Botanical Studies (2007) 48: 239-242.

Authentication of *Actinidia macrosperma* using PCR-RFLP based on *trn*K sequences

Yun-Peng ZHAO, Ying-Xiong QIU, Wei GONG, Jian-Hua LI, and Cheng-Xin FU*

Research Program for Resource Botany and Phytochemistry, Lab. of Systematic & Evolutionary Botany and Biodiversity, College of Life Sciences, Zhejiang University, Hangzhou, 310058, P. R. China

(Received September 1, 2006; Accepted December 14, 2006)

ABSTRACT. "Cat ginseng", the dried root of *Actinidia macrosperma*, is a famous traditional Chinese medicine against cancers in eastern China. The roots of some other species of the genus *Actinidia* such as *A. valvata* and *A. melanandra* have also been used as fake "cat ginseng", but they have little therapeutic value. However, identification of the original plants used to make the crude drugs is difficult, especially during the vegetation period. In this study we developed molecular markers for the determination and authentication of *A. macrosperma.* The restriction digestion of the chloroplast *trn*K region using endonucleases *Dde*I and *Dra*I produces two unique patterns in *A. macrosperma*, and in the *mat*K sequences, there are 11 sites unique to *A. macrosperma*. The molecular markers provide an effective and accurate identification and authentication of *A. macrosperma* in *Actinidia*.

Keywords: *Actinidia macrosperma*; *Actinidia valvata*; PCR-RFLP; Molecular marker.

INTRODUCTION

"Cat ginseng" is one of the most commonly used traditional Chinese medicines (TCM) for anti-tumor therapy in East China (Jiangsu New Medicine College, 1984). It first attracted people's interest as a catnip (Tucker and Tucker, 1988). Recently, three compounds, i.e. dihydronepetalactone, iridomyrmecin, and dihydroactinidiolide, have proven to be responsible for the interesting response (Zhao et al., 2006a). *Actinidia valvata* had been considered the source of "Cat ginseng" before our recent studies confirmed *A. macrosprma* as the genuine source (Zhao, 2006). It was not surprising that *A. macrosperma* and *A. valvata* had been mistaken for each other given the similarity in their morphological and anatomical characters (Liang, 1984). A phylogenetic analysis based on sequences of *mat*K and ITS also suggested that *A. macrosperma* and *A. valvata* were sister species (Li et al., 2002). Besides *A. valvata*, two more species (*A. melanandra*, *A. chinensis*) have been misidentified as *A. macrosperma*, and their roots have also been used as *"*cat ginseng*"* in China*.* The confusion may have compromised the therapeutic value of this TCM and jeopardized genuine resources for raw material production.

DNA markers, including PCR-RFLP and DNA sequences, are useful for the identification and standardization of TCM (Yang et al., 2001). The chloroplast *trn*D-*trn*T region digested by endonuclease *Hinf*I and *Dde*I has shown a specific pattern in *Sinopodophyllum hexandrum* that is distinct from species of *Dysosma*. We have used the PCR-RFLP data to differentiate *S. hexandrum* (Gong et al., 2006). The objective of this study was to differentiate *A. macrosperma* from other *Actinidia* species using PCR-RFLP data and DNA sequences of chloroplast *trn*K region.

MATERIALS AND METHODS

Seventeen samples were used in this study representing ten species and four sections of *Actinidia* (Table 1), and their voucher specimens are deposited in the Herbarium of Zhejiang University (HZU). DNAs were extracted from silica-gel dried leaves using a modified CTAB method (Doyle, 1991). PCR (polymerase chain reaction) was conducted using primers W83040 (5′-GGG TTG CCC GGG ACT CGA AC-3′) and W83041 (5′-CAA CGG TAG AGT ACT CGG CTT TTA-3′) for *trn*K (Demesure et al., 1995). A 50 μl PCR amplification run contained 50 ng template DNA, 5 μl 10×buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primer and 2.0 U *Taq* DNA polymerase. The PCR reaction was performed using a PTC-100 PCR DNA Thermal Cycler (Bio-Rad, USA), and the cycling program included an initial 4 min denaturation at 94°C, which was followed by 35 amplification cycles with 1 min denaturation at 94°C, 1 min annealing at 65°C, and a 1.5 min extension at 72°C. A final extension step of 7

*Corresponding author: E-mail: cxfu@zju.edu.cn; Phone: +86-571-8820-6607; Fax: +86-571-8643-2273.

