Analyses on Progenitor Donors of the Cultivated Allotetraploid Cottons Revealed by GISH

Xiao Shuiping1,2, Wang Kunbo2, Yang Lei1, Ke Xingsheng1, Wang Chunying2, Liu Xinwen1, Sun Liangqing1, Yang Shaoqun1, Liu Fang2, Chen Yi1

1 Cotton Research Institute of Jiangxi Province/ Poyang Lake Cotton Experiment Station, CARS, Jiujiang, Jiangxi 332105, China
2 Institute of Cotton Research of CAAS / State Key Laboratory of Cotton Biology, Anyang, Henan 455000, China

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Abstract GISH (Genomic in situ hybridization) of the mitotic metaphase chromosomes of two cultivated tetraploid cotton (AD)1 (G hirsutum) and (AD)2 (G barbadense) with all 3 diploid A cotton gDNA(genomic DNA) as probes, blocking with ssDNA(salmon sperm DNA) respectively. The hybridization signals were dected distribute in the A sub-genome chromosomes of (AD)1, and (AD)2, besides, three pairs of crimson signals were also detected only with the A14 gDNA probe which named GISH-NORs. GISH of (AD)1 and (AD)2 with all 13 diploid D cotton gDNA as probes, blocking with ssDNA respectively, except the D6 (Gossypium) gDNA probe generated the hybridization signals in all the chromosomes of (AD)1 and (AD)2, the other 12 diploid D gDNA probes only generated the signals on the D sub-genome chromosomes of (AD)1 and (AD)2, the D6 gDNA probe was very specific. And three pairs of strong GISH-NORs were detected with all 13 diploid D genome species gDNA probes, the intensity of their GISH-NORs were much brighter than the A14 gDNA probe. These results visually confirmed the amphidiploid origin of the allotetraploid cotton species. DA (distinguishing ability ) values of each gDNA probe generated were calculated basing on the above GISH results. It showed that the DA value of A14 gDNA probe was the biggest in all 3 diploid A genomes both in (AD)1 and (AD)2 GISH, and this indicated that A14 genome was most likely to be the A sub-genome progenitor donor of (AD)1 and (AD)2, while the D3,4 (G davidsonii ) and D6 (G raimondii) genome species were most likely to be the D sub-genome progenitor donor of (AD)1, and (AD)2 respectively. And this further confirmed that tetraploid cottons are polyphylectic.

Keywords Gossypium; Ghirsutum L.; Gbarbadense L.; Progenitor; GISH; GISH-NOR

Introduction
Gossypium genus belongs to the Malvaceae family, which contains 46 diploid (2n = 2× = 26,) species and 5 tetraploid (2n = 4× = 52). The Gossypium genus are believed to have originated from a common ancestor approximately 5~10 million years ago, of which eight diploid genomes, designated as A to G and K, have been found across Africa, Asia Australia and North America (Phillips,1963; Wendel .1989; Fryxell,1992; Dejoodeed and Wendel, 1992), and 5 tetraploid species were named (AD)1 (G hirsutum), (AD)2 (G barbadense), (AD)3 (G tomentosum), (AD)4 (G mustelinum) and (AD)6 (G darwinii) respectively(Wendel .1989). At present, (AD)1 and (AD)2 are major cultivated species, which are extensive cultivated in the world. Previous studies had shown that diploid cotton A and D genome species are the respective donor species of A and D sub-genome of the allotetraploid cotton species, and the A sub-genome chromosomes are longer than the D sub-genome chromosomes(Phillips,1966; Endrizzi,1985).

The Fluorescence in situ hybridization (FISH) technology was introduced to plant research in 1985 (Rayburn,1985), which has applied to various aspects such as repeat sequences positioning, chromosome identification, cell genetic map construction, the origin of polyploid genome evolution and phylogenetic relationships and so on (Snowdon et al., 1997 ; Tang et al., 2000; Jiang and Gill,2006; Wang k et al.,2008; Nemeth et al., 2015; Melo et al., 2015).
FISH technology have developed successfully on other crops (Mukai et al., 1993; Cheng et al., 2002; Xiong et al., 2004). But research on cotton far lags behind those, mainly because cotton cell has more density cytoplasm, more hard cell wall, more number of chromosomes and smaller size compare with other crops, which lead to cotton cell processing more difficult in prepare of high quality chromosome slides. In recent years, with the breakthrough of the bottleneck technology, cotton FISH research is developing rapidly (Liu et al., 2005; Wang et al., 2009; Wu et al., 2008; Gan et al., 2013; Zhang et al., 2014; Cui et al., 2015).

