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EFFECTS OF ENVIRONMENTAL FACTORS ON THE POPULATION GENETIC DIVERSITY IN THE CHINESE ENDANGERED AND ENDEMIC MEDICINAL PLANT *NOTOPTERYGIUM INCISUM*

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Abstract

Correlation between environmental factors and genetic diversity of 23 populations throughout the distribution range of *Notopterygium incisum* was examined using nuclear internal transcribed spacer gene fragment (ITS) and chloroplast DNA (cpDNA) rpl20-rps12 intergenic spacer. A total of 635 nucleotides of ITS were sequenced from 343 individuals. 26 haplotypes were defined by 26 variable sites. A total of 772 nucleotides of rpl20-rps12were sequenced from 253 individuals. 30 haplotypes were defined by 26 variable sites. Based on cpDNA rpl20-rps12 region, the correlations between the haplotype diversity, nucleotide diversity and environmental factors were all non-significant. However, based on ITS sequences, a significant positive relationship was observed between haplotype diversity and ammonium nitrogen, annual average rainfall and elevation, as well as between nucleotide diversity and ammonium nitrogen and annual average rainfall. Synchronously, significant negative relationships were observed between haplotype diversity and latitude and variation coefficient of rainfall, as well as between nucleotide diversity and latitude. Latitude, elevation, annual average rainfall, variation coefficient of rainfall and ammonium nitrogen were the dominant factors that influenced the genetic diversity of *N. incisum*. The genetic diversity of *N. incisum* tended to increase in the environment with lower latitude, more rainfall, more ammonium nitrogen, higher elevation and a more stable climate.

Key words: ITS, cpDNA, Haplotype diversity, Nucleotide diversity.

Introduction

Biological phenotype, physiological characters are affected by the combination of their own genetic material and the environment. Due to the spatial and temporal changes of ecological factors, natural selection acts on the gene flow or the new genetic variation, which will produce different ecological genes, physiological, and phenotypic changes (Merrell, 1981). Scientists demonstrated the relationship between the variation of the molecular level of plant population and the environment (Nevo *et al*., 1998; Semagn *et al*., 2000), and they concluded that genetic diversity was related to environmental adaptation. Carroll *et al*., (2007) reports that once the gene flow is positively affected by complex ecological factors, it will promote the process of biological diversification in a short time scale. Therefore, the role of environmental factors cannot be ignored.

To date, previous studies of the effect of environmental factors on the genetic diversity reported that geographic variation of genetic diversity was associated with environmental factors such as latitude and longitude, altitude, temperature, precipitation and wind speed in blooming period (Huang *et al*., 2005; Zhao *et al*., 2006; Huang *et al*., 2007; Yang *et al*., 2008; Qu *et al*., 2012; Zhou *et al*., 2012). However, different study had different views, for example, the genetic diversity of *Cordyceps sinenesis* was positively correlated with latitude (Chen *et al*., 1997), while the genetic diversity of *Camellia japeniea* was correlated to longitude and was not affected by latitude (Li *et al*., 1996). Meanwhile, research on *Alectoris chukar* showed that population genetic diversity was negatively correlated to the annual precipitation mean (Randi *et al*., 1994). The genetic diversity of chukars of Longzhong Loess Plateauwas positively correlated with longitude, temperature and precipitation, and negatively correlated with latitude, altitude, the coefficient of variation of the temperature and precipitation, which indicated that the genetic diversity was higher in a stable environment (Huang *et al*., 2005).Therefore, further studies about the effect of environmental factors on the genetic diversity are required to uncover the environment role in the evolutionary process.

Notopterygium incisum, belonging to the family Umbelliferae, is an endangered and endemic perennial herb in China (Wang *et al*., 1996; Pu *et al*., 2000). It is distributed mainly in Qinghai, Gansu and Sichuan Province in China (Zhou *et al*., 2003). It grows among alpine shrubs, sub-alpine shrubs and alpine forest margins between 1700 m and 4500 m. As the traditional Chinese medicine, roots of *N. incisum* can be used medicinally for cold, headache, rheumatic pain and heart head blood-vessel disease (National Pharmacopoeia Committee, 2010). However, *N. incisum* is highly vulnerable to environmental changes. According to a previous report and our field survey, the habitats of this species have been severely deteriorated and fragmented largely due to anthropogenic activities (e.g., deforestation, over-exploitation), and its natural populations have shrunk to small sizes. This situation has been further aggravated by its unique biological traits, such as low seed germination

rates under natural conditions and low rate of natural regeneration. Therefore, it has been listed as the second protected wild plant in the revision of the "China's list of rare and endangered plants" in 1987. In 2005, the IUCN Red List of Threatened Species classified this species as vulnerable (Wang and Xie, 2005). Previous studies have mainly focused on its anatomy (Wang *et al*., 1996), systematics (Pu *et al*., 2000), ecology (Jiang *et al*., 2005) and pharmacognosy (Ji *et al*., 1997; Yang *et al*., 2006; Zhang and Yang., 2008), genetic diversity (Yang *et al*., 2015; Yang *et al*., 2015), little is known about the effects of environmental factors on the population genetic diversity. In this study, sequence variation of nrDNA and cpDNA rpl20-rps12 noncoding spacers were utilized to analyses the correlation between the population genetic diversity and environmental factors of *N. incisum.* The purpose of this investigation was to examine how the environmental factors influence the genetic diversity of *N. incisum* and to check whether there is any differences of the influence of environmental factors on different genes?

Materials and Methods

Plant materials: A total of 352 individuals, which corresponded to 23 populations of *N. incisum*, were sampled across 3 provinces in China; Qinghai, Gansu, and Sichuan Provinces (Table 2). This sampling strategy covered most of its presently known populations (Ma *et al.,* 2010). Individuals were sampled from the studied populations. Fresh leaves were collected, dried in a ziplock bag with silica gel, transported back to the laboratory and kept in -80°C freezer. The neighboring individuals were at least 50 m apart, so as to avoid re-sampling from the same individual. Corresponding to each population, parameters such as longitude, latitude and altitude were recorded for further analysis.

DNA extraction, PCR amplification and sequencing: Genomic DNA of single individual was extracted following the improved CTAB protocol (Doyle, 1991). The concentration and quality of DNA samples were checked on 0.8% agarose gel by a comparison with lambda DNA standards and with UV spectroscopy. The DNA samples were diluted to approximately 10 ng/μL with a 0.1% TE buffer (10mM pH 8.0 Tris-HCl; 1mM pH 8.0 EDTA) for the subsequent use.

Amplification of the nrITS regions, including the 5.8S gene, was conducted using the previously published universal primers ITS1 and ITS4 (White *et al.,* 1990). The rpl20-rps12 non-coding region of chloroplast DNA was amplified using a pair of universal primers (Hamilton, 1999). All PCR amplification was carried out in a total volume of 50 μL containing 10ng template DNA, 5 μl 10 × PCR reaction buffer, 5 μL $MgCl_2$ (25 mM), 5 μldNTP mix (10 mM), 10 pmole of each primer, and 2 U of Taq polymerase. Amplification of genomic DNA was performed on a MJ Thermal Cycler under the following reaction conditions: initial denaturation for 3 min at 95°C followed by 35 cycles of 30 sec at 94°C(denaturation), 30 sec min at 51-52°C (annealing temperature 51°C for nrITS and 52°C for rpl20-rps12, respectively), and 45 sec at 72°C (extension) with a final extension at72°C for 10 min. PCR products were resolved electrophoretically on 1.5% agarose gels run at 200 V in 1×TAE, visualized by staining with ethidium bromide, and photographed under ultraviolet light.

All successfully amplified DNA fragments were purified using a TIAN quick Midi Purification Kit according to the recommended protocol (TIANGEN), prior to sequencing. Then, PCR products were sequenced directly using an ABI3730XL automated DNA sequencer, applying the PCR-primers as sequencing primers. Sequence electropherograms were edited using Chromas version 2.33 (http://www.technelysium.com.au/chromas.html).

Sequence analysis

DNA sequences were aligned with Clustal X 1.81 (Thompson *et al*., 1997) and refined manually. Nucleotide diversity (π) and haplotype diversity (h) of each population were calculated using the program DNA Sequence Polymorphism (DnaSP) (Rozas *et al.,* 2003).

