Genotyping and species identification of *Fritillaria* by DNA chips

TSOI Pui-yan[1] , WOO Hok-sin[1] , WONG Man-sau[2] , CHEN Shi-lin[2] ,
FONG Wan-fung[1] , XIAO Pei-gen[2,3*] , YANG Meng-su[1*]

(1 . *Applied Research Centre for Genomic Technologies , and Department of Biology and Chemistry , City University of Hong Kong , Kowloon , Hong Kong , China ; 2 . Department of Applied Biology and Chemical Technology Hong Kong Polytechnic University , Kowloon , Hong Kong , China ; 3 . Institute of Medicinal Plant Development , Chinese Academy of Medical Sciences and Peking Union Medical College , Beijing 100094 , China*)

Abstract: **Aim** To investigate the genetic polymorphism of several species of *Fritillaria* and to develop a DNA chip for the genotyping and identification of the origin of various species of *Fritillaria* at molecular level . **Methods** Genomic DNA from bulbs of several *Fritillaria* species was extracted and the polymorphisms of the D2 and D3 regions inside the 26S rDNA gene were identified by direct sequencing . Oligonucleotide probes specific for these polymorphisms were designed and printed on the poly-lysine coated slides to prepare the DNA chip . PCR products from the *Fritillaria* species were labeled with fluorescence by incorporation of dye-labeled dideoxyribonucleotides and hybridized to the immobilized probes on the chip . **Results** The polymorphisms were used as markers for discrimination among various species . Specific oligonucleotide probes were designed and immobilized on a DNA chip . Differentiation of the various *Fritillaria* species was accomplished based on hybridization of fluorescent labeled PCR products with the DNA chip . **Conclusion** The results demonstrated the reliability of using DNA chips to identify different species of *Fritillaria* , and the DNA chip technology can provide a rapid , high throughput tool for genotyping and quality assurance of the plant species verification .

Key words: DNA chip ; genotyping ; *Fritillaria* ; species identification
CLC number: R965 **Document code**: A **Article ID**: 0513 - 4870(2003)03 - 0185 - 06

DNA芯片技术用于贝母的基因分型和种类鉴别

蔡佩欣[1] , 胡学善[1] , 黄文秀[2] , 陈士林[2] , 方宏勋[1] , 肖培根[2,3] , 杨梦苏[1]

(1 . 香港城市大学 基因组科技应用研究中心 ; 2 . 香港理工大学 应用生物学与化学技术系 ;
3 . 中国医学科学院 ,中国协和医科大学 药用植物研究所 , 北京 100094)

摘要 : 目的 通过对贝母几个种遗传多态性的研究来开发在分子水平上用于鉴别贝母基因型及不同种类的 DNA 芯片技术 。方法 用 PCR 扩增法和 DNA 直接测序法确定核苷酸多态性 ,用 DNA 芯片进行基因检测 。结果 首先提取来自多种贝母根茎的基因组 DNA ,对 26S rDNA 基因 D2 与 D3 区的多态性片段进行扩增和测序 ,然后将不同种属多态性片段的特异性寡核苷探针点置于经多聚赖氨酸处理包被的芯片 。用来自不同种贝母的荧光素标记的 PCR 产物与 DNA 芯片进行杂交 ,可在芯片特定位置检测到不同种贝母的荧光信号 。结论 本研究显示 DNA 芯片技术可为植物种属的验证与质量控制提供一种快速 ,高通量的检测工具 。

关键词 : DNA 芯片 ; 基因型检测 ; 贝母 ; 种类鉴别

Received date : 2002-03-22 .
Foundation item : City University of Hong Kong (9600001) .
* Corresponding author
Xiao PG Tel : 86 - 10 - 63011294 , Fax : 86 - 10 - 63038753 ,
E-mail : XiaoPG@public .bta .net
Yang MS Tel :86 - 852 - 27887797 , Fax :86 - 852 - 27887406 ,
E-mail : bhmyang@cityu .edu .hk