At present, about the great theories on *Gossypium* such as origin of species, evolution and classification had already formed the basic agreement. However, there still exist dissensions on the specific donors of the 5 tetraploid cotton species, and the germplasm disproportionation in the process of origin, and the homologous degree between different genome species of *Gossypium* or within one genome species (Liu et al., 2003; Wang KB et al., 2008). Our laboratory has made a great progress on using FISH technology to research the origin and evolution of cotton genomes and the interspecific genetic relationship or the genonomy of *Gossypium* (Wang et al., 1999; Wang et al., 2001; Liu et al., 2005; Wu et al., 2008; Gan et al., 2013).

In this study, GISH (Genomic in situ hybridization, one of the FISH technology) was used to the two cultivated alltetraploid cottons somatic metaphase chromosomes of (AD)$_1$ and (AD)$_2$, and used All 3 diploid A and 13 diploid D cotton gDNA (genomic DNA) as probes respectively, blocking with ssDNA (salmon sperm DNA). The results showed that the red hybridization signals were mainly distributed on the longer 13 pairs of A sub-genome chromosomes of (AD)$_1$ and (AD)$_2$ (as shown in Figure 1), and this visually confirmed the allodiploid origin of the tetraploid cottons (Wendel et al., 2002). Besides, three pairs of crimson signals were detected only with the A$_{1,a}$ gDNA probe both in (AD)$_1$ and (AD)$_2$ (Figure 1-c, f), which were named “GISH-NORs” (Liu et al., 2005), of which one in the A sub-genome chromosomes (green arrows), and two in the D sub-genome chromosomes (white arrows), and it significantly different from the A$_1$ and A$_2$ gDNA probes, the specific distributions about GISH-NORs were shown in Table 1.
Figure 1 GISH of mitotic metaphase chromosomes of two cultivated allotetraploid cotton species *Ghirsutum* (AD)₁ and *Gharbadense* (AD)₂ with all 3 A diploid cotton gDNA probes respectively (all probes were labeled with digoxigenin, and red fluorescence signals were observed on A sub-genome chromosomes in each image). Fig.1-a To 1-c: GISH of *Ghirsutum var. zhong* 16 with diploid A genome species *Gherbaceum var. hongxing* (A₁), *Garboreum var. shixiya* 1 (A₂), *Gherbaceum wild species arfrium* (A₁₁) gDNA probe respectively, blocking with ssDNA (In Fig.1-c, six arrows showed GISH-NORs, of which two green arrows denote the GISH-NORs in A sub-genome chromosomes and other four white arrows denote the GISH-NORs in D sub-genome chromosomes of (AD)₁). Fig.1-d To 1-f: GISH of *Gharbadense var. Xinhai* 7 also with diploid A genome species A₁, A₂, and A₁₁ gDNA probe respectively, blocking with ssDNA (In Fig.1-f, six arrows also showed the signals of GISH-NOR, of which two green arrows denote the GISH-NORs in A sub-genome chromosomes and four white arrows denote in D sub-genome).

Bars=10μm

| Table 1 Distribution of GISH-NORs in (AD)₁ and (AD)₂ by GISH with all A and D diploid gDNA probes |
|---|---|---|---|---|---|
| Target | gDNA Probe | Chromosome | Pairs | Size | Intensity | Position |
| (AD)₁ | A₁₁ | A sub-genome | 1 | Major | weak | terminal |
| | A₁ | D sub-genome | 2 | Major | weak | terminal |
| all 13 diploid D genome species | A₁₁ | A sub-genome | 0 | Major | | |
| | A₁ | D sub-genome | 0 | Major | | |
| (AD)₂ | A₁, A₂ | A sub-genome | 1 | Major | strong | terminal |
| | A₁ | D sub-genome | 2 | Major | strong | terminal |
| all 13 diploid D genome species | A₁₁ | A sub-genome | 0 | Major | | |
| | A₁ | D sub-genome | 0 | Major | | |

