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APPLICATION OF BIOMOLECULAR TECHNIQUES IN TREE VARIETY IDENTIFICATION IN CHINA

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APPLICATION OF BIOMOLECULAR TECHNIQUES IN TREE VARIETY IDENTIFICATION IN CHINA

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INTRODUCTION

1. China is a relatively new member of UPOV. The "Regulation on protection of new plant varieties" came into effect on October 1, 1997, and China acceded to UPOV on April 23, 1999. The "Rules for Implementation of the Regulations of the People's Republic of China on the protection of new varieties of plants (Forestry Part)" was promulgated on August 10, 1999. The plant variety protection (PVP) system of China is somewhat unique because it consists of two, parallel PVP subsystems for agricultural and forestry plants, which are respectively administered by the Ministry of Agriculture (MOA) and the State Forestry Administration (SFA). The SFA is responsible for forest trees, dry fruit trees, woody ornamental plants, bamboo, woody vines, woody plants for oil, drinks and condiment production and woody medicinal plants.

2. A legal system has been established for new variety certification, registration, protection and management. To effectively implement the PVP system in China, it needs accurate, reliable and standardized methods to distinguish different varieties. These methods should not be affected by environmental factors and the developmental stage of the plant. However, very often the difference is not distinguishable in morphological traits and differences can only be found at the molecular level.

3. Genetic markers have been a useful tool for studying genetic diversity of plants and distinguishing different plant taxa. Genetic markers including morphological markers, cytological markers, biochemical markers and molecular markers are used in plant variety identification. Identification of plant varieties has traditionally been based on morphological and phylogenic traits, biological characteristics and especially on the traits of economic value. However, long cultivation histories, lack of written records and hybridization among many plant cultivars of different species make it difficult to identify varieties only by taxonomic methods. Moreover, the phenotypes of many different varieties are hard to distinguish morphologically and cytologically, even by protein markers, due to inbreeding of the related germplasm resources.

4. During the last 20 years, the world has experienced a rapid development of biochemical and molecular techniques, which use isozyme/allozyme or DNA markers to analyze genetic parameters. These new techniques have greatly facilitated genetic studies of plant species. Particularly, biochemical and molecular techniques have shown great potential for identifying plant varieties, and consequently have received increasing attention for plant variety protection.

5. In this paper, we present a general overview of genetic studies of plant species carried out in China using biochemical and molecular techniques, and the potential of biochemical and molecular techniques in the identification of plant varieties for the PVP system.

APPLICATION OF BIOCHEMICAL AND MOLECULAR TECHNIQUES IN FOREST TREES IN CHINA

Development of Biochemical and Molecular Techniques

6. Many studies were carried out using isozyme markers during the period 1970-1980. Since the 1990s, the emergence of DNA molecular markers provided a reliable way of identifying species at the molecular level. As molecular markers are direct products of genes, they can be used to detect differences at gene level, leading to increased use of molecular markers in plant variety identification. At the end of 1980 and in the early 1990s, without phenotypic and environment influences, a number of DNA markers, such as RFLP, RAPD, AFLP, SSR, SCAR, were widely used in genetic studies of crops. DNA markers are very suitable for studying genetic diversity, phylogenetic relationship, mating system, genetic mapping, QTL and marker assisted selection. Currently, the most widely-used DNA markers in plant (particularly forest trees) studies, particularly in variety identification, are SSR and ISSR markers.

Applications of Biochemical and Molecular Techniques

Genetic diversity and classification

7. Since the 1980s, because of their low cost and ease of use, isozyme markers have been used to identify different species within same genus; for example, in *Cupressus* (Jian & Wang, 1986), *Magnolia* L. (Zhao *et al*, 1994), *Dysosma* (Su & Liu, 1996), *Actinidi* (Yao *et al*, 2003), *Cephalotaxu* (Chen *et al*, 2003a), *Paulownia* (Chen *et al*, 2003b) and *Ilex* L. (Fu *et al*, 2004). In recent years, SSR and/or ISSR have been frequently used to analyze genetic diversity in forest trees, such as *Ginkgo biloba* (Ge *et al*, 2003), *Abies beshanzuensis* M. H. Wu (Ai *et al*, 2005), *Populus deltoids* (Li *et al*, 2006), Aspen hybrid (Zhang *et al,* 2006a), and *Populus nigra* (Zhang *et al*, 2006b) in China. These molecular markers have also been used to identify species within the same genus: for example, *Bauhinia purpurea*, *B. blakeana* and *B. variegate* (Luo, 2006).

Variety identification

8. Nowadays, biochemical and molecular techniques have been used to identify varieties of forest trees in China, mostly poplar trees. As showed in Table 1, many studies have been carried out to identify varieties (cultivars or clones) of forest trees by isozyme markers since 1982. Since the early twentieth century, more and more research has been reported on variety identification of forest trees by DNA markers, such as RAPD, SSR, and ISSR. Some research focused on the optimization of the ISSR reaction system. The concentration of Mg2+, Taq DNA polymerase, dNTPs, primer and genome DNA were screened to optimize the ISSR amplification system in *Callistemon rigidus* R.Br. (Li *et al*, 2006), *Torrey ajackii* Jin &Li, (2006), *Populus tomentosa* Carr. (Feng *et al*, 2006), *Tilia amurensis* (Wei *et al*, 2006).

Table 1: Application of biochemical and molecular techniques in identification of different taxonomic units in China

No.	Species	Markers	Purpose	Literature sources
1	*Populus nigra*, *P. deltoids* and *Populus* x *euramericana*	isozyme	clone identification	Wang *et al* (1982)
2	*Populus* L.	isozyme	clone identification	Wang *et al*(1995)
3	*Ziziphus jujuba* Mill.	isozyme	variety identification	Li *et al*(1995)
4	*Chimonanthus praecox* (L.) Link	isozyme	variety identification	Chen(1995)
5	*Ammopiptanthus nanus* and *A. mongolicus*	isozyme	variety identification	Wei & Shi (1995)
6	*Populus tomantosa*	isozyme	clone identification	Ru *et al*(1998)
7	*Salix psamniphila*	isozyme	clone identification	Wu *et al*(2001)
8	*Toona sinensis*	isozyme	variety identification	Tu *et al*(2002)
9	*Actinidi*	isozyme	variety identification	Wang *et al*(2003)
10	*Hibiscus*	isozyme	variety identification	Zhuang *et al*(2004)
11	*Punica granatum*	isozyme	variety identification	Xu *et al*(2006)
12	*Ginkgo biloba*	isozyme	variety identification	Gao (2005)
13	*Castanea mollissima* Bl.	RAPD	variety identification	Gao (1999)
14	*Rosa hybrida* L.	RAPD	variety identification	Wang *et al*(2001)
15	*Rosa rugosa, Rosa chinensis*	RAPD	variety identification	Chen *et al*(2002)
16	*Hevea brasishensis*	RAPD	variety identification	Sun (2005)
17	*Populus* L.	SSR	variety identification	Liang *et al*(2005)
18	*Juglans regia* L.	SSR	variety identification	Yang(2006)
19	*Camelia sinensis*	SSR	variety identification	Jin(2006)
20	*Michelia tsoi*	ISSR	cultivar identification	Qiu *et al* (2002a)
21	*Osmanthus fragrans*	ISSR	cultivar identification	Qiu *et al* (2004) and Hu *et al* (2004)
22	*Ginkgo biloba*	ISSR	variety identification	Shen *et al* (2005)
23	*Morus alba* L.	ISSR	variety identification	Zhao *et al* (2006)
24	*Paeonia lactiflora* Pall	ISSR	variety identification	Yu *et al*,2006

9. Wang *et al* (1982) analyzed 13 clones of Aigioros poplars by electrophoresis and considered that isozyme analysis was feasible to identify Aigioros poplar. Clear differences have been found in peroxide isozyme patterns in *Populus nigra*, *P. deltoids* and their hybrids *Populus x euramericana*. The Aigioros clones differ not only vary in band number but also differ in band Rf value.