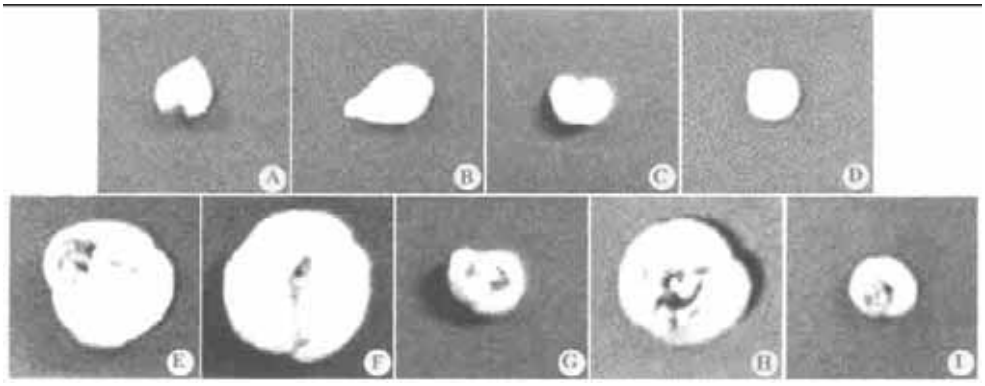
Beimu refers to the bulb of various species of the Lily Family (*Fritillaria liliaceae*) . It is traditionally used as an antitussive agent in Chinese herbal medicine[1] . This herb causes bronchodilation and inhibition of mucosal and salivary secretions . *Fritillaria cirrhosa* is one source of medicinally effective Beimu with high market value . There are almost 100 species of *Fritillaria* in the world ,

many of which show similar appearance but variable medicinal properties and market values[1] . For example , due to lack of adequate quantity of *Fritillaria cirrhosa* , some other species such as *Fritillaria thunbergii* have become extensively utilized as substitutes . Because it is difficult to distinguish different species and varieties of *Fritillaria* available in the market based on their morphologies , it is important to develop methods for the identification of the origin of various species of *Fritillaria* at molecular level .

Previous studies described the use of 5S rRNA spacer domain[2] and 18S ribosomal RNA genes[3] as genetic markers for identification of different species of *Fritillaria* . However , the existence of inter-spacer polymorphism of 5S rRNA spacer domain in the same species and the few phylogenetically informative characters of 18S rRNA gene[4] prevent the genotyping of individual species . Recently , the phylogenetic potential of highly conserved 26S rDNA (≈3.4 kb) sequences in plants has been demonstrated[5] . The twelve domains (D1 - D12) inside the 26S rDNA gene are highly variable among different species . The diversity of these domains can be used for species identification purpose . In this study , the D2 and D3 regions (≈320 bases) of the 26S rDNA gene from several *Fritillaria* species were selected for identification of polymorphisms among the species . A DNA chip consisting of a microarray of immobilized oligonucleotide probes on a glass slide was developed for the detection of these single nucleotide polymorphisms[6-9] .

Materials and methods

Plant materials and DNA polymorphisms The dried bulbs of nine species of *Fritillaria* standards were used in the study , including *F . cirrhosa* , *F . thunbergii* var . *chekiangensis* , *F . ussuriensis* , *F . delavayi* , *F . pallidiflora* , *F . unibracteata* (three variants) , and an unidentified species (Figure 1) . Genomic DNA was extracted according to published procedures[2] . The primers used in the amplification of D2 - D3 domain of 26S rRNA gene for *Fritillaria* were N-nc26S2 (forward primer : 5′-GAG TCG GGT TGT TTG GGA-3′) and 950 rev (reverse primer : 5′-GCT ATC CTG AGG GAA ACT TC) . Cycling conditions consisted of an initial 5 min at 94 ℃ followed by denaturing at 94 ℃ for 1 min , annealing at 55 ℃ for 1 min , elongation at 72 ℃ for 2 min with 30 cycles and final extension for 5 min at 72 ℃ . For sequencing reactions , about 50 ng of purified PCR products were mixed with 3.2 pmol of the N-nc26S2 primer and a solution containing dye-labeled terminators (ddNTPs) and DNA polymerase (AmpliTaq , Applied Biosystems) . The reaction condition consisted of a thermal cycle of 96 ℃ for 30 s , 50 ℃ for 15 s and 60 ℃ for 4 min with a total run of 25 cycles . The purified DNA products were then sequenced by using DNA automatic sequencer ABI377 (PE Applied Biosystems) .