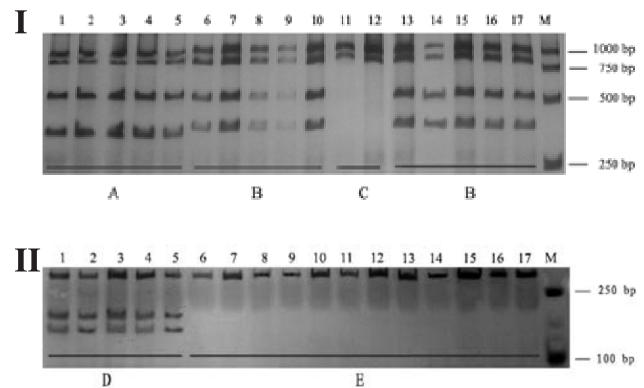
min at 72°C was used following the final circle. The PCR products were run in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Sequences of *mat*K (major part of *trn*K) were downloaded from GenBank, and subsequently analyzed using Sequencher Software 4.0.5 for restriction maps. The PCR products of the *trn*K region were digested with the three restriction endonuleases *Dde*I, *Dra*I, and *Rsa*I according to the manufacturer's instructions (Promega Corporation, USA). The resulting restriction digests were separated on a 6% denaturing polyacrylamide gel and were silver-stained (Brant et al., 1991). The size of the fragments was estimated by comparison with a DL-2000 DNA ladder (TaKaRa Biotechnology [Dalian] Co., Ltd). The sequences were aligned using CLUSTAL software 1.81 (Chenna et al., 2003).

RESULTS AND DISCUSSION

The *trn*K region was about 2580 base pairs (bp) long for all tested species. Three restriction patterns were obtained using *Dde*I (A, B, and C in Figure 1-I). The A pattern was unique to *A. macrosperma*; the C pattern appeared in *A. arguta* var. *purpurea* and *A. melanandra*; and the B pattern was shared by the other species (*A. valvata*, *A. polygama*, *A. maloides*, *A. callosa* var. *discolor*, *A. hemsleyana*, *A. eriantha*, *A. chinensis*). Two restriction patterns were observed using *Dra*I (D and E in Figure 1-II). The pattern D with two specific fragments of 200 bp and 170 bp was specific to *A. macrosperma*. The digestion pattern of the remaining species (E) was different from that of *A. macrosperma*. The restriction pattern of the *trn*K region with *Rsa*I in *A. macrosperma* was different from that shown in *A. valvata*, but was identical to that of most remaining species (Figure 2). At the DNA sequence level, *A. macrosperma* differed from other species at 11 sites while *A. valvata* and *A. polygama* were identical in all sites except for one (1239 bp).

The results of *mat*K (major part of *trn*K) sequence alignment (Table 2) indicate site mutation includes transversion and transition of a single base, and no base



Figrue 1. 6% silver-stained polyacrylamide gels showing PCR-RFLP profiles of cpDNA *trn*K with *Dde*I and *Dra*I in *A. macrosperma* and closely related species. I: *Dde*I digestion; II: *Dra*I digestion. 1-5, *A. macrosperma*; 6-9, *A. valvata*; 10, *A. polygama*; 11, *A. arguta* var. *purpurea*; 12, *A. melanandra*; 13, *A. maloides*; 14, *A. callosa* var. *discolor*; 15, *A. hemsleyana*; 16, *A. eriantha*; 17, *A. chinensis*; M, DL-2000 DNA ladder. A, B, C, D, E: different digestion patterns.