Environmental factors: The climatic factors (annual average temperature, average annual rainfall), terrain factor (Longitude, latitude and altitude) and soil factors (ammonium nitrogen, nitrate nitrogen, organic matter, total nitrogen, total phosphate, total potassium, and available phosphorous and potassium) were used as environment factors. Longitude, latitude and altitude were recorded corresponding to each sampled population. Soil was sampled from the studied populations. Five 10~20 cm soil layer soil samples including mixed amount, dried indoor air were collected. Then, 8 indicators of soil factors were measured. Climatic data over 30 years of sampling localities were collected from National Climatic Data Center (Table 3). Variation coefficient of temperature and rainfall were calculated from average temperature and average rainfall, respectively.

Statistical analysis

All statistical analyses were carried out by the SPSS statistical software package (SPSS Inc., Chicago, IL, USA), and relationships between variables were expressed by Pear's correlation coefficient treating all tests as two tailed.

Results

The nucleotide and haplotype diversity: A total of 635 bp of the nrDNA ITS region was resolved in 343 plants from 23 populations of *N. incisum* revealing 26 polymorphic sites (4.09%), of which 11 sites were parsimony informative. Among the 26 variable sites, 25 were caused by point mutations, and 1 were the results of insertions or deletions (indels) (Table 1). From these polymorphic sites a total of 26 haplotypes (N1-N26) were identified. Complete cpDNA rpl20-rps12 sequences were obtained from 253 individuals of *N. incisum*, each being 772bp in length. 26 variable sites were identified (3.37%) among 253 rpl20-rps12 sequences, including 11 parsimony informative sites and 15 singleton variablesites. 30 haplotypes were defined and their DNA sequences were submitted to GenBank (KU886103- KU886132).

Table 1. Variable sites of ITS sequences from 26 nrDNA haplotype of *N. incisum* were identified.

Mutation position	ITS1-ITS4																								
	13	42	43	44	66	108	126	183	186	187	192	199	207	413	414	423	502	523	524	556	568	597	613	618	628	629
N1	G	C	C	C	G	G	G	C	A	T	T	G	T	G	A	C	G	A	A	G	T	A	C	G	C	G
N2	G	C	C	C	G	G	A	C	A	T	T	G	T	A	G	C	A	A	A	G	T	A	C	G	C	G
N3	G	C	C	C	G	G	G	C	A	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N4	G	C	C	C	A	G	G	C	A	T	T	G	T	G	G	A	A	A	A	G	T	A	C	G	C	G
N5	G	C	C	C	A	G	G	C	C	G	C	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N6	G	C	C	C	A	G	G	C	C	G	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N7	G	C	C	C	A	G	G	C	C	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N8	G	C	C	C	A	G	G	C	C	T	T	G	T	G	G	A	A	A	A	G	T	A	C	G	C	G
N9	G	C	C	C	A	G	G	C	A	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N10	A	C	C	C	A	G	G	C	A	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N11	G	C	C	C	A	G	G	C	A	T	T	G	A	G	G	C	A	A	A	G	T	A	C	G	C	G
N12	G	C	C	C	A	A	G	C	A	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N13	G	C	C	C	A	G	G	G	A	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N14	G	C	C	C	G	G	G	G	A	T	T	G	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N15	G	C	C	C	G	G	G	C	A	T	T	A	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N16	G	C	C	C	A	G	G	C	A	T	T	A	T	G	G	C	A	A	A	G	T	A	C	G	C	G
N17	G	C	C	C	G	G	G	C	A	T	T	G	T	G	G	C	A	G	A	G	T	A	C	G	C	G
N18	G	C	C	C	G	G	G	C	A	T	T	G	T	G	G	C	G	G	A	G	T	A	C	G	C	G
N19	G	C	C	C	G	G	G	C	A	T	T	A	T	G	G	C	G	A	A	G	T	A	C	G	C	G
N20	G	C	C	C	A	G	G	C	A	T	T	G	T	G	G	C	G	A	A	G	T	A	C	G	C	G
N21	G	T	-	T	A	G	G	C	A	T	T	G	T	G	G	C	G	A	A	G	T	A	C	G	C	G
N22	G	C	C	T	A	G	G	C	A	T	T	G	T	G	G	C	G	A	A	G	T	A	C	G	C	G
N23	G	C	C	T	G	G	G	C	A	T	T	G	T	G	G	C	G	A	A	G	T	A	C	G	C	G
N24	G	C	C	C	G	G	G	C	A	T	T	G	T	G	G	C	G	A	A	G	T	A	C	G	C	G
N25	G	C	C	C	G	G	G	C	A	T	T	G	T	G	G	C	G	A	A	A	T	A	C	G	C	G
N26	G	C	C	C	G	G	G	C	A	T	T	G	T	G	G	C	G	A	T	G	C	T	T	T	T	T