A: *F . cirrhosa* ; B: *F . unibracteana* (Jianbei) ; C: *F . thunbergii* var . *chekiangensis* ; D: *F . ussuriensis* ; E: *F . delavayi* ; F: *F . pallidiflora* ; G: *Fritillaria* (unidentified) ; H: *F . unibracteata* (Tibet) ; I: *F . unibracteata* (pearl)

Figure 1 The simple morphologies of different species of *Fritillaria* available in the commercial market and used in this study

Fabrication of DNA chips Six oligonucleotide probes with different lengths (14 - 23 nt) were designed to be complementary to their particular amplicons . The probes are F1291O-CP (5′-CAC TCT TTG ACT CTC TTT TCA AA-3′) , F56-CP (5′-CTT ACT CAA ATC CAT CCG AA-3′) , F6-CP (5′-CGA CGA TCG ATT TGC A-3′) , F7-CP (5′-AGG CCT TTG ACC GG-3′) , F8-CP (5′-CCA GTG CTT CCC TTT CA-3′) and FRIT-CP (5′-CCT TGG TCC GTG TTT C-3′) . Each of the probes was mixed with a spotting solution[10] and then printed twice

on the poly-lysine glass slide by an array printer (SPBIO, Hitachi).

Fluorescence labeling of PCR products and hybridization The purified PCR products were labeled with TAMRA-labeled ddCTP with DNA polymerase in the presence of several primers corresponding to the polymorphisms: F56 (5′-TTC GGA TGG ATT TGA GTA AG-3′), F6 (5′-TGC AAA TCG ATC GTC G-3′), F7 (5′-CCG GTC AAA GGC CT-3′), F8 (5′-TGA AAG GGA AGC ACT GG-3′), F12910 (5′-TTT GAA AAG AGA GTC AAA GAG TG-3′) and FRIT (5′-GAA ACA CGG ACC AAG G-3′). Labeling reaction was carried out for a total of 30 thermal cycles, each cycle consisting of 30 s of denaturation at 96 ℃, 30 s of annealing at 50 ℃, and 1 min of extension at 60 ℃. The fluorescence-labeled mixture was sequentially mixed with a 12x hybridization buffer and 0.8 % SDS. Hybridization of DNA chips was performed for 4 h at 50 ℃. The slides were then rinsed with 2x SSC with 0.1 % SDS, 2x SSC and 0.2x SSC. Fluorescent signals of the hybridized DNA chips were detected by using a fluorescence microarray scanner (ScanArray 4 000, GSI Lumonics) at a pixel resolution of 10 μm.

Results and discussion

1 DNA Sequencing and polymorphism of *Fritillaria*

The optimal PCR condition for successful amplification of the D2 and D3 regions (≈320 bases) of the 26S rDNA gene from genomic DNA of all nine species of *Fritillaria* was achieved by using an annealing temperature of 55 ℃. The PCR products were then analyzed by direct DNA sequencing. The sequence variations of the D2 - D3 domain of the different *Fritillaria* species are shown in Figure 2. Because of the possible existence of inter-spacer polymorphism within the multiple repeats of the 26S rDNA among a single species, or the possible contamination of a sample by different species, it is advisable that genomic DNA samples from individual bulb, not a mixture of several bulbs, be used for sequencing as well as genotyping applications.

