Study on the Genetic Variation of *Polygonum multiflorum* Thunb species by sequences analysis of chloroplast *matK* gene

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Abstract: Object: To investigate the relationship between the variation of chloroplast *matK* gene sequence and the geographical distribution of *Polygonum multiflorum* Thunb. Method: PCR direct sequencing was applied to detect the *matK* sequences of 17 samples of *Polygonum multiflorum* Thunb collected from 15 populations. Results: The *matK* gene sequences were 1271 bp in length. Multiple sequence alignment showed that there were 12 variable sites in *matK* sequences and the *Polygonum multiflorum* Thunb smples were divided into four groups. Conclusion: These relatively variable sites in *matK* gene provided potential information for identifying *Polygonum multiflorum* species at the DNA level. The phylogenetic relationship of *Polygonum multiflorum* Thunb was well correlated with their geographical distribution.

Keywords: *polygonum multiflorum* Thunb; polygonaceae; phylogenetic relationship; *matK* gene; DNA sequencing

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何首乌叶绿体*matK*基因序列分析

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摘要：目的 通过DNA序列变异，初步研究何首乌品种的亲缘关系及其与地理分布的相关性。**方法** 采用PCR直接测序技术对来源于何首乌15个居群的17个样品的叶绿体*matK*基因进行测序分析研究。**结果** 何首乌的*matK*基因长度均为1271 bp，根据排序比较,何首乌17个样品间存在12个变异位点,用UPGMA（不加权的算术平均对群法）法构建的系统分支树将其分成四支。**结论** *matK*基因序列可变位点为何首乌品种的归并提供了分子依据，且品种的亲缘关系与地理分布呈良好的相关性。

关键词：何首乌；蓼科；亲缘关系；*matK*基因；DNA测序

Polygonum multiflorum Thunb is used for the treatment of anemia, swirl, deobstruent, antipyrotic, insomnia, amnesia and atheroma etc in Chinese traditional medicine [1,2]. In the Chinese Pharmacopoeia, *Polygonum multiflorum* Thunb is prescribed as the dried rhizome and root of *Polygonum multiflorum* Thunb from the family Polygonaceae, which contains lecithin, anthraquinones, 2,3,5,4'- tetrahydroxystilbene-2-O-*β*-D-glucoside and so on[3-4]. Recently, the demands of *Polygonum multiflorum* increase quickly and their safe use has become a critical issue because of the quality problems, their misuse and the confusions such as multiple sources, regional custom-herbs similarity in appearance. With the development of tissue culture propagation and asexual reproduction, new cultivars were achieved from some variants, different phenotypical individuals [5-6]. However, there is little information available about genetic relationships of cultivars, wilds and breeds.

Traditional authentication of *Polygonum multiflorum* Thunb relies on the investigation of sensory

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characteristics, such as its shape, color, texture and odor, the accuracy of which greatly depends on the examiner's experiences. The identification methods are mainly based on their histological and chemical characteristics, which are greatly influenced by the plant growth and environmental conditions. Molecular techniques are proved to be a more efficient means for evaluating genetic diversity in higher plant [7-10] than the above-mentioned methods. The purpose of the study was to provide a new method for efficiently clarifying the genetic relation of the cultivars and the wild *Polygonum multiflorum* Thunb by molecular techniques.

Materials and methods

Plant material

18 Specimens including *Reynoutria japonica Houtt* were examined. The plant specimens used in this study were identified by Prof. Xing F. W and deposited in the Liuhuaqiao hospital. The collection data was summarized in Table1.