10. RAPD markers have been used to study genetic diversity of chestnut trees (Gao, 1999). The standardized procedure for RAPD analysis and the fingerprints database of chestnut was established for chestnut species, which provided a good basis for chestnut breeding and a precise and reliable tool for identifying chestnut varieties. Sun (2005) analyzed the fingerprints of 39 rubber tree varieties using RAPD markers; 31 varieties were identified by RAPD fingerprinting and 8 varieties were not identified.

11. SSRs are widely used to construct plant fingerprints because of their rich polymorphism and co-dominance. More and more studies have shown that it is possible to construct fingerprints for fruit trees and for new tree species by SSR. SSRs are regarded as a very efficient method for identifying plant varieties because the SSR fingerprinting can reveal the unique feature of many varieties. SSR markers have been used in identifying varieties of crops such as wheat, barley, rice, corn, soybean (Yan et al, 2003), potato, rape, peanut, pear, apple etc.

12. A study was conducted on 67 commercial apple varieties using SSR markers for identification of the varieties (Wang, 2005a). 219 loci were detected in 25 varieties of the Golden Delicious family. Between 2 and 17 loci were detected for each pair of primers and the average number of detectable loci was 8.1. 12 pairs of useful primers were obtained, any of which was enough to distinguish more than 13 varieties from each other. A total of 25 varieties were identified. Cao (2006) analyzed the genetic diversity of 41 pear cultivars belonging to 5 cultivated species and inter-specific hybrids using SSR markers. The results showed that SSR analysis was very reliable and was unaffected by plant material and time. Primer BGT23b produced 15 putative alleles in the 41 cultivars and could distinguish between 30 cultivars. Two pairs of primer (BGT23b and CH02D11) could distinguish all of the pear cultivars except the mutant cultivars.

13. Liang *et al* (2005) used SSR to determine the genomic DNA variations and genetic relationships in *Populus* L. 10 cultivars were used in SSR analysis. A total of 10 SSR primers were selected to determine the genetic diversity and genetic relationships. 122 DNA bands were amplified, 114 of which were polymorphic (93.44 %). The average number of DNA bands amplified by each primer was 12.2. DNA profiles based on SSR markers revealed potential diagnostic fingerprints for various individual cultivars.

14. ISSR markers are regarded as stable and highly repeatable, compared with RAPD markers. Feng et al (2005) studied the factors affecting ISSR results of apricot trees. Through adjusting template DNA concentration, Mg^{2+} concentration, primer concentration, dNTP and Taq polymerase contents and annealing temperature, the PCR amplification conditions were optimized. Meanwhile, a set of 42 ISSR primer pairs were also studied to screen the suitable primers for assessing the genetic diversity of 12 apricot accessions. Shen *et al* (2005) studied the DNA fingerprints of the main *Ginkgo biloba* varieties cultivated all over China, using ISSR markers. Results indicated that ISSR markers were highly effective for identifying 13 varieties. Using only primers ISSR46 and ISSR44 , 13 varieties were identified based on 11 polymorphic loci. Zhao *et al* (2006) found ISSR markers were very effective in distinguishing mulberry varieties. The ISSR fingerprints of 24 mulberry cultivars were constructed. A total of 80 bands were produced using 17 primers and 40 bands showed polymorphism. From the bands amplified, there were three independent ways to identify the mulberry varieties, such as unique ISSR markers, unique band patterns and a combination of band patterns provided by different primers.

EXAMPLES OF PROVIDING SUPPLEMENTARY EVIDENCE FOR GRANTING VARIETY RIGHTS

15. Molecular markers can be used to obtain evidence for DUS tests; however, providing such supplementary information on distinguishing varieties may be controversial. The following is an example of the use of SSR markers to obtain supplementary evidence to identify poplar varieties (Figure 1)) carried out by the Nanjing Forestry University. Four SSR

markers (ORNL-394, ORNL-288, ORNL-287, ORNL-365) were used to assay the six poplar clonal varieties.

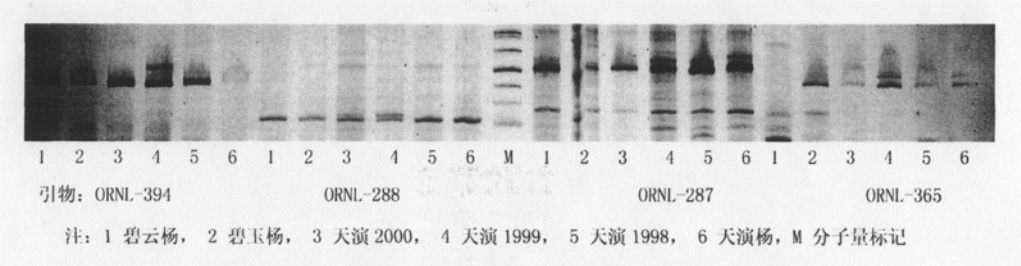

Figure 1: SSR markers used to distinguish poplar varieties: The channels 1, 2, 3, 4, 5, 6 and M respectively denote varieties of 'Biyun', 'Biyu', 'Tianyan 2000', 'Tianyan 1999', Tianyan 1998' and 'Tianyan' as control (from which the new clones were developed).

16. The following conclusions can be drawn from the four SSR markers shown above:

- ORNL-365 distinguishes: 'Biyun' from 'Biyu' and from the Tianyan series; 'Biyu' from 'Tianyan 2000' and 'Tianyan'; and 'Tianyan 1998' from 'Tianyan';
- Both ORNL-394 and ORNL-288 distinguish 'Tianyan 1999' from the other 3 clones of the Tianyan series.
- ORNL-287 distinguishes 'Tianyan 2000' from 'Tianyan 1999', 'Tianyan 1998' and the 'Tianyan (the original variety used as the control in the analysis)';
- ORNL-287 distinguishes 'Biyu' from 'Tianyan 1998';
- All four markers distinguish 'Biyu' from 'Tianyan 1999'

17. The above evidence from the four SSR markers indicates that the six poplar varieties are different from each other. This example proves that, for varieties that are controversial with respect to distinctness, biochemical and molecular techniques may be employed as a supplementary tool to identify the varieties. This would be an important use of biochemical and molecular techniques for the PVP system in China, particularly for varieties of vegetatively propagated plants.

DISCUSSION AND RECOMMENDATIONS

18. Although biochemical and molecular techniques have been widely used in genetic studies and variety identification of plant varieties in China, most of those applications were in agricultural crops and fruit trees, with only a few cases for forest tree species. This could be because breeding new varieties of forest trees needs a much longer period, leading to fewer new varieties of trees being developed in comparison to crops. However, with the rapid expansion of the market for ornamental trees, which brings great economic potential, it is envisaged that a number of new ornamental tree varieties will be bred in future, and the potential of using biochemical and molecular techniques in the identification of new varieties of tree species (ornamental trees in particular) will be great. The techniques described above can be easily applied if efforts can be made to standardize the testing procedures and methodologies.

19. We have established two molecular labs for the application of biochemical and molecular techniques in the SFA PVP system. Current activities are focused on the

development of specific biochemical and molecular techniques for specific plants and on DNA profiling for the establishment of DNA-fingerprint databases. Although biochemical and molecular techniques have great potential in DUS testing and variety profiling, there is still much to do to make the application of biochemical and molecular techniques meet the requirements of UPOV. Standardization of biochemical and molecular techniques is needed to avoid systematic errors caused by the use of different markers, materials and laboratory procedures, and, therefore, to improve the reducibility of biochemical and molecular techniques among different labs and repeatability within the same laboratory.

20. The PVP office of the State Forestry Administration will set up a national working group on the application of biochemical and molecular techniques in the PVP system, aiming to study the problems and potential of the application of biochemical and molecular techniques in China's PVP system and to develop the standards needed for the application of biochemical and molecular techniques in DUS examination, variety identification and profiling.

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