Table 2. Details of the sample sites of *N. incisum.*

Population code	Location	Sample size		
		Total	ITS	rpl20-rps12
Q1	Ledu city, Qinghai Province	19	19	9
Q2	Huzhu city, Qinghai Province	15	15	10
Q3	Menyuan city, Qinghai Province	16	16	13
Q4	Maqin city, Qinghai Province	18	18	12
Q5	Banma city, Qinghai Province	21	21	12
G2	Minle city, Gansu Province	11	10	11
G3	Shandan city, Gansu Province	19	19	17
G4	Yuzhong city, Gansu Province	16	16	7
G5	Hezuo city, Gansu Province	17	17	16
G6	Zhuoni city, Gansu Province	14	13	14
G7	Lintan city, Gansu Province	11	11	10
G8	Maqu city, Gansu Province	13	11	13
G9	Luqu city, Gansu Province	16	11	16
S1	Ruoergai city, Sichun Province	14	14	10
S2	Hongyuan city, Sichun Province	17	17	9
S3	Aba city, Sichun Province	11	11	8
S4	Maerkang city, Sichun Province	14	14	11
S5	Jinchuan city, Sichun Province	16	16	11
S6	Daofu city, Sichun Province	19	19	10
S7	Ganzi city, Sichun Province	16	16	10
S8	Luhuo city, Sichun Province	15	15	7
S9	Danba city, Sichun Province	10	10	6
S10	Xiaojin city, Sichun Province	14	14	11
Total		**352**	**343**	**253**

Levels of haplotype and nucleotide diversity were investigated at both the nuclear ITS and chloroplast rpl20-rps12 across all the 31 natural populations of *N. incisum*. On the whole, levels of total haplotype diversity and overall nucleotide diversity detected at the locus rpl20-rps12 (h=0.6448; π=0.00144) were lower than at the locus ITS (h=0.658; π=0.00208) (Table 4). Genetic diversity varied largely from one population to another both at the locus ITS and at the locus rpl20-rps12. Haplotype diversity ranged from zero to 0.8897, and nucleotide diversity varied from zero to 0.008819 at the locus ITS (Table 4). Among the studied populations, the G7 population exhibited the most abundant nucleotide diversity (π=0.008819) and the S2 population exhibited the most abundant haplotype diversity (h=0.8897), while Q1, Q2, G2, G4, G5, G6 and G9 populations possessed the lowest levels of genetic variability (h=0.0000; π=0.0000). At the locus rpl20-rps12 haplotype diversity ranged from zero to 0.8889, and nucleotide diversity varied from zero to 0.009536. The S3 population exhibited the most abundant nucleotide diversity (π=0.009536) and the Q1, Q2 populations exhibited the most abundant haplotype diversity (h=0.8889), while Q3 and S1populations possessed the lowest levels of genetic variability (h=0.0000; π=0.0000) (Table 4).

The correlation between genetic diversity and environment factors: The results of environmental factors and its correlation with genetic diversity were listed in Table 3 and 5, respectively. The results showed that haplotype diversity and nucleotide diversity of the nrDNA ITS region of *N. incisum* increased with increasing ammonium nitrogen and annual average rainfall (p<0.05) (Table 5). Synchronously, haplotype and nucleotide diversity of the nrDNA ITS region showed negative correlation with increasing latitude (p<0.05) (Table 5). The haplotype diversity of the nrDNA ITS region was increased with increasing elevation and decreased with increasing VCR. Other correlations were non-significant (p>0.05).