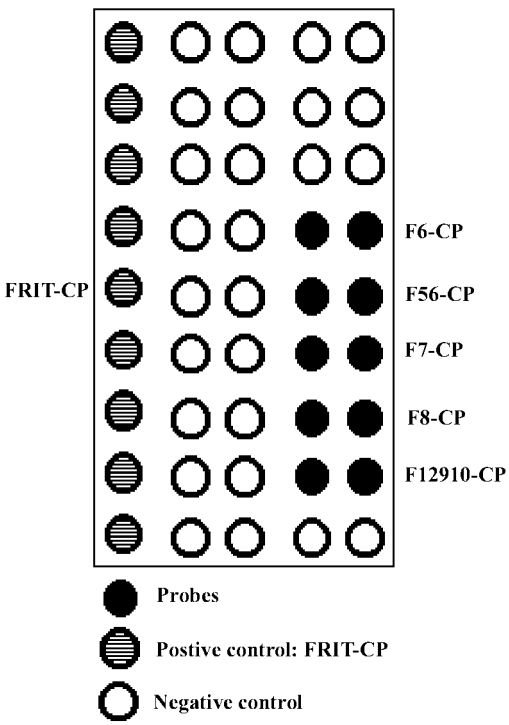
```
I2         1   GGC-GGTAAA CTCCGTCCAA GGCT-AAATA CGG-GCGAGA GACCGATAGC GAACAAGTAC
B3         1   ...-...... .......... ....-..... ...-..... .......... ..........
B2         1   ...-N..... ..N....... ....-..... ...-..... .......... ..........
H          1   N..-...... .......... ....-..... ...-..... .......... ..........
B1         1   ...-...... .......... ....-..... ...-..... .......... ..........
A          1   ...T...... ..N....... ....-..... ...-..... .......... ..........
I3         1   ...-...... .......... ....-..... ...-..... .......... ..........
Control    1   ...-...G.. T......... ....-..... ...-..... .......... ..........
F          1   ..T-...... T.T.A..T.. A...-..... .T.-..CG.. .......... .C.......G
D          1   ...C...... T.T.A..T.. A...-..... .T.-..CG.. .......... .C.......G
E          1   ...T...... T.T.A..T.. A...-..... .T.-..CG.. .......... .C.......G
I1         1   ..GT...... T.T.A..T.. A...-..... .T.-..CG.. .......... .C.......G
G          1   ..N-...... T.T.A..T.. A...-..... .T.-..CG.. .......... .C.......G
C          1   ...T...... TN.GA.NT.. A..NT....N .T.T..CN.. .......... .GN......G

I2        58   CGCGAGGGAA AGATGAAAAG GACTTTGAAA AGAGAGTCAA AGAGTGCTTG AAATTGCCGG
B3        58   .......... .......... .......... .......... .......... ..........
B2        58   .......... .......... .......... .......... .......... ..........
H         58   .......... .......... .......... .......... .......... ..........
B1        58   .......... .......... .......... .......... .......... ..........
A         59   .......... .......... .......... .......... .......... ..........
I3        58   ..N....... .......... .......... .......... .......... ..........
Control   58   .......... .......... .......... .......... .......... ......T...
F         58   A.T..TC... .......... C......... .......T.. .C..CA.G.. ......TT.A
D         59   A.T..TC... .......... C......... .......T.. .C..CA.G.. ......TT.A
E         59   A.T..TC... .......... C......... .......T.. .C..CA.G.. ......TT.A
I1        59   A.T..TC... .......... C......... .......T.. .C..CA.G.. ......TT.A
G         58   A.T..TC... .......... C......... .......T.. .C..CA.G.. ......TT.A
C         61   ANTC.TC.N. N......... C......N.. .N.....T.. .C..C..G.. ......T..A
```

Figure 2 Sequence alignment for selected regions of the D2 - D3 domain of the 26S rDNA gene from different *Fritillaria* species

2 DNA chip design and target hybridization

Based on the sequence alignment of all *Fritillaria* samples (Figure 2) , the sequence at 365 - 381 was found to be unique among all nine species investigated. A universal primer FRIT was designed and expected to anneal to all the DNA templates. Six oligonucleotide probes with different lengths and sequences were designed based on the specific sequence polymorphisms identified above, and immobilized on the poly-lysine coated glass slide to prepare the array according to the pattern shown in Figure 3. For the target DNA, purified PCR products were labeled with TAMRA-labeled ddCTP with DNA polymerase in the presence of several extension primers corresponding to the immobilized probes.



FRIT: 5′-GAA ACA CGG ACC AAG G-3′
Fl2910: 5′-TTT GAA AAG AGA GTC AAA GAG TG-3′
F56: 5′-TTC GGA TGG ATT TGA GTA AG-3′
F6: 5′-TGC AAA TCG ATC GTC G-3′
F7: 5′-CCG GTC AAA GGC CT-3′
F8: 5′-TGA AAG GGA AGC ACT GG-3′
FRIT-CP: 5′-CCT TGG TCC GTG TTT G-3′
Fl2910-CP: 5′-CAC TCT TTG ACT CTC TTT TCA AA-3′
F56-CP: 5′-CTT ACT CAA ATC CAT CCG AA-3′
F6-CP: 5′-CGA CGA TCG ATT TGC A-3′
F7-CP: 5′-AGG CCT TTG ACC GG-3′
F8-CP: 5′-CCA GTG CTT CCC TTT CA-3′

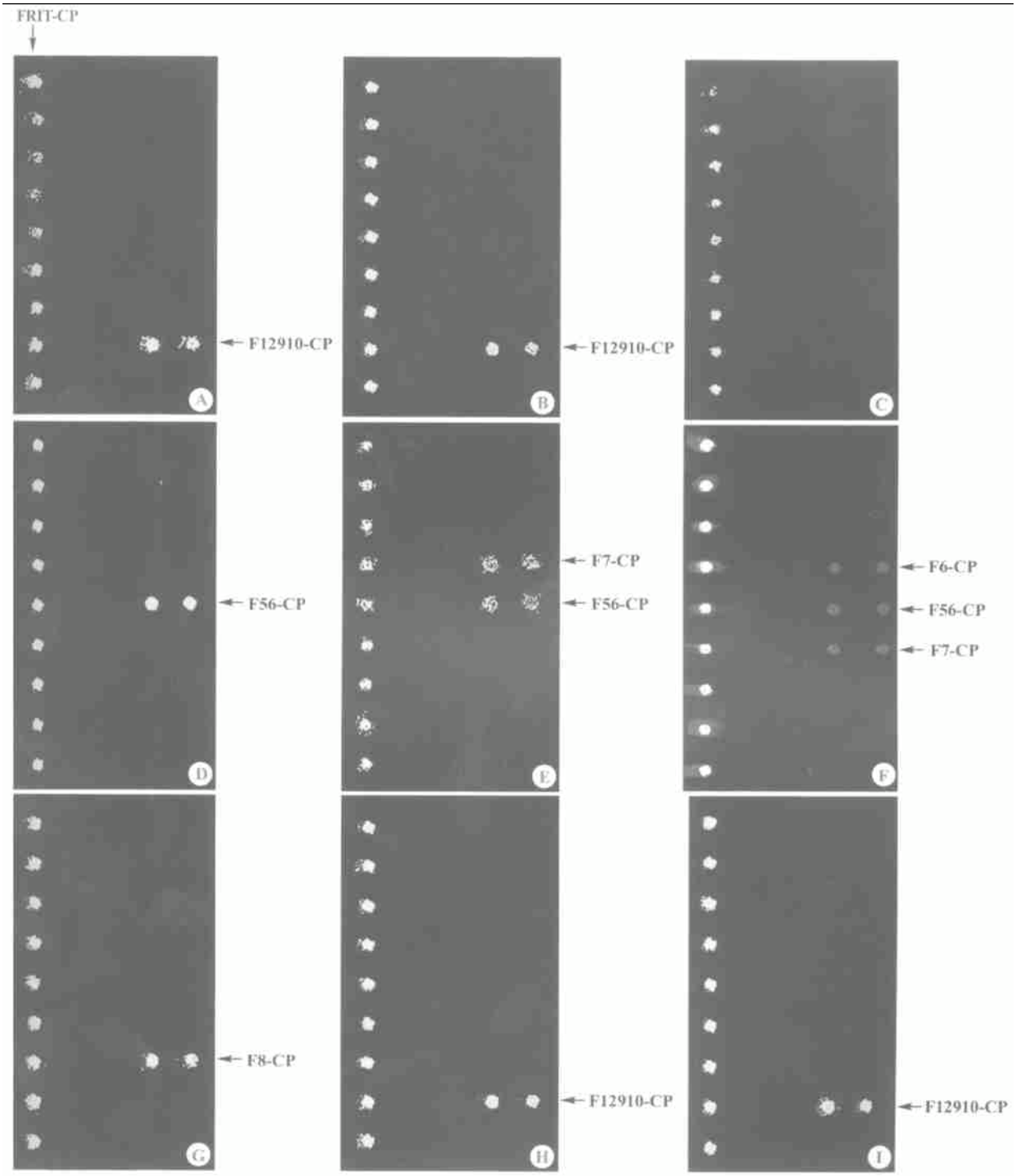
Figure 3 Microarray design and sequences of primers and probes for the *Fritillaria* DNA chip

The fluorescence-labeled PCR products of various species of *Fritillaria* were hybridized to the DNA chip and the microarray images were acquired by using a fluorescence scanner. The universal primer FRIT would anneal to all the DNA templates and undergo fluorescence labeling by incorporation of TAMRA-labeled ddCTP at the 3′-end of the primer. Therefore, fluorescent signals were observed at the FRIT-CP positions for all species (Figure 4A - I) , indicating the presence of a *Fritillaria* species. A general survey of the images showed that each *Fritillaria* species has a unique pattern of hybridization signals.

3 Species identification of *Fritillaria* by DNA chips

A comparison between various species of *Fritillaria* showed that the sequence of *F. cirrhosa* is highly homologous to the three variants of *F. unibracteata*, "Jianbei" (99.4 %) , "Tibet" (98 %) and "Pearl" (99.3 %) . Because of the great similarity between these four species at the molecular level, a single primer (Fl2910) and its corresponding probe (Fl2910-CP) were designed. Therefore, fluorescence signals were observed at the Fl2910-CP position as expected for *F. cirrhosa* and these three variants of *F. unibracteata* (Figure 4A, B, H and I) . Since no specific probe was designed for *F. thunbergii* var. *chekiangensis* species, only the FRIT signal was recorded and this species served as a negative control in the experiment (Figure 4C) .