Table 1. Plants used in this study and their accession numbers in Genbank.

No.	Infra-genus classification	Species	Locality	Specimen No.	Accession No.
1	Sect. *Leiocarpae*	*A. macrosperma*	Hangzhou, Zhejiang	A2004057	AF322621
2		*A. macrosperma*	Fuyang, Zhejiang	A2003005	
3		*A. macrosperma*	Fuyang, Zhejiang	A2003006	
4		*A. macrosperma*	Fuyang, Zhejiang	A2003022	
5		*A. macrosperma*	Wuhan, Hubei	A2004071	
6		*A. valvata*	Wuning, Jiangxi	A2003020	AF322602
7		*A. valvata*	Linan, Zhejiang	A2004066	
8		*A. valvata*	Mt. Tianmu, Zhejiang	A2004078	
9		*A. valvata*	Wuhan, Hubei	A2003010	
10		*A. polygama*	Wuhan, Hubei	A2004072	AF322601
11		*A. arguta* var. *purpurea*	Mt. Baishanzu, zhejiang	A2003001	-
12		*A. melanandra*	Wuning, Jiangxi	A2003022	AF322600
13		*A. maloides*	Mt. Emei, Sichuan	A2004047	-
14	Sect. *Maculatae*	*A. callosa* var. *discolor*	Linan, Zhejiang	A2004065	AF322614
15	Sect. *Strigosae*	*A. hemsleyana*	Mt. Baishanzu, Zhejiang	A2003002	AF322608
16	Sect. *Stellatae*	*A. eriantha*	Mt. Baishanzu, Zhejiang	A2003003	AF322616
17		*A. chinensis*	Wuning, Jiangxi	A2003021	U61324

Table 2. Variable sites of cpDNA *mat*K sequence from *Actinidia* spp.

Species	bp																			
	25	31	122	256	361	369	600	603	676	761	778	782	796	918	1031	1109	1239	1407	1408	1453
A. macrosperma	**A**	G	T	C	**A**	**C**	**T**	A	**T**	**T**	**T**	**G**	A	G	C	A	T	**G**	**T**	**A**
A. valvata	G	**A**	**G**	**G**	C	A	C	**C**	C	G	G	T	**G**	**A**	**A**	**C**	**C**	A	G	G
A. polygama	G	**A**	**G**	**G**	C	A	C	**C**	C	G	G	T	**G**	**A**	**A**	**C**	T	A	G	G
A. melanandra	G	G	T	C	C	A	C	A	C	G	G	T	A	G	C	A	T	A	G	G
A. callosa var. *discolor*	G	G	T	C	C	A	C	A	C	G	G	T	A	G	C	A	T	A	G	G
A. hemsleyana	G	G	T	C	C	A	C	A	C	G	G	T	A	G	C	A	T	A	G	G
A. eriantha	G	G	T	C	C	A	C	A	C	G	G	T	A	G	C	A	T	A	G	G
A. chinensis	G	G	T	C	C	A	C	A	C	G										

insertions or deletions were found in the studied squences. There are eleven mutative sites in the *mat*K sequence of *A. macrosperma*. The mutative sites of *A. valvata* and *A. polygama* are almost identical, only differing at 1239 bp, at which thymine was transited into cytosine in *A. valvata*.