1.2 GISH of (AD)₁ and (AD)₂ with all 13 diploid D cotton gDNA as probes respectively

Except the *D₆*(*G.gossypioiides*) gDNA probe, GISH of the somatic metaphase chromosomes of (AD)₁ and (AD)₂ both with other 12 diploid D gDNA as probe respectively, blocking with ssDNA (As shown in Figure 2 and 3), the red fluorescence signals were observed on the short D sub-genome chromosomes of (AD)₁ and (AD)₂. In addition, three pairs of major GISH-NORs were detected, of which one in the longer A sub-genome chromosomes (green arrows showed in each image), and other two in the D sub-genome chromosomes (white arrows in each image), and the specific distribution about GISH-NORs were shown in Table 1. However, there were some differences on the fluorescence signal strength generated by each D genome species probe. GISH of (AD)₁ and (AD)₂ with D₆ gDNA probe respectively, the red fluorescence signals were not only distribute on the D sub-genome chromosomes but also on A sub-genomes (As shown in Figure 2-h and Figure 3-h), and we could not distinguish the A and D sub-genome of (AD)₁ and (AD)₂, and three pairs of major GISH-NORs were also observed. This showed a great difference between the *D₆* genome and the other 12 D genomes, and the result illustrated that diploid *D₆* genome may contain plenty of A genome repeat sequences, it is a very special genome species in the diploid D genomes.

1.3 DA value analysis based on GISH of (AD)₁ and (AD)₂ with diploid A, D genome species probes

DA (distinguishing ability) value reflect the genetic relationship between the diploid A or D genomes and the tetraploid genomes (Markova et al., 2007). DA values (As shown in Table 2) generated by A₁, A₂ and A₁₁ gDNA probes to (AD)₁ was 0.361, 0.358 and 0.369 respectively. It showed the A₁₁ gDNA possess the strongest ability to recognize the A sub-genome chromosomes of (AD)₁, while the A₁ and A₂ gDNA possess same ability to recognize the A sub-genome chromosomes of (AD)₁, that’s to say the A₁₁ genome had closer genetic relationship with the A sub-genome of (AD)₁. And DA values generated by A₁, A₂ and A₁₁ gDNA probes to (AD)₂ were 0.343, 0.346 and 0.352 respectively. the results also showed the A₁₁ gDNA possess the strongest ability to recognize the A sub-genome chromosomes of (AD)₂ therefore, the A₁₁ genome also had closer genetic relationship with the A
The sub-genome of \((AD)_2\).

Figure 2 GISH of \((AD)_1\) with all 13 D genome species as probes (all probes were labeled with digoxigenin, and red fluorescence signals were observed on D sub-genome chromosomes in each Fig). Mitotic metaphase chromosomes of \((AD)_1\) with \(G\) \(thurberi\) \((D_1)\) (a), \(G\) \(armourianum\) \((D_2)\) (b), \(G\) \(harknessii\) \((D_2)\) (c), \(G\) \(klotzschianum\) \((D_3)\) (d), \(G\) \(davidsonii\) \((D_3)\) (e), \(G\) \(aridum\) \((D_4)\) (f), \(G\) \(raimondii\) \((D_5)\) (g), \(G\) \(gossypiodies\) \((D_6)\) (h), \(G\) \(lobatum\) \((D_7)\) (i), \(G\) \(trilobum\) \((D_8)\) (j), \(G\) \(laxum\) \((D_9)\) (k), \(G\) \(turneri\) \((D_{10})\) (l), \(G\) \(schwendimanii\) \((D_{11})\) (m) gDNA as probe respectively, blocking with ssDNA (In each Fig, there are six arrows, all arrows show signals of GISH-NORs, of which two green arrows denote the GISH-NORs in A sub-genome and four white arrows denote the GISH-NORs in D sub-genome). Bars=10µm.

DA values of \((AD)_1\) generated by all 13 diploid D gDNA probes respectively were shown in Table 2. Because of the hybridization signals generated by \(D_6\) gDNA probe were distributed on both A and D sub-genome chromosomes of \((AD)_1\), therefore the DA value generated by \(D_6\) gDNA probe (DA value was 0.402) was much higher than the other 12 D gDNA probes in \((AD)_1\). Except the \(D_6\) gDNA probe, the highest DA value was 0.286 generated by \(D_{3,4}\) gDNA probe, and then followed by \(D_8\), \(D_9\), \(D_{1,11}\), \(D_{2,1}\), \(D_{2,2}\), \(D_9\), \(D_{3,4}\), \(D_9\), \(D_{10}\) and \(D_7\) probes. This showed that the \(D_{3,4}\) gDNA probe possess the strongest ability to recognize the D sub-genome chromosomes.
of (AD)₁, while the other D gDNA probes show weaker ability to recognize the D sub-genome chromosomes of (AD)₁, therefore the D₃-d genome had closer genetic relationship with the D sub-genome of (AD)₁.

