene diversity index (H)
1	106.67	53	98.15	0.19	13	36.61	39	72.22	0.50
2	94.92	13	24.07	0.23	14	36.37	20	37.04	0.33
3	75.63	35	64.81	0.48	15	34.93	29	53.70	0.44
4	72.52	19	35.19	0.32	16	34.58	21	38.89	0.35
5	69.90	21	38.89	0.34	17	33.79	10	18.52	0.16
6	65.50	54	100.00	0.00	18	32.73	45	83.33	0.48
7	46.00	38	70.37	0.50	19	32.02	20	37.04	0.34
8	45.08	16	29.63	0.28	20	30.86	49	90.74	0.39
9	43.92	42	77.78	0.49	21	30.68	10	18.52	0.18
10	43.44	19	35.19	0.32	22	28.58	38	70.37	0.50
11	42.12	24	44.44	0.38	23	26.39	43	79.63	0.48
12	41.39	13	24.07	0.23	24	25.19	28	51.85	0.42

3.5 Clustering analysis Clustering analysis results of bands of water-solubility and salt-solubility proteins from 54 tested materials via UP-GMA (Fig. 2) showed that genetic similarity coefficients of all tested materials changed from 0.60 to 0.91, illustrating that genetic difference of *P. sativum* from different districts and counties of Tibet was larger. No. 23 and No. 24, No. 53 and No. 54 *P. sativum* materials had the maximum similarity coefficient (0.894), showing that the two groups of materials had small genetic difference and the most close relationship. At similarity coefficient GS value, 54 materials were all clustered into one class at 0.6 level and 8 classes at 0.696 level: class I had 1 material; class Ⅱ had 1 material; class Ⅲ had 8 materials, which was divided into two larger subgroups; class Ⅳ had 1 material; class Ⅴ had 3 materials; class Ⅵ had 13 materials, which was divided into two larger subgroups; class Ⅶ had 2 materials; class Ⅷ had 24 materials, which was the largest group. At GS level of 0.76, 54 materials could be divided into 7 subunits. By clustering analysis, it was found that local and traditional species at different altitudes of Tibet did not cluster together, but they had cross clustering, showing that genetic distance of proteins from the tested materials had unobvious relationship with geographical origin.

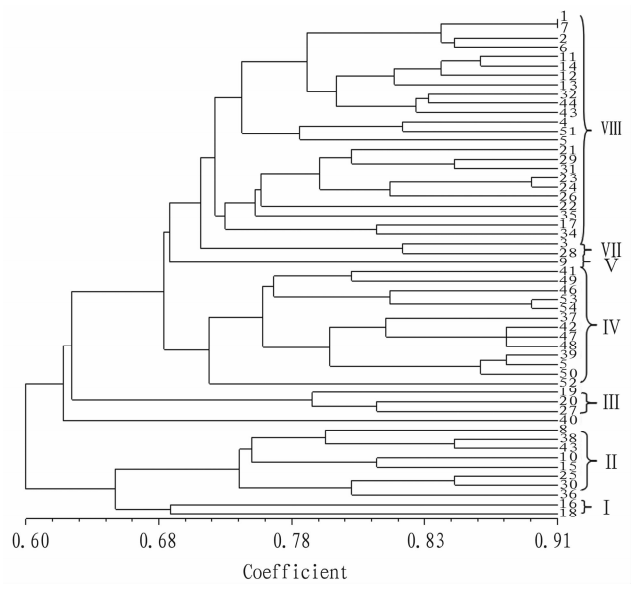

Fig. 2 Belt type of N-J phylogenetic tree of water-solubility and salt-solubility protein subunits from 54 pea materials