PCR-RFLP has been applied successfully to the discrimination of medicinal plants and their adulterants (*Panax* spp., Ngan et al., 1999; *Codonopsis* spp., Fu et al., 1999; *Dendrobium chrysanthum* and *D. fimbriatum*, Zhang et al., 2005; *Fritillaria pallidiflora*, Wang et al., 2005; *Salvia divinorum*, Bertea et al., 2006; *Sinopodophyllum hexandrum*, Gong et al., 2006). In this study, our samples represent all four sections of *Actinidia* (Liang, 1984), most species of Sect. *Leicarpae*, to which *A. macrosperma* belongs, and different populations of *A. macrosperma* and *A. valvata*. Patterns A and D are present in all samples of *A. macrosperma*, suggesting that both *Dde*I and *Dra*I can be applied to the differentiation of *A. macrosperma* from its related species. In addition, sequences of the *mat*K gene in *A. macrosperma* are distinctive at eleven sites. Therefore, the chloroplast DNA markers developed here can be used in the effective and accurate identification and authentication of *A. macrosperma* and contribute to its quality control and raw material production.

Acknowledgements. The authors are grateful to Wuhan Botanical Garden, Chinese Academy of Sciences for kindly providing three *Actinidia* samples. This work was supported by Science & Technology Bureau of Zhejiang Province (2006C13077) and China Postdoctoral Science Foundation (20070411194).

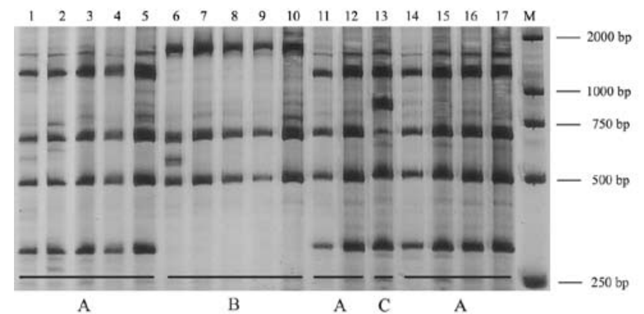


Figure 2. 6% silver-stained polyacrylamide gels showing PCR-RFLP profiles of cpDNA *trn*K with *Rsa*I in *A. macrosperma* and its closely related species. 1-5, *A. macrosperma*; 6-9, *A. valvata*; 10, *A. polygama*; 11, *A. arguta* var. *purpurea*; 12, *A. melanandra*; 13, *A. maloides*; 14, *A. callosa* var. *discolor*; 15, *A. hemsleyana*; 16, *A. eriantha*; 17, *A. chinensis*; M, DL-2000 DNA ladder. A, B, C: 3 different digestion patterns.

LITERATURE CITED

Bertea, C.M., P. Luciano, S. Bossi, F. Leoni, C. Baiocchi, and C. Medana. 2006. PCR and PCR-RFLP of the 5S-rRNA-NTS region and salvinorin A analyses for the rapid and unequivocal determination of *Salvia divinorum*. Phytochemistry **67:** 371-378.

Brant, J.B., C.A. Gustavo, and M.G. Peter. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem. **196:** 80-83.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T.J. Gibson, and D.G. Higgins. 2003. Multiple sequence alignment with the Clustal series of programs. Nucl. Acids Res. **31:** 3497-3500.

Doyle, J.J. 1991. DNA protocels for plants-CTAB total DNA isolation. *In* G.M. Hewitt and A. Johnston (eds.), Molecular Techniques in Taxonomy. Berlin: Springer Verlag, pp. 283-294.

Demesure, B., N. Sodzi, and R.J. Petit. 1995. A set of universal primers for amplification of non-coding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. **4:** 129-131.

Fu, R.Z., J. Wang, Y.B. Zhang, Z.T. Wang, P.P. But, and N. Li. 1999. Differentiation of medicinal *Codonopsis* species from adulterants by polymerase chain reaction-restriction fragment length polymorphism. Planta Med. **65:** 648-650.

Gong, W., C.X. Fu, Y.P. Luo, and Y.X. Qiu. 2006. Molecular

Identification of *Sinopodophyllum hexandrum* and *Dysosma* Species using cpDNA Sequences and PCR-RFLP Markers. Planta Med. **72:** 650-652.