The correlations between the haplotype diversity and nucleotide diversity of the cpDNA rpl20-rps12 region and the environmental factors were all non- significant (p>0.05) (Table 5).

Table 3. Environmental factors in each regions of *N. incisum* populations.

Population	Terrain factors			Climatic factors				Soil factors							
	Latitude (°N)	Longitude (°E)	Elevation (m)	AAT (°C) X ± SD	VCT	AAR (mm) X ± SD	VCR	Ammonium nitrogen mg/kg	Nitrate nitrogen mg/kg	Organic matter g/kg	Total nitrogen g/kg	Total phosphate g/kg	Total potassium g/kg	Available phosphorous mg/kg	Available potassium mg/kg
Q1	36.70	102.39	2935	1.17±0.59	0.5018	367.41±51.24	0.1395	25.33	17.06	287.40	8.70	0.85	11.8	3.45	469.88
Q2	36.93	102.38	2735	3.27±0.57	0.1755	352.46±47.15	0.1378	20.34	19.30	325.1	8.10	0.65	12.30	5.40	410.81
Q3	37.37	102.01	3216	-0.28±0.61	-2.1343	333.61±61.53	0.1844	35.45	12.92	217.60	6.10	0.64	15.60	3.74	241.06
Q4	34.61	100.22	4012	-0.33±0.64	-1.9570	483.45±58.26	0.1226	37.29	12.94	217.90	7.90	0.70	12.40	2.39	247.46
Q5	32.74	100.77	3600	1.95±0.57	0.2933	665.63±81.03	0.1217	30.24	11.51	193.80	6.70	0.92	17.10	2.85	479.44
G2	38.09	100.93	3408	-1.60±0.62	-0.3881	292.21±70.85	0.2424	22.25	36.16	175.42	7.32	0.64	19.68	8.68	262.50
G3	34.62	101.44	2765	0.31±0.64	2.0702	523.18±59.23	0.1132	16.71	49.08	188.71	6.95	0.68	16.86	8.30	362.50
G4	35.73	104.03	2958	0.80±0.61	0.7596	414.59±58.50	0.1411	17.36	3.00	108.56	3.88	0.72	17.97	5.26	250.00
G5	35.23	103.08	3164	1.58±0.58	0.3667	494.19±63.88	0.1293	14.70	41.85	116.17	5.79	1.09	19.85	9.07	387.50
G6	34.60	103.08	3025	2.76±0.58	0.2105	552.66±67.47	0.1221	22.15	29.64	159.80	6.39	1.09	17.66	8.99	250.00
G7	34.90	103.69	2946	2.46±0.56	0.2283	519.21±67.23	0.1295	25.48	49.91	231.82	8.38	1.03	17.90	8.81	287.50
G8	34.10	101.89	3683	0.058±0.65	11.3304	583.63±68.50	0.1174	33.73	52.07	357.16	13.06	1.03	13.83	20.64	375.00
G9	34.54	102.53	3683	0.073±0.65	8.9310	553.56±68.28	0.1233	17.26	36.02	154.45	6.98	1.14	16.61	6.64	150.00
S1	33.31	103.23	3757	1.13±0.60	0.5324	678.94±82.93	0.1221	34.08	11.50	211.04	9.44	1.29	17.44	9.20	300.00
S2	32.43	102.63	3963	0.74±0.55	0.7384	757.49±93.12	0.1229	34.72	4.06	152.40	6.73	1.11	20.01	5.64	170.00
S3	32.92	101.57	3493	2.26±0.60	0.2634	687.84±79.16	0.1511	35.30	7.95	125.06	5.60	0.91	20.76	7.50	437.50
S4	31.79	102.22	3693	1.78±0.59	0.3295	779.36±93.31	0.1197	32.11	9.05	115.65	5.91	0.87	26.42	6.32	165.00
S5	31.46	101.80	3808	1.83±0.47	0.2562	764.72±87.08	0.1139	34.87	5.15	146.67	5.21	0.88	18.60	8.47	275.00
S6	31.01	101.01	3846	3.77±0.53	0.1399	724.28±84.00	0.1160	35.105	3.99	145.70	4.98	0.85	17.38	4.91	350.00
S7	31.52	99.98	3886	3.67±0.56	0.1522	654.32±82.35	0.1259	28.745	16.25	261.43	13.91	1.44	14.42	16.13	312.50
S8	31.60	100.71	3445	3.63±0.51	0.1395	691.20±81.81	0.1184	34.96	8.52	181.65	7.50	1.10	18.92	10.36	562.50
S9	30.64	101.64	3844	1.48±0.48	0.3238	803.04±94.45	0.1176	34.335	5.98	159.72	8.03	1.40	18.84	8.38	180.00
S10	31.52	102.38	3654	2.03±0.47	0.2328	800.81±91.02	0.1137	34.22	6.78	141.16	6.46	1.06	21.46	6.72	275.00

