Pak. J. Bot., 47(1): 103-107, 2015.

KARYOTYPES AND FISH DETECTION OF 5S AND 45S RDNA LOCI IN CHINESE MEDICINAL PLANT *ATRACTYLODES LANCEA* SUBSP. *LUOTIANENSIS*: CYTOLOGICAL EVIDENCE FOR THE NEW TAXONOMIC UNIT

YUAN-SHENG DUAN[1, 2], BIN ZHU [1], SHAO-HUA SHU, ZAI-YUN LI[1] AND MO WANG[1*]

[1]*Institute of Medicinal Plants, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China*

[2]*College of Biological and Chemical Engineering, Huanggang Polytechnic University, Huanggang 438002, Hubei Province, P.R. China*

**Corresponding author e-mail: wangmo@mail.hzau.edu.cn; Tel.: +86 27 87280543*

Abstract

Atractylodes lancea (Thunb.) DC. in the *Asteraceae* family produces the atractylodes rhizome which is widely used as a traditional medicine in China. The subspecies *A. lancea* (Thunb.) DC subsp. *Luotianensis* distributed in mountainous Luotian and Yingshan regions in Hubei Province presented distinct morphology and superior medicinal quality. This study firstly reported the chromosome karyotype of this subspecies and the detection of 5S and 45S rDNA loci by fluorescent *in situ* hybridization. The karyotype was 2*n*=24=12m+12sm (2SAT). A single locus of 5S rDNA and two loci of 45S rDNA loci were identified and separated on different chromosomes. Its one pair of the satellited chromosomes rather than two pairs in other *Atractylodes* species yet still with 2*n*=24 occurred likely after its occupation of this geographic location. The evidence of karyotype differentiation of this subspecies native to the area is useful for elucidating the genome structure and identifying chromosomes.

Key words: Karyotypes, fish detection, rDNA, *Atractylodes lancea, Luotianensis*.

Introduction

Chromosome karyotypes can give information about taxonomic relationships, genetic aberrations, and the evolutionary origins of species (Young *et al*., 2012; Wang *et al*., 2013). But the establishment of a standard karyotype is usually difficult for the plants with the chromosomes of small and similar sizes and with the lack of distinguishing features for individual chromosomes. The genus *Atractylodes* belongs to the *Carlininae* subtribe, the *Cynareae* tribe in the *Asteraceae* family and includes about eight species, mainly *Atractylodