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# Comparative genetic and chemical profiling performed on *Alstonia scholaris* in China and its implications to standardization of Traditional Chinese Medicine

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**Validity of traditional Chinese medicine (TCM) is oppugned very often because of the bugs of quality, effectiveness, and repeatability. Standardization of TCMs is one way to make a change. How to realize standardization of TCMs and with what measures to shape the quality control system, is provoking much debate in the TCM field. Genetic and chemical profiling were comparatively performed on *Alstonia scholaris* in China through amplified fragment length polymorphism (AFLP) analysis and alkaloid high performance liquid chromatography (HPLC) scanning, aiming to evaluate the attributes of chemical and DNA fingerprinting as applicable tools for quality control of TCMs and explore theoretic strategy of TCM standardization. Each individual of *A. schlolaris* displayed a unique AFLP or HPLC profile, indicating well individual-distinguishing ability of the two techniques. Patterns of variance structure disclosed by the HPLC and AFLP profiling were similar, and more than 50% of alkaloid loci were kept constant across any single population, suggesting a genetic basis of the alkaloid secondary metabolism in the plant and justifying DNA fingerprinting as a qualified identifier for quality control of the TCM. Compared to the alkaloid HPLC scanning, the AFLP analysis produced much more loci with lower polymorphic loci percentage, suggesting that AFLP can be more statistically informative and with moderate sensitivity. In passing, a strategy pursuing genetically identical TCMs by popularizing plantation of selected germplasm of medicinal plants was suggested for TCM standardization.**

**Key words:** Traditional Chinese Medicine (TCM), standardization, *Alstonia scholaris*, alkaloid scanning, genetic profiling.

## INTRODUCTION

*Alstonia scholaris* is an evergreen tree with white perfumed flowers, growing in mixed forests and village groves from 200 to 1000 m a.s.l. in the tropical and subtropical areas such as Southern Yunnan and South-western Guangxi, and cultivated in Fujian, Guangdong, Hainan, Hunan, Taiwan and as an ornamental in China (Li et al., 1995). It has been widely used in traditional Chinese medicine (TCM) to treat various diseases such as headache, influenza, malaria, bronchitis and pneumonia (Zhou et al., 2005). Various alkaloids from its bark and leaves were confirmed to be the active ingredients (Khyade and Vaikos, 2009; Shang et al., 2010).

Morphologically, the species is a ‘good’ species, very homogenous without much difference recognized between populations from different geography areas, and easily distinguished from other *Alstonia* species. It represents a useful simple model to test the difference between chemical and DNA fingerprinting as two marker

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systems to identify medicinal plants, since taxon with multiple morphological variation will make the comparison more complicated and elusive.

Traditional Chinese medicine (TCM) has been seeing an increasing business volume in China in recent years. According to PricewaterhouseCoopers (2009), sales revenue for TCM products in China reached approximately US$ 21 billion in 2007, accounting for around 40% of the total pharmaceutical market in China. In 2008, TCM represented, in terms of sales volume, around two-thirds of drug sales in China. The widespread use of TCM had already posed substantial competition to the conventional drug industry, and sales in TCM might see a further increase (PricewaterhouseCoopers, 2009). TCM is not only one of the important historical cultural heritages in the world, but also one of the important scientific heritages of humans. The long history of application of TCM by Chinese is in nature a history of clinic experiment of TCMs directly upon human bodies. The effectiveness and safety of TCM is rationally based. This is the very reason that TCM has been seeing an increasing business volume in China. However, TCM still faces many challenges, one of which is to address the inconsistencies found in its manufacturing processes (Business Monitor International, 2008). Raw materials for many TCMs are from wild resources in current practice. A stockpile of a specific TCM in a drugstore might come from different geographical area and might differ in heredity and chemical constituents. This is one of the reasons that the patients often complain about the quality, effectiveness and repeatability of TCMs and that some countries refuse TCM usage. Standardization of TCMs is the most important way to make the quality of TCMs appropriately controlled, qualifying TCM to serve people in modern society, and relieving TCM of the present embarrassed situation. How to realize standardization of TCMs and with what measures to shape the quality control system is provoking much debate in the TCM field. Chemical and DNA fingerprinting, as two applicable tools to identify medicinal plants, are often the focus of this kind of controversies. What are the attributes of these two kinds of marker? How effective are the two measures when used as identifier? How about their distinguishing ability? Are they perfect identifiers throughout the whole industrial production process of a specific TCM? Such questions have remained to be resolved since phytochemical and genetic studies are often separately performed on different medicinal plants by different investigators. Systematic comparative studies of phytochemical and genetic diversity of medicinal plants are still limited.

Therefore, in this paper, we used genetic and chemical profiling as a framework to look at the genetic and alkaloid variation within *A. scholaris* in China, evaluated the attributes of chemical and DNA fingerprinting as applicable tools for quality control of TCMs and explored theoretic strategy to realize TCM standardization.

## MATERIALS AND METHODS

### Taxa and sampling

Four wild geographical populations of *A. scholaris* were selected, with each population being at least 200 km away from its closest neighbor (Table 1). The sampling strategy was to include most variations of *A. scholaris* in China and allowing detection of chemical and genetic variation within and among populations. During the early fall of 2005, 15 flowering individuals were sampled randomly for each population at intervals of at least 10 m. All studied materials are listed in Table 1, and all vouchers were deposited in the herbarium of Kunming Institute of Botany, CAS (KUN).

### DNA extraction and amplified fragment length polymorphism (AFLP) experiment

Total genomic DNA was extracted from dried leaf material according to the CTAB protocol of Doyle and Doyle (1987), treated with RNAase (30 min at 37°C), and adjusted to a concentration of ca. 25 ng/µl. The AFLP analysis followed the protocol described by Vos et al. (1995) with minor modifications. 250 ng/10 µl of genomic DNA were restricted with the enzyme combination *Mse*I and *Eco*RI and ligated to *Mse*I and *Eco*RI adapters. Pre-amplifications were performed in an ICYCLER thermal cycler [BioRad] using primer pairs with a single selective nucleotide (*Eco*RI-A/*Mse*I-A). Sixteen different primer combinations with three selective nucleotides were tested for the selective amplification. On the basis of consistency of results and the number of scoreable bands, two primer combinations were chosen to carry out selective amplification for all samples, namely *Eco*RI-AGA/*Mse*I-AAG and *Eco*RI-AAC/*Mse*I-ACC. The products of final amplification were denatured and loaded on 6% polyacrylamide denaturing gels with 100 bp DNA Ladder [Promega] as a standard. The bands were sequentially visualized by silver staining (Figure 1) (Bassam et al., 1991). The gels were scanned on an A3 sized scanner and bands were visually scored as present or absent with the aid of LabWorks Analysis Software version 4.0 (UVP). Only bands that could be scored confidently were recorded.

### Total alkaloid extraction and HPLC analysis

The dried and powdered leaves of *A. scholaris* (5.0 g) were extracted with MeOH (each 25 ml) five times at room temperature each, for 24 h, and the solvent was evaporated *in vacuo*. The residue was dissolved in 1% HCl, and the acidic solution was adjusted from pH 9 to 10 with ammonia. The basic solution was partitioned with EtOAc, and then EtOAc layer was evaporated *in vacuo* to afford total alkaloids which were subjected to further analysis. HPLC analysis was carried out on a Waters 2695 separation module equipped with a Waters 2996 photodiode array detector and Millenium32 software using a Xterra RP18 column (4.6 × 250 mm, 5 um) and a solvent system of 20:80 MeCN/0.5% $CF_3COOH$-$H_2O$ isocratic, with a flow rate of 1 ml/min. The column temperature was 35°C and 40 min running time for each sample. Peaks in the HPLC profiles were visually scored as present or absent.

### Statistical analysis

Based on the constructed present/absent data matrix, the molecular diversity indices, total number of loci, percentage of polymorphic loci, mean pairwise differences and average gene/alkaloid diversity over loci were generated by Arlequin 2.01. An Analysis of Molecular

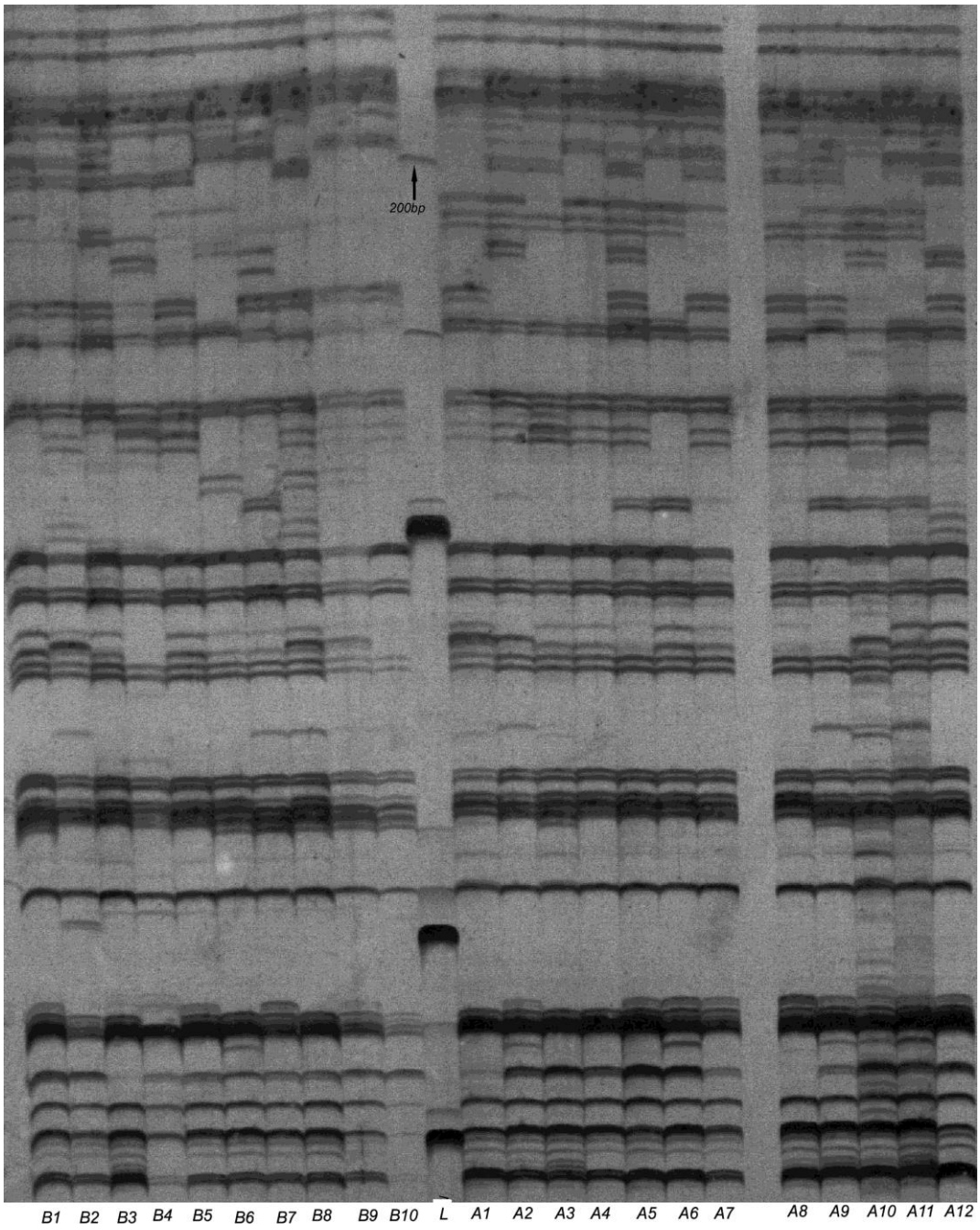


**Figure 1.** Example of AFLP profile obtained for *Alstonia scholaris* with the primer combination *Eco*RI-AGA/*Mse*I-AAG. B1-A12, codes for individuals. B1= the first individual of population B. L= 100 bp DNA ladder [Promega].

**Table 1.** Material resources, vouchers, and population codes.

| Taxon | Population code | Location | Voucher | Sample size |
|---|---|---|---|---|
| *Alstonia scholaris* | A | Kengma | ZZY2005002 | 15 |
| | B | Malipo | LS2005001 | 15 |
| | C | Chiangcheng | ZZY2005001 | 15 |
| | D | Jinping | LS2005002 | 15 |

**Table 2.** Biodiversity within the *Alstonia scholaris*, which was evaluated by number and percentage of polymorphic loci, mean pairwise difference within population, and average diversity over loci (with 5% allowance of missing data; standard deviation in parentheses).

| population | Number of polymorphic loci | | Percentage of polymorphic loci | | Mean number of pairwise differences | | Average diversity over loci | |
|---|---|---|---|---|---|---|---|---|
| | Gen. | Alk. | Gen. | Alk. | Gen. | Alk. | Gen. | Alk. |
| A | 41 | 10 | 31.80 | 31.3 | 15.90 (7.48) | 3.87 (2.10) | 0.12 (0.07) | 0.12 (0.07) |
| B | 41 | 8 | 31.80 | 25 | 14.55 (6.87) | 3.51 (1.94) | 0.11 (0.06) | 0.11 (0.07) |
| C | 46 | 15 | 35.70 | 46.90 | 16.44 (7.72) | 5.31 (2.78) | 0.13 (0.07) | 0.17 (0.10) |
| D | 43 | 14 | 33.30 | 43.80 | 15.79 (7.43) | 5.40 (2.82) | 0.12 (0.06) | 0.17 (0.10) |
| Total | 54 | 21 | 41.80 | 65.60 | 18.19 (8.17) | 5.93 (2.89) | 0.14 (0.07) | 0.19 (0.10) |

Total loci are 129 for AFLP and 32 for alkaloid profile. Percentage of polymorphic loci = Number of polymorphic loci/total loci; Gen. = genetic statistics based on AFLP profiling; Alk. = statistics base on total alkaloid profiling.

**Table 3.** AMOVA-designed variance among and within populations of *Alstonia scholaris*.

| Source of variation | D. F. | Sum of squares | | Variance components | | Percentage of variation | |
|---|---|---|---|---|---|---|---|
| | | Gen. | Alk. | Gen. | Alk. | Gen. | Alk. |
| Among populations | 3 | 97.78 | 34.25 | 1.65 | 0.92 | 17.40 | 28.82 |
| Within populations | 56 | 483.80 | 81.40 | 7.83 | 2.26 | 82.60 | 71.18 |
| Total | 59 | 536.58 | 115.65 | 9.48 | 3.18 | - | - |

Gen. = Genetic statistics based on AFLP profiling; Alk. = statistics base on total alkaloid profiling.

Variance (AMOVA; Excoffier et al. 1992) was also performed using Arlequin 2.01 with 1000 permutations and the same general settings as for the calculation of molecular diversity dices. The structure was defined to partition the total variance into components among populations and among individuals within populations. The variance components of AFLP were also used to calculate the fixation index Fst.

## RESULTS

Overall genetic/alkaloid variation for *A. scholaris* obtained by high performance liquid chromatography (HPLC) and AFLP analysis was evaluated by total number of loci, number and percentage of polymorphic loci, mean pairwise differences, and average gene/alkaloid diversity over loci (Table 2). Each individual displayed a unique AFLP and HPLC profile in the analysis, indicating extensive genetic/alkaloid variation within *A. scholaris*. For genetic variation, a total of 129 reliable loci were recorded with two primer combinations, of which 54 loci (42%) were polymorphic at species level and 75 bands were shared by all individuals. The percentages of polymorphic loci at population level were lower than that at species level, ranging from 32 to 36%. While for alkaloid variation, a total of only 32 peaks were recorded, of which 21 peaks were being polymorphic at species level and 11 peaks shared by all individuals. The mean value of pairwise differences at species level was 18 loci in the AFLP analysis and 6 peaks in the HPLC analysis. The average diversity over loci at species level was 0.14 for the amplified DNA fragments and 0.19 for the alkaloids. With respect to variance components within and among populations, *A. scholaris* displayed similar patterns in the AFLP and the HPLC profiling. AMOVA analysis attributed 17% of total genetic variance (29% of alkaloid variation) among the populations and 83% (71% of alkaloid variation) within the populations (Table 3). Moreover, the average genetic fixation index between populations was estimated to be 0.17.

## DISCUSSION

### Diversity pattern displayed by *A. scholaris*

*A. scholaris* is a ‘good’ species, very homogenous without much difference recognized between populations from different geography areas, and easily distinguished from other *Alstonia* species. Nevertheless, the AFLP and HPLC analysis disclosed considerable genetic and alkaloid variation within the species. Each individual presents a unique AFLP and HPLC profile. With respect to structure of variance, the *A. scholaris* displayed limited divergence and strong gene flow among populations. Only little portion of total variance resided among populations whether in AFLP or HPLC analysis, indicating a strong out-crossing tendency of the species. This meets the expectation of the species. *A. scholaris* is a perennial

tree with height of 20 to 40 m, which can facilitate the long-distance dissemination of pollen and seeds. Its strongly perfumed flowers are very attractive to insects, which can promote out crossing. Its pendulous fruits can stay on the branches for a long time and keep releasing the numerous small seeds. The seeds, bearing a tuft of hairs 7 to 13 mm long at each end, can easily float in the air (Li et al., 1995). These characteristics together make long-distance dispersal of the seeds possible, promoting populations in different geographical areas to be homogenized. Therefore, it is not surprising if strong gene flow is detected among populations of the species.