Jiangsu New Medicine College. 1984. Dictionary of Chinese Traditional Medicines. Shanghai: Shanghai Science and Technology Press, 2205.

Li, J.Q., H.W. Huang, and T. Sang. 2002. Molecular phylogeny and infrageneric classification of *Actinidia* (Actinidiaceae). Syst. Bot. **27:** 408-415.

Liang, C.F. 1984. *Actinidia*. *In* K.M. Feng (ed.), Flora Reipublicae Popularis Sinicae. Beijing: Science Press, pp. 196-268.

Ngan, F., P. Shaw, P. But, and J. Wang. 1999. Molecular authentication of *Panax* species. Phytochemistry **50:** 787-791.

Tucker, A.O. and S.S. Tucker. 1988. Catnip and the catnip response. Econ. Bot. **42:** 214-231.

Wang, C.Z., P. Li, J.Y. Ding, G.Q. Jin, and C.S. Yuan. 2005. Identification of *Fritillaria pallidiflora* using diagnostic PCR and PCR-RFLP based on nuclear ribosomal DNA internal transcribed spacer sequences. Planta Med. **71:** 384-386.

Yang, M., D.M. Zhang, J.Q. Liu, and J.H. Zheng. 2001. A molecular marker that is specific to medicinal rhubarb based on chloroplast *trn*L/*trn*F sequences. Planta Med. **67:** 784-786.

Zhang, T., L.S. Xu, Z.T. Wang, K.Y. Zhou, N. Zhang, and Y.F. Shi. 2005. Molecular identification of medicinal plants: *Dendrobium chrysanthum*, *D. fimbriatum* and their morphologically allied species by PCR-RFLP analyses. Acta Pharm. Sin. **40:** 728-733.

Zhao, Y.P. 2006. Studies on pharmarcognosic identification and relationship between fingerprints and bioactivity of *Actinidia macrosperma* and its related species [Dissertation]. Hangzhou: Zhejiang University.

Zhao, Y.P., X.Y. Wang, Y. Lu, Z.C. Wang, C.X. Fu, and S.Y. Chen. 2006a. Essential oil of *Actinidia macrosperma*, a catnip response kiwi endemic to China. J. Zhejiang Univ. Sci. B **7:** 708-712.

Zhao, Y.P., Y.J. Zhong, Y. Lu, Z.C. Wang, S.Y. Chen, and C.X. Fu. 2006b. Pharmacognostic discrimination on stems of nine *Actinidia* species. Chin. Pharm. J. **41:** 1053-1057.

抗腫瘤中草藥大籽獼猴桃及其形態相似種的 PCR-RFLP 鑒別

趙雲鵬　邱英雄　龔　唯　李建華　傅承新

浙江大學生命科學學院植物系統進化與生物多樣性實驗室 植物資源學與植物化學研究團組

貓人參是獼猴桃屬植物大籽獼猴桃（*Actinidia macrosperma*）的根莖，是中國華東地區常用的抗腫瘤中草藥。同屬的鑷合獼猴桃（*A. valvata*）、黑蕊獼猴桃（*A. melanandra*）等常被混用作貓人參，嚴重混淆了貓人參正品的種源，危害了貓人參的藥效。然而大籽獼猴桃及其混淆品原植物的鑒別較困難，營養生長期尤難鑒別。本文採用 PCR-RFLP 法，用限制性內切酶 *Dde*I、*Dra*I 消化葉綠體 *trn*K 序列，獲得了大籽獼猴桃的特異性消化式樣，可作為鑒別貓人參正品種源大籽獼猴桃的分子標記，用於貓人參的 GAP 生產。進一步的序列比對結果表明，供試材料序列位點變異均爲單一鹼基顛換或轉換，不存在鹼基或片段的插入或缺失，大籽獼猴桃有 11 個點突變位點。

關鍵詞：大籽獼猴桃；鑷合獼猴桃；PCR-RFLP；分子標記。