Figure 3 GISH of (AD)₂ with all 13 D genome species as probes (all probes were labeled with digoxigenin, and red fluorescence signals were observed on D sub-genome chromosomes in each Fig, all generated GISH-NORs). Mitotic metaphase chromosomes of (AD)₂ with G. thurberi (D₁) (a), G. armourianum (D₂₁) (b), G. harknessii (D₂₂) (c), G. klotzschianum (D₃₄) (d), G. davidsonii (D₃₈) (e), G. aridum (D₄) (f), G. raimondii (D₅) (g), G. gossypiodies (D₆) (h), G. lobatum (D₇) (i), G. trilobum (D₈) (j), G. laxum (D₉) (k), G. turneri (D₁₀) (l), G. schwendimanii (D₁₁) (m) gDNA as probe respectively, blocking with ssDNA (In each Fig, there are six arrows, all arrows show the GISH-NORs, of which two green arrows denote the GISH-NORs in A sub-genome while other four white arrows denote the GISH-NORs in D sub-genome). Bars=10μm.

DA values of (AD)₂ generated by all 13 diploid D gDNA probes also be shown in Table 2. Except the D₆ gDNA probe (DA value was 0.387), the highest DA value generated by D₅ gDNA probe was 0.263, and then followed by D₃-d, D₁, D₄, D₂₁, D₂₂, D₁₁, D₈, D₁₄, D₁₀, D₉ and D₇ probes. which was also higher than those of the other 12 D gDNA probes. The results showed that the D₅ gDNA possess the strongest ability to recognize the D sub-genome...
chromosomes of (AD)$_2$, while the other 12 D gDNA show weaker ability to recognize the D sub-genome chromosomes of (AD)$_2$, so the D$_2$ genome species had closer genetic relationship with the D sub-genome of (AD)$_2$.

Table 2 DA values of all diploid A, D genome species probes generated by GISH of (AD)$_1$ and (AD)$_2$

| Probe                                      | (AD)$_1$ | (AD)$_2$ |
|--------------------------------------------|----------|----------|
| *G. herbaceum* (*A$_1$*)                   | 0.361    | 0.343    |
| *G. arboreum* (*A$_2$*)                    | 0.358    | 0.346    |
| *G. herbaceum* wild species (*A$_{1-4}$*)  | 0.369    | 0.352    |
| *G. thurberi* (*D$_1$*)                    | 0.258    | 0.247    |
| *G. armourianum* (*D$_{2-1}$*)             | 0.136    | 0.189    |
| *G. harknessii* (*D$_{2-2}$*)              | 0.102    | 0.111    |
| *G. klotzschianum* (*D$_{1-4}$*)           | 0.087    | 0.071    |
| *G. davidsonii* (*D$_{1-4}$*)              | 0.286    | 0.252    |
| *G. aridum* (*D$_4$*)                      | 0.274    | 0.241    |
| *G. raimondii* (*D$_3$*)                   | 0.255    | 0.263    |
| *G. gossypiodes* (*D$_6$*)                 | 0.402    | 0.387    |
| *G. lobatum* (*D$_7$*)                     | 0.081    | 0.044    |
| *G. trilobum* (*D$_8$*)                    | 0.095    | 0.076    |
| *G. laxum* (*D$_9$*)                       | 0.085    | 0.058    |
| *G. turneri* (*D$_{10}$*)                  | 0.083    | 0.065    |
| *G. schwendimaniii* (*D$_{11}$*)           | 0.231    | 0.098    |

2 Discussion

2.1 The A sub-genome progenitor of (AD)$_1$ and (AD)$_2$

Since the discovery that allotetraploid *Gossypium* genomes contain both A and D genomes, investigators had attempted to look for which one of the modern diploid A and D genome species can be best served as the progenitor genome donors of allopolyploid cottons.