3.6 Genetic diversity difference among different germplasm materials at different altitudes According to altitude of *P. sativum* collection site, 54 *P. sativum* germplasm resources were divided into 4 ecological environmental groups: altitude lower than 3000 m, altitude of 3000 – 3500 m, altitude of 3500 – 4000 and altitude >4000 m. Genetic diversity of *P. sativum* resources at each altitude was shown as Table 6. Results showed that total band number of protein electrophoresis from 54 materials in 4 eco-environmental groups were 67; total polymorphic band was 60; percentage of polymorphic band was 89.55; total genetic diversity index(H) was 0.34; average genetic diversity index in the group was 0.27. Diversity indexes of 4 ecological groups had certain difference, and number of polymorphic band changed from 39 to 55, with the mean of 50.5; percentage of polymorphic band changed from 58.21 to 89.55; Shannon information index changed from 0.33 to 0.52, with the mean of 0.43; effective number of alleles (ne) changed from 1.41 to 1.62, with the mean of 1.52; the proportion of effective allelic variation(ne/na) changed from 0.86 to 0.89, with the mean of 0.87; gene diversity index (H) changed from 0.18 to 0.38, with the mean of 0.27. Gene diversity index among groups of 54 pea materials (Ht) was 0.3223; gene diversity index in the group (Hs) was 0.2929; average coefficient of gene variation (Gst) was 0.0913; gene flow of group estimation (Nm) was 4.7971. Comprehensively seen from each index, each diversity index was the maximum at the altitude of 2500 – 4000 m, showing that the most rich genetic variation of Tibet *P. sativum* germplasm was mainly distributed in ecological region of 3500 – 4000 m, followed by the region >4000 m.

Table 6 Genetic diversity analysis of *P. sativum* ecological group at different altitudes

Altitude//m	Number of accessions	Observed allele number (na)	Effective number of alleles (ne)	Gene diversity index (H)	ne/na	Shannon's information index (I)	The number of polymorphic loci	The percentage of polymorphic loci
<3000	4	1.58	1.41	0.23	0.89	0.33	39.00	58.21
3000 – 3500	8	1.72	1.50	0.18	0.87	0.41	48.00	71.64
3500 – 4000	33	1.90	1.62	0.35	0.85	0.52	60.00	89.55
>4000	9	1.82	1.57	0.31	0.86	0.46	55.00	82.09
Average		1.75	1.52	0.27	0.87	0.43	50.50	75.37
Total	54	1.90	1.60	0.34	0.84	0.50	60.00	89.55

Note: na showed observed number of alleles; ne showed effective number of alleles; H showed genetic diversity index; I showed Shannon's information index.

4 Discussions

The research on genetic variation in species is conducive to analysis of gene pool, breeding of new varieties, identification of relationship and similarity in species[7]. Using protein, DNA molecular marker, cytogenetics, biochemistry and phenotypic characteristics, genetic variation and variety identification were conducted[27-30]. In this paper, by analyzing genetic diversity of proteins from 54 Tibet *P. sativum*, it was found that gene diversity index of water-solubility protein was between 0 and 0.5, while gene diversity index of salt-solubility protein was between 0 and 0.5. It was clear that Tibet *P. sativum* germplasm had rich genetic variation. When sufficiently using these germplasm resources, it would efficiently broaden genetic background of *P. sativum* breeding, and provide the basis for improvement and breeding of *P. sativum* germplasm resource. According to band difference of water-solubility and salt-solubility proteins, 54 *P. sativum* germplasm resources were divided into 7 classes at the similarity coefficient of 0.76, and obvious regional distribution was not shown among different classes, which was consistent with Wang Lixia *et al.* [31-32]. Maybe it was related to special geographical environment in Tibet region. In long-time environment adaption process of species, the phenomenon of some properties tending to be same could appear, causing the intersection of property among different species[33]. Meanwhile in eco-environment with different altitudes, genetic diversity of *P. sativum* germplasm resources showed certain rule. The result was similar to the report of Wang Aihua et al[34]. on genetic diversity of Tibet wild barley by using RAPD and ISSR. Correlation analysis between geographical ecological factors and protein content of pea found that longitude showed significantly positive correlation with crude protein content of pea; latitude showed positive correlation with crude protein; altitude did not show correlation with altitude (Table 2). The results illustrated that crude protein of pea had certain increase from east Tibet to central Tibet to post Tibet; with latitude increased, crude protein content of pea had an increasing trend; with altitude increased, total protein of pea had a slightly declining trend. Gene inheritance diversity of 54 *P. sativum* germplasm resources (H) was 0.34, indicating that 34% of genetic variation occurred among all tested materials, while residu-

al 64% of genetic variation occurred in the group. Yeh *et al.* [35] thought Gst of 0.151 – 0.250 represented large genetic difference. Based on the range, 4 geographical ecological groups of Tibet *P. sativum* showed weaker differentiation. In this paper, the estimated gene flow Nm was 4.7971. Wright [36] thought that Nm lower than 1 indicated group started to differentiate because of genetic drift, while Mcdermott *et al.* [37] thought that Nm lower than 0.5 indicated wide genetic diversity was generated in the group because of gene drift. In the test, Nm was far higher than 1, indicating that Tibet *P. sativum* in different ecological groups was in differentiation process.

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