Table 4. Haplotype diversity and nucleotide diversity of *N. incisum* populations.

Population code	ITS		rpl20 -rps12	
	***h*±SD**	**π±SD**	***h*±SD**	**π±SD**
Q1	0.0000 ± 0.0000	0.0000 ± 0.0000	0.8889 ± 0.0910	0.004279 ± 0.002754
Q2	0.0000 ± 0.0000	0.0000 ± 0.0000	0.8889 ± 0.0754	0.003535 ± 0.002316
Q3	0.1250 ± 0.1064	0.0001772 ± 0.001361	0.0000 ± 0.0000	0.0000 ± 0.0000
Q4	0.3660 ± 0.1124	0.004611 ± 0.002834	0.3182 ± 0.1637	0.000873 ± 0.000809
Q5	0.0000 ± 0.0000	0.0000 ± 0.0000	0.3182 ± 0.1637	0.000616 ± 0.000648
G2	0.0000 ± 0.0000	0.0000 ± 0.0000	0.7091 ± 0.1366	0.002550 ± 0.001763
G3	0.1053 ± 0.0920	0.001160 ± 0.001006	0.3309 ± 0.1426	0.000906 ± 0.000808
G4	0.0000 ± 0.0000	0.0000 ± 0.0000	0.2857 ± 0.1964	0.001123 ± 0.001024
G5	0.0000 ± 0.0000	0.0000 ± 0.0000	0.2417 ± 0.1353	0.000939 ± 0.000830
G6	0.0000 ± 0.0000	0.0000 ± 0.0000	0.8571 ± 0.0557	0.002765 ± 0.001839
G7	0.5636 ± 0.1340	0.008819 ± 0.005174	0.3778 ± 0.1813	0.000729 ± 0.000734
G8	0.4727 ± 0.1617	0.005784 ± 0.003573	0.7564 ± 0.0974	0.002467 ± 0.001691
G9	0.0000 ± 0.0000	0.0000 ± 0.0000	0.6417 ± 0.1032	0.009206 ± 0.005112
S1	0.7582 ± 0.0841	0.001938 ± 0.001467	0.0000 ± 0.0000	0.0000 ± 0.0000
S2	0.8897 ± 0.0542	0.004724 ± 0.002903	0.6389 ± 0.1258	0.001019 ± 0.000925
S3	0.8333 ± 0.0691	0.002529 ± 0.001811	0.4286 ± 0.1687	0.009536 ± 0.005693
S4	0.5055 ± 0.1581	0.001852 ± 0.001419	0.7273 ± 0.0679	0.002002 ± 0.001464
S5	0.7750 ± 0.0626	0.005630 ± 0.003379	0.4727 ± 0.1617	0.000906 ± 0.000836
S6	0.6842 ± 0.0917	0.005507 ± 0.003280	0.7333 ± 0.1199	0.002880 ± 0.001962
S7	0.1250 ± 0.1064	0.000197 ± 0.000356	0.6444 ± 0.1518	0.005003 ± 0.003107
S8	0.4667 ± 0.1478	0.003150 ± 0.002107	0.4762 ± 0.1713	0.000625 ± 0.000697
S9	0.6444 ± 0.1518	0.007174 ± 0.004355	0.7333 ± 0.1552	0.002534 ± 0.001921
S10	0.3956 ± 0.1588	0.001506 ± 0.001225	0.7818 ± 0.1073	0.002665 ± 0.001826
Total	0.658 ± 0.086	0.00208 ± 0.00023	0.6448 ± 0.032	0.00144 ± 0.00014