Which one of the diploid A genome species was the really donor of the A sub-genome of allopolyploid cottons? Many previous researches suggested that *G. herbaceum* (*A$_1$*) was the donor or the similar ancestors of the allopolyploid A sub-genome (Beasley, 1940; Gerstel, 1953; Phillips, 1963; 1964). However, the subsequent research had shown that there existed much differences between *G. herbaceum* (*A$_1$*) and the A sub-genome of allopolyploid cotton, whether in the chromosome or molecular level (Wendel, 1989; Wendel and Albert, 1992; Cronn, et al., 1996). And cell cytogenetic and comparative mapping research also revealed that there existed at least two large translocations between their genomes (Gerstel, 1953; Small, et al., 1998; Liu and Wendel, 2001). Branch taxonomy analyses of the molecular sequences had showed that *G. herbaceum*(*A$_1$*) was not the actual progenitor of the A sub-genome of allotetraploid cottons (Endrizzi, et al., 1985; Wendel and Cronn, 2002). In the evolutionary process of allotetraploid cottons, *G. herbaceum*(*A$_1$*) and *G. arboreum*(*A$_2$*) were the phylogenetically sisters between each other and hence were genealogical equidistant to the A sub-genome of the allotetraploid cottons (Cronn et al., 1996; Liu and Wendel, 2001; Wendel, 1989; Wendel and Albert, 1992).

In our experiment, GISH of (AD)$_1$ and (AD)$_2$ both with all 3 A genome gDNA as probes, 13 pairs of A sub-genome chromosomes were painted with red fluorescence signals, and the DA value was very similar between A$_1$ and A$_2$ gDNA probe, there was no significant difference between A$_1$ and A$_2$ both in GISH of (AD)$_1$ and (AD)$_2$, while the DA value of A$_{1-4}$ gDNA probe was higher than A$_1$ and A$_2$ gDNA probe, the A$_{1-4}$ gDNA had the strongest ability to recognize the A sub-genome chromosomes of (AD)$_1$ and (AD)$_2$, so we considered that A$_{1-4}$ genome...
species was most likely to be the A sub-genome progenitor donor of allotetraploid cotton (AD)₁ and (AD)₆.

The A₁₋₆ genome had been divided into the variant of A₁ genome in the taxonomy (Stewart, 1987). Many researches always used the varieties of A₁ and A₂ genome species to study the origination and evolution of the A sub-genome of allotetraploid cottons, but they seldom and nearly didn’t use the A₁₋₆ genome as the research materials, and even some researchers took the A₁₋₆ and A₁ genome to lump together (Stewart, 1987; Wendel, 1989; Wendel and Albert, 1992; Wendel et al., 1994, 1995; Wendel and Wessler, 2000; Wendel and Cronn, 2002; Wang et al., 2004). In this experiment, when using A₁₋₆ gDNA as probe we found that the GISH result was obviously distinguished from the A₁ gDNA probe, and Wang et al.(1995) had also found the karyotype parameters exist significant differences between A₁ and A₁₋₆ genome. The A₁₋₆ genome made a strong independence from A₁ genome, and we suggested that A₁₋₆ genome should be given the “species” level in the classification of *gossypium*, that’s means the A₁₋₆ genome possessed the same status with A₁ and A₂ genome.

2.2 The D sub-genome progenitor of (AD)₁ and (AD)₆

Since the discovery that allotetraploid *Gossypium* species contain two genomes whose progenitors presently occur in different hemispheres, investigators had attempted to provide pieces to the puzzle of the polyploid origin. A diverse array of tools had been used in an effort to examine this issue, from early study methods by comparative morphology, cytology, cytogenetic, comparative phytochemistry, and protein electrophoretic methods to modern phylogenetic investigations using DNA sequencing of homologous genes. Several investigators proposed that allotetraploid cottons formed more than once, they suggested the best models of the potential D subgenome ancestral donor of allotetraploid cottons were D₁(G. thurberii), D₃₋₄(G. davidsonii), D₃₋₄ (G. klotzschianum) and D₄(G. raimondii), so they considered the allotetraploid cottons are polyphylectic (Kammacher, 1960; Sherwin, 1970; Johnson, 1975; Umbeck, 1985; Stewart, 1987; Da and Bertrand, 1995). However, Most authors proposed that allotetraploid cottons formed only once, and that D₃ was more similar to the D subgenome of allotetraploid cottons than other D genome diploids, they considered the allotetraploid cottons are monophyletic (Phillips, 1963, 1964, 1966; Endrizzi et al., 1985; Wendel, 1989; Cronn et al., 1996; Seelanan et al., 1997; Small and Wendel, 1999, 2000; Wendel and Albert et al., 1992; Wendel et al., 1995; Wendel and Cronn, 2002; Admas, 2003).