Table 1 The used plant specimens and their accession numbers of *matK* gene sequences

Date of collection	Taxon	Locality	Statement	Code No.	GenBank accession no. of *matK* gene
2005.9.27	*Polygonum multiflorum* Thunb	Deqing County, Guangdong Prov., China,	Cult.	DX	EF153684
2005.9.28		Deqing County,Guangdong Prov., China,	Cult.	JD	EF153694
2005.9.29		Deqing County, Guangdong Prov., China,	Cult.	BK	EF159150
2005.10.06		Lianzhou County, Guangdong Prov., China,	Wild.	LZ	EF153692
2005.10.17		Suixi County, Guangdong Prov., China,	Cult.	SX	EF153697
2005.11.17		Arboretum of South China, Guangdong Prov., China,	Wild.	HN	EF153690
2005.11.22		Xianhu Arboretum of Shenzhen China , Guangdong Prov, China,	Wild.	XH	EF153687
2005.11.23		Arboretum of Dongguan, Guangdong Prov., China,	Cult.	DG	EF153696
2005.12.15		Jingxi County, Guangxi Prov., China	Wild.	JX	EF153686
2005.12.16		Debao County, Guangxi Prov., China	Wild.	DB1 DB2	EF153688 EF153689
2005.12.17		Guangxi Medicinal Plant Botanical Garden, Guangxi Prov., China	Wild.	GX	EF153695
2006.1.10		Xishuangbanna Tropical Botanical Garden, Yunnan Prov., China	Wild.	YN	EF153691
2006.4.7		Enshi County, Hubei Prov., China	Wild.	ES	EF153693
2006.4.9		Yichang City, Hubei Prov., China	Wild.	YC1 YC2	EF153685 EF153699
2006.9.12		Xinjian County, Jiangxi Prov., China	Cult.	JXJ	EF153698
2006.4.7	*Reynoutria japonica Houtt.*	Enshi County,Hubei Prov.,China	Wild.	ESH	EF153700

DNA extraction & PCR amplification of partial *matK* gene

Plant DNA was extracted by the use of the modified CTAB method [11]. PCR amplifications of the partial *matK* gene was performed using 10~100 ng of total DNA as a template in 50 μL of reaction mixture which consisted of 10 μL 5×Primer STAR^{TM} Buffer (Mg^{2+} plus), 0.2 mmol/L of each dNTP, 0.25 μmol/L of each primer, and 1.0 U of Primer STAR^{TM} HS DNA Polymerase (Takara, Japan). The primer of *matK* gene was as follows: *matK*AF (5'-CTA TAT CCA CTT ATC TTT CAG GAG T-3') and *matK*8R (5'-AAA GTT CTA GCA CAA GAA AGT CGA-3') [12]. PCR amplifications were carried out under the following cycling conditions: hot start at 98 ℃

for 10 s, followed by 30 cycles at 98 ℃ for 10 s, 45 ℃ for 30 s, and 72 ℃ for 2 min, and final extension at 72 ℃ for 15 min. Part volume of the resulted PCR product (1/10) was detected by 1.0% agarose gel electrophoresis and then the remaining part was sent to the Invitrogen Company for sequencing.

Sequencing and Phylogenetic Analysis

Each sequence was determined by Invitrogen Company and were assembled and aligned by the CLUSTAL X program (Version 1.83, Thompson et al, U.S.A.). *Matk* gene sequences were defined by a comparison with the sequence of *Rheum palmatum*. (GenBank no. AB115669). The phylogenetic tree was constructed by UPGMA using the MEGA program (Version 3.1, Sudhir et al, U.S.A.). Branch length was calculated by Kimura's two parameters method, *Reynoutria japonica Houtt.* of the family Polygonaceae as an outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of the topology of the consensus tree.

Results

Total DNAs with high quality were isolated from the samples (Fig.1). The PCR products of mat K gene showed a single band in the electrophoresis profile, approximately 1.2 kb in length. (Fig.2)

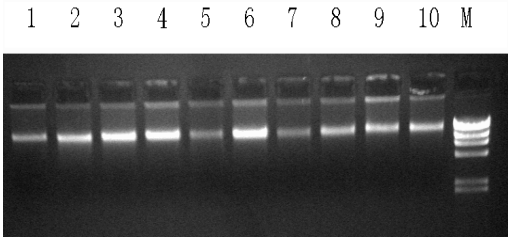

Fig.1 Agarose Gel Electrophoresis of DNA

M: λ—HindⅢ

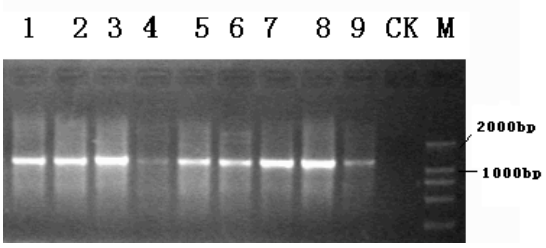

Fig.2 Agarose Gel Electrophoresis of PCR Products of *matK* Gene

M: DL2000

For the purpose of clarifying the phylogenetic relationships between the cultivated and wild *Polygonum multiflorum*, the sequence analysis of 17 species was conducted on *matK* gene region, which was easily amplified by PCR with the universal primers. The nucleotide sequence data of partial *matK* gene was deposited in GenBank nucleotide sequence databases with the accession numbers shown in Table 2.

Table 2 Comparison of the *matK* Gene among 17 Specimens of Polygonum multiflorum Thunb

Code No.	Nucleotide position											
	35	89	138	164	212	298	315	426	561	609	693	921
DX	C	C	A	A	C	C	C	G	G	C	C	A
JX	*	*	*	*	*	*	*	*	*	*	*	*
XH	*	*	*	*	*	*	*	*	*	*	*	*
DB1	*	*	*	*	*	*	*	*	*	*	*	*
DB2	*	*	*	*	*	*	*	*	*	*	*	*
HN	*	*	*	*	*	*	*	*	*	*	*	*
YN	T	*	*	*	A	*	A	A	*	T	T	*
LZ	*	A	*	T	A	*	*	*	*	*	*	*
ES	*	*	*	*	A	T	A	*	A	*	*	C
JD	*	*	*	*	*	*	*	*	*	*	*	*
GX	*	*	*	*	*	*	*	*	*	*	*	*
DG	*	*	*	*	*	*	*	*	*	*	*	*
SX	*	*	*	*	*	*	*	*	*	*	*	*
JXJ	*	*	*	T	A	*	*	*	*	*	*	*
BK	*	*	*	*	*	*	*	*	*	*	*	*
YC1	*	*	C	*	A	*	A	*	*	T	*	*
YC2	*	*	C	*	A	*	A	*	*	T	*	*

The partial *matK* gene was found to be 1271 bp in length. A comparison of the sequences of 17 specimens in table 2 showed 12 sites of nucleotide substitutions among seven specimens. DX, JX, XH, DB1, DB2, HN, JD, GX, SX, BK had a similar sequence. The nucleotide substitutions of YC1, YC2 existed at positions 138, 212, 315 and 609 with cytosine, adenine, adenine and thymine, respectively. The nucleotide substitutions in YN were 35(T), 212(A), 315(A), 426(A), 609(T) and 693(T) and those in LZ were 89(A) 164(T) and 212(A). In ES, the nucleotide substitutions were 212(A), 298(T), 315(A), 561(A) and 859(C) and those in JXJ were 164(T) and 212(A).

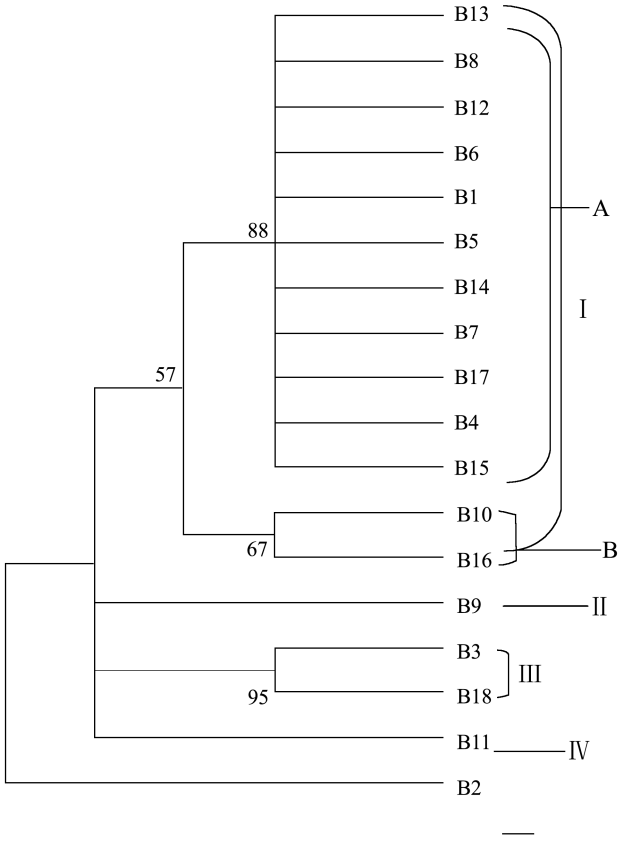

Fig.3 Phylogenetic Tree of Heshouwu Species Constructed on the Basis of Parial *matK* Gene using the UPGMA method

Branch length was calculated by Kimura's two parameters method, *Reynoutria japonica Houtt.* of the family *Polygonaceae* as an outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of the topology of the consensus tree

B1: DX; B2: ESH; B3:YC1; B4: JX; B5: XH; B6:DB1; B7:DB2; B8: HN; B9: YN; B10: LZ; B11: ES; B12: JD; 13: GX; B14: DG; B15: SX; B16: JXJ; B17: BK; B18:YC2

The phylogenetic tree reconstructed based on the *matK* gene sequences by UPGMA showed that the specimens came from Guangdong and Guangxi provinces were in clade Ⅰwhich were divided into two subclades in the phylogenetic tree (A and B). The wild *Polygonum multiflorum* Thunb of Yunnan belonged to the clade Ⅱ. Accordingly, specimens from Hubei province were classified in clade Ⅲ and Ⅳ. Clade Ⅰ, clade Ⅲ, subclade A and subclade B were well supported by high bootstrap values of 57%, 95%, 88% and 67%, respectively. (Fig.3)

Discussion

Molecular techniques provided an efficient means for evaluating genetic diversity in higher plant. Information of cp DNA variations had been widely used in pedigree analysis and population differentiation. Ribosomal RNA maturase (*matK*) was found in the noncoding region of the lysine-tRNA coding region in cpDNA. The evolution rate of this open reading frame region was comparatively fast [9]. *Polygonum multiflorum* Thunb countrywidly spread in China and even into some places in Japan.

The phylogenetic relation of *Polygonum multiflorum* based on the *matK* gene showed that all the examined specimens could be divided into four clades. The sequences of *matK* gene in the examined specimens had many differentiations, which correlated with the geographical distributions of the species.

Analysis of *MatK* gene sequences of 17 specimens (1271 bp) showed that no nucleotide substitution and variation were in the sequence of GX, HN, JD, DB1, DX, XH, DG, DB2, BK, JX and SX. However, plants growing in neighboring areas had the same or similar *matK* gene sequences, due to the maternal inheritance of the *matK* gene. Based on the analysis results of *matK* gene sequences, the samples collected from Guangdong and Guangxi provinces were classified in clade Ⅰwhich were divided into two subclades in the phylogenetic tree (A and B). The cultivated specimen from Deqing County of Guangdong province was the major geoherbs in Chinese traditional medicine with a more than 100-year cultivation history. A comparison of the other species from Guangdong with those from Guangxi provinces (including the cultivated and wild species) showed the

partial *matK* genes of those species were similar, which might be due to the same origin and the growth in the similar environment as Guangdong and Guangxi province were neighbors in geography. Comparison of the sequence of partial *matK* gene revealed that the key different nucleotides YN existed at position 35,426,693 of YN, 138 of YC1 and YC2, 298, 561 and 859 of ES and LZ, and 164 of JXJ. So *mat*K gene sequence provided valuable information for the identification of geographical subdivision of the plant.

Nucleotide sequencing of plastid *matK* gene could provide novel information for origin identification of *Polygonum multiflorum* species due to its higher mutation rate. In conclusion, each group of *Polygonum multiflorum* Thunb species was found to have a unique sequence pattern in the *matK* gene region, so that they could be easily distinguished at the DNA level and genetic variation of *matK* gene was influenced by the geographical distributions of species, which provided valuable information for identification of *Polygonum multiflorum* Thunb species breeds and for geographical subdivision of the plant.

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