Table 5. Relationship coefficient between genetic diversity of *N. incisum* and environmental factors

Environmental factors	ITS		rpl20 -rps12	
	h	**π**	***h***	**π**
Latitude	-0.644**	-0.461*	-0.133	-0.032
Longitude	-0.027	-0.032	-0.108	-0.105
Elevation	0.575**	0.379	0.024	0.074
AAT	0.150	0.009	0.176	0.069
VCT	-0.221	-0.050	-0.319	-0.316
AAR	0.694**	0.402*	0.062	0.026
VCR	-0.396*	-0.297	-0.122	-0.019
Ammonium nitrogen	0.734**	0.525*	-0.063	-0.145
Nitrate nitrogen	-0.377	0.000	0.019	0.034
Organic matter	-0.195	0.057	0.203	-0.023
Total nitrogen	-0.030	0.114	0.193	0.095
Total phosphate	0.307	0.231	0.105	0.164
Total potassium	0.372	0.080	-0.024	-0.043
Available phosphorous	0.123	0.207	0.170	0.093
Availablepotassium	-0.141	-0.155	-0.045	-0.006

Discussion

In this study, correlation between environmental factors and genetic diversity of 23 populations of *N. incisum* throughout their distribution ranges was examined using nuclear ITS gene fragment and chloroplast DNA (cpDNA) rpl20-rps12intergenic spacer. At the locus ITS, the G7 population exhibited the most abundant nucleotide diversity (π=0.008819) and the S2 population possessed the most abundant haplotype diversity (h=0.8897). However, at the locus rpl20-rps12 the S3 population had the most abundant nucleotide diversity (π=0.009536) and the Q1, Q2 populations demonstrated the most abundant haplotype diversity (h=0.8889). The population genetic diversity level was inconsistent with two kinds of molecular markers, illustrating that the population genetic diversity of *N. incisum* was influenced by natural selection. In addition, a significant relationship was observed between nucleotide diversity and latitude, ammonium nitrogen and annual average rainfall, as well as between haplotype diversity and latitude, elevation, annual average rainfall and variation coefficient of annual average rainfall based on ITS sequences. However, non-significant relationship was observed between genetic diversity and environment based on cpDNArpl20-rps12 sequences. This is indicated that the ITS gene and rpl20-rps12 gene of *N. incisum* were affected by different environmental factors. The main reason is that natural selection can lead to different model of different gene genetic diversity, namely selection pressure has different role for different region (Hao *et al.*, 2004; Le Corre *et al.*, 2002; Xie *et al.*, 2018).

Climate is one of the most important drivers of local adaptation in plant species (Mosca *et al*., 2012); climate change has the potential to alter the morpho-biochemical and molecular processes of plants as well as others ecosystem processes (Jan *et el*., 2017, Gul *et al*., 2017; Jan *et al*., 2016; Avolio *et al*., 2013; Gilani *et al*., 2011; Kayani *et al*., 2011; Hamayun *et al*., 2010). In this study, genetic diversity of *N.incisum* is correlated positively and significantly with annual average rainfall, suggesting that rainfall is an important factor that influences the genetic diversity of *N. incisum*, i.e. the more rainfall, the higher genetic diversity of *N. incisum*, which is consistent with the results of genetic diversity of *Medicago ruthenica* (Li *et al*., 2014). The reason is that *N. incisum*, as a kind of alpine plants, mainly grows on the slope of alpine and subalpine habitat. *N. incisum*, suitable for cold and humid environment, may be limited by low rainfall, resulting in a decline in its genetic diversity.