We compared the GISH signals and DA values of (AD)₁ and (AD)₂ chromosomes generated by different diploid D genome species. The DA values of D₃₋₄(G. gossypioide) were much higher than those of other D genome species both in (AD)₁ and (AD)₂, but the signals were distributed on both A⁻ and D subgenome chromosomes of (AD)₁ and (AD)₂, and the signal intensity was much weaker than that produced by other D genome species such as D₃₋₄ (G. davidsonii) and D₂ (G. aridum) in (AD)₁, or D₁ (G. thurberii), D₃₋₄ (G. davidsonii) and G. raimondii (D₂) in (AD)₂. Therefore, the D₆ genome species cannot be the D subgenome progenitor donor of (AD)₁ and (AD)₂.

The signal intensity in D₃₋₄ probe was more stronger than any other D genome species probes in GISH of (AD)₁, and the DA value of D₃₋₄ probe was much higher than other D genome species too. This indicated that D₃₋₄ genome species has the strongest ability to recognize the D subgenome chromosomes of (AD)₁, so we suggested that D₃₋₄ but not D₁ was the possible D subgenome progenitor donor of (AD)₁.

And on the other hand, the signal intensity in D₅ probe was more stronger than any other D genome probes in GISH of (AD)₂, and the DA value of D₅ probe was also much higher than other D genome probes. This indicated that D₅ genome species has the strongest ability to recognize the D subgenome chromosomes of (AD)₂, so we thought that the D₅ is the possible D subgenome progenitor donor of (AD)₂.

Previous GISH studies had shown that diploid D₁ and D₃₋₄ genome species was the D sub-genome progenitor donor of (AD)₁ (G. mustelinum) and (AD)₂ (G. darwinii) respectively (Wu et al., 2010; 2013), combined with the results of this experiment, allotetraploid cottons maybe formed with different D genome species as the progenitor donor, these results supported the hypothesis that allotetraploid cottons are polyphyletic.
3 Materials and Methods

We used allotetraploid cultivated cotton species (2n = 4x = 52) (AD)1 (G. hirsutum var. Zhongmiansuo 16) and (AD)2 (G. barbadense var. Xinhai 7) as target chromosomes. The probes were made from diploid cotton (2n = 2x = 26) genomic DNA (gDNA), including all 3 A genome species G. herbaceum var. hongxing (A1), and G. arboreum var. shixiya 1 (A2), and G. herbaceum wild var. arfrium (A1, A2); and all 13 D genome species, including G. thurberi (D1), G. armouriunn (D2, 1), G. harknessii (D2, 2), G. davidsonii (D2, 3), G. klotzschianum (D2, 4), G. aridum (D2, 5), G. raimondii (D3), G. gossypiodies (D2), G. lobatum (D3), G. trilobum (D3), G. laxum (D3), G. turneri (D10) and G. schwendianni (D11). All of the plant materials are being taken from National Wild Cotton Germplasm Nursery in Sanya city, Hainan, China.

Total gDNA was extracted and purified from immature leaves using the CTAB method (Song et al., 1999). The purified total gDNA was cut off with 120℃ 10 min, and examined with 0.8% agarose gel electrophoresis for their fragment size, which was generally appropriate in 300-600bp, and then tagged with markers. The gDNA probes were labeled with DIG-High-Prime labeling system (Roche Company, Germany), according to the standard operating procedures. The preparation of mitotic metaphase chromosomes which derived from the root tip cells and the procedure of GISH referenced to the method of Wang (Wang et al., 1999).

The hybridization signals were observed using a fluorescence microscope (Ziess Axioskop 2 plus). Images were captured by ISIS (in situ imaging system) software by adjusting their brightness and contrast. And also used this software to calculate the DA (distinguishing ability) value which was described by Markova et al (2007). And used Adobe Photoshop 7.0 software makes the plate.

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