## Attributes of DNA and chemical profiling

Chemical fingerprinting is based on chemical constituents which are the physical foundation of medicinal function of a TCM, while DNA fingerprinting is an in-direct reflection of the chemical constituents. Therefore, chemical fingerprinting apparently seems in authority on quality control of TCMs, which is the main argument of phyto-chemists supporting chemical fingerprinting as the only legitimate tool. As expected, more than 50% of alkaloid loci were kept constant across any single population (Table 2), and the patterns of variance structure disclosed by the alkaloid scanning was similar to that by AFLP profiling (Table 3), suggesting that the alkaloid secondary metabolism in the plant can be genetically based on nature. The genetic basis of the alkaloid secondary metabolism justifies DNA fingerprinting as a qualified tool for quality control of the TCM.

Each individual of *A. schlolaris* displayed a unique AFLP or HPLC profile. This implies that both AFLP and alkaloid scanning method possess well individual-distinguishing ability, powerful enough to be identifier of TCMs. Nevertheless, there are still some subtle differences between the two kinds of marker system that should be kept in mind. First, with two primer combinations, the AFLP analysis produced 129 reliable loci, whereas the alkaloid scanning produced only 32 information loci which could not be increased further by adjusting experimental parameters. This suggests that AFLP method can produce much more loci for succedent statistical analysis, which will be helpful for eliminating influence of experimental error and other random factors and therefore improve the confidence coefficient. Secondly, polymorphic loci percentage for the limited alkaloid loci in the HPLC profiling is higher than that in the AFLP profiling. This can be explained as the result of environmental plasticity of secondary metabolism and indicates that chemical scanning is more sensitive. Thirdly, DNA fingerprinting like AFLP cannot play role on TCMs in the form of chemical extract, while chemical scanning can play role on TCMs in various dosage forms, having a wider application range. Therefore, with respect to information capacity and moderate sensitivity, genetic fingerprinting such as AFLP is a preferred identifier especially in the case of identifying plant materials.

## Strategy to standardize TCM

Chemical compounds are the physical foundation of function of TCM. Therefore, the most ambitious intention of TCM standardization is to make a specific TCM chemically completely identical so that TCM can meet the standard of modern medicine. Frustratingly, a ‘good’ species like *A. scholaris* bears considerable genetic and chemical variation all the same, not to say other species with multiple varieties or ecological races. Pursuing chemically identical TCM is practically doomed to fail, because no two identical biological individuals exist in the world owing to environmental plasticity and individual history, even for twin brothers or clones which are genetically identical. Does this mean that standardization of TCM is impossible or we should give up TCM thoroughly? Of course the answer is no. The key point is that we should adopt an appropriate standard with appropriate flexibility. In fact, it is one of the logic foundations of TCM pharmaceutics that different individuals of a species bear similar chemical constituents and function similarly as medicine. What should be done is to control the strictness when to define or delimit a specific TCM.

In view of the alkaloid diversity displayed by *A. scholaris* and other complex species like *Spiraea japonica* (Zhang et al., 2006), it can be confirmed that much chemical variation exist within traditional defined TCMs. However, the identity control of many TCMs is routinely based on traditional morphological classification, which might ignore the much variance residing within a species. In addition, it has often happened that several closely related and morphologically undistinguishable species or varieties are un-discerned and used as substituted agents of a trueborn TCM, which makes things worse. This practice is one of the root reasons hindering further healthy development of TCM. Homonym of TCMs will make it impossible for doctors or pharmacologists to accurately evaluate the aimed TCM by recording and comparing the curative effect or bio-activity of the TCM among different cases, different periods and different recipe. Also, it will be difficult for doctors or pharmacologists to exchange their experiences or views on a specific TCM or on a recipe. Therefore, identity control of TCMs ought to be strengthened in TCM practice. On another hand, ambition to make a TCM chemically completely identical is unpractical and also unnecessary in practice. From perspective of long-term development of TCM, pursuing genetically identical TCMs is a less ambitious and achievable goal and may be adequate. Advocating plantation and popularization of germplasm selected is the way to gradually achieve the goal.

## Conclusion

Conclusively, it was confirmed that much genetic and chemical variation exist within traditional defined TCMs. Identity control of traditionally defined TCMs ought to be strengthened and standardization is necessary for further development of TCM. In practice, pursuing genetically identical TCM is a less ambitious and achievable goal. Plantation and popularization of germplasm selected is the way to achieve the goal gradually. For identity control of TCMs, genetic and chemical fingerprinting are two powerful tools with several advantageous and disadvantageous aspects, respectively. With respect to information capacity and moderate sensitivity, genetic fingerprinting like AFLP is a preferred identifier especially in the case of identifying plant materials.

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