Besides rainfall, the result based on ITS sequences showed that genetic diversity of *N. incisum* had a significant negative correlation with the latitude, suggesting that latitude had obvious influence on genetic diversity of *N. incisum*, which was the opposite to the result of *Cordyceps sinensis* (Chen *et al*., 1997). The correlation analysis showed a significant negative correlation between latitude and annual average rainfall (R^2=0.8608, p<0.01), indicating the higher latitude, the lower annual average rainfall. Low rainfall limits the population genetic diversity of *N. incisum*. In addition, the result also showed that haplotype diversity of *N. incisum* was correlated positively and significantly with altitude, which might be because of excessive excavation in recent years that made the optimal distributionof regional population of *N. incisum* to extinct. The survived *N. incisum* are distributed across the regions where altitudes are high (Zhou *et al*., 2003). The regions with high altitude were less affected by human disturbance, and the community and population structures were reasonable. The gene flow between populations was stable, which had high genetic diversity. However the regions with low altitude and with an easy access were frequently disturbed by human activities, which had caused a drastic decrease in the number of individuals and a reduction in genetic diversity (Zhou *et al*., 2016).

Variation rate of climate factors were a standard used to measure the stability of climate. The higher of the variation coefficient of annual average temperature, annual average rainfall, annual average relative humidity or annual average relative sunshine duration, the lower stable climate, and genetic diversity might also be correlated with stability of climate (Qu & Liu, 2012; Zhou *et al*., 2012; Huang *et al*., 2007; Huang *et al*., 2005; Vanhala *et al*., 2004). In this study, all measures of genetic diversity are correlated negatively with variation coefficients of rainfall (significant) and temperature (non-significant, but existing the trend), indicating that higher genetic diversity of *N. incisum* is observed in those regions with more stable climate (Table 3). Natural selection will tend to adapt a population to local environment conditions. Many kinds of genotypes can exist due to the environment pressure relaxation in more stable climatic areas, so genetic diversity is higher. In unstable climatic areas, however, only the individuals with genotypes adapted to changeable conditions can survive; thus, the population possesses lower genetic diversity (Zhou *et al*., 2012; Huang *et al*., 2007; Huang *et al*., 2005; Vanhala *et al*., 2004).

In previous studies, the different views on the relationship between genetic diversity and soil factors were detected (Wang *et al*., 2006; Hong *et al*., 2006; Hu *et al*., 2001; Li & Peng, 2001). However, their finding is not supported by our findings on the relationship between genetic diversity and soil factors in this study. A significant positive correlation was observed between genetic diversity and ammonium nitrogen, suggesting that the more of the content of ammonium nitrogen, the higher was genetic diversity of *N. incisum*, which indicated that ammonia nitrogen, might have played an important role in maintaining the genetic diversity of *N. incisum*. Form the above analysis; we can see that the relationship between plant species genetic diversity and soil factors may not be the same in different regions and different plants. Different plants have different genetic adaptation mechanisms for different habitats due to the difference in biological and ecological characteristics (such as life, life history traits), and have different ways to maintain its genetic diversity.

In summary, the genetic diversity of nrDNA ITS region of *N. incisum* was influenced by the environmental factors. That is a significant positive relationship was observed between haplotype diversity and ammonium nitrogen, annual average rainfall and elevation, as well as

between nucleotide diversity and ammonium nitrogen and annual average rainfall. Synchronously, significant negative relationships were observed between haplotype diversity and latitude and variation coefficient of rainfall, as well as between nucleotide diversity and latitude. Latitude, elevation, annual average rainfall, variation coefficient of rainfall and ammonium nitrogen was the dominant factors that influenced the genetic diversity of *N. incisum*. The genetic diversity of *N. incisum* tended to increase in the environment with lower latitude, more rainfall, more ammonium nitrogen, higher elevation and a more stable climate.

In this study, the research on the relationship between environmental factors and genetic diversity was preliminary tried, mainly related to topographic, climatic and soil factors. Comprehensively considering the various environmental factors which influenced the population genetic diversity, provided a theoretical basis for revealing the environmental adaptation mechanism of population of *N. incisum*. In fact, due to the complicated natural conditions, there are many other environment factors influencing the genetic diversity of *N. incisum*, such as site conditions, wind speed, the micro environment, and many others. In this study, only some environmental factors were analyzed, and the effect of slope, slope direction and so on were not completely eliminated which was one of the limiting factors of the field experiment design. In addition, only nuclear genes and parts of nuclear genes fragment were analyzed which were probably not comprehensive for revealing more details on population environment adaptability. To further reveal the correlation between genetic diversity of *N. incisum* and environment factors, more molecular markers and more environmental factors must be investigated to study the relationship between the genetic diversity and environmental adaptability.

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