Since the sequences of *F. ussuriensis*, *F. delavayi*, *F. pallidiflora* and *F.* var. *chekiangensis* exhibit only about 75 % similarity with that of *F. cirrhosa*, it was possible to design a set of specific primers and immobilized probes to distinguish these species. Positive signals at F56-CP were observed for both *F. ussuriensis* (Figure 4D) and *F. delavayi* (Figure 4E) , because F56-CP probe was designed based on the sequence at 467 - 486 position of *F. ussuriensis* while the same sequence exists for *F. delavayi*. F6-CP (covering nucleotides 580 - 595) was designed specifically for *F. delavayi* (Figure 4E) and the combination of both F56-CP and F6-CP serves to distinguish the two species. *F. pallidiflora* and *F. sp.* (unidentified) exhibited positive signals at both F7-CP and F8-CP positions (Figure 4F and G) , while non-specific signals were observed for *F. pallidiflora* on F56-CP and F6-CP positions due to partial sequence matching between the F56 and F6 primers and respective regions of *F. pallidiflora* (Figure 4F) . Therefore, *F. pallidiflora* and *F. sp.* (unidentified) can be distinguished based on the combination of the four probes.



A: *F. cirrhosa*; B: *F. unibracteata* (Jianbei); C: *F. thunbergii* var. *chekiangensis*; D: *F. ussuriensis*; E: *F. delavayi*; F: *F. pallidiflora*; G: *F. sp.* (unidentified); H: *F. unibracteata* (Tibet); I: *F. unibracteata* (pearl)

Figure 4 Image shows the hybridization results of various species of *Fritillaria* with the DNA chip

Conclusion

The work described in this paper represents one of the first applications of DNA chip technology for simultaneous genotyping and species identification of medicinal plants. Single nucleotide polymorphisms were used to distinguish different *Fritillaria* species based on the variation in sequences on D2 - D3 region of 26S rDNA gene. The DNA chip genotyping method is based on the ability of immobilized DNA probes to hybridize with their complementary sequences. The results demonstrate the reliability of using DNA chip technology to identify different species of *Fritillaria* for quality

assurance and this technology will be further explored to provide a rapid, highly efficient screening tool to supplement the conventional chemical methods for herbal drug authentication.

References:

[1] Huang KC. Antitussive Herbs [A]. Huang KC. *The Pharmacology of Chinese Herbs* [M]. Washington, D.C.: CRC Press, 1999 .275 - 176.

[2] Cai ZH, Li P, Dong TTX, *et al*. Molecular diversity of 5S-rRNA spacer domain in *Fritillaria* species revealed by PCR analysis [J]. *Planta Med*, 1999 ,**65** :360 - 364.

[3] Hamby KR, Zimmer EA. Ribosomal RNA as a phylogenetic tool in plant systematics [A]. Soltis PS, Soltis DE, Doyle J. *Molecular Systematics of Plants* [M]. New York: Chapman and Hall, 1992 .50 - 91.

[4] Soltis DE, Soltis PS. Phylogenetic relationships in Saxifragacae Sensu Lato: a comparison of topologies based on 18S rDNA and RBCL sequences [J]. *Am J Bot*, 1997 ,**84** (4) :504 - 522.

[5] Kuzoff RK, Sweere JA, Soltis DE, *et al*. The phylogenetic potential of entire 26S rDNA sequences in plants [J]. *Mol Biol Evol*, 1998 ,**15**(3) :251 - 263.

[6] Cronin MT, Pho M, Dutta D, *et al*. Utilization of new technologies in drug trials and discovery [J]. *Drug Metab Dispos*, 2001 ,**29**(4) :586 - 590.

[7] Raitio M, Lindroos K, Laukkanen M, *et al*. Y-chromosomal SNPs in Finno-Ugric-speaking populations analyzed by minisequencing on microarrays [J]. *Genome Res*, 2001 ,**11** (3) :471 - 482.

[8] Shi MM. Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies [J]. *Clin Chem*, 2001 ,**47**(2) :164 - 172.

[9] Lindroos K, Liljedahl U, Raitio M, *et al*. Minisequencing on oligonucleotide microarrays: comparison of immobilisation chemistries [J]. *Nucleic Acids Res*, 2001 ,**29**(13) :E69.

[10] Yang M, Miao JM. Methods for fabrication of medium-density DNA chips [P]. *PRC Pat*: Application No. 99104666, 1999-08, Publication No.1273362, 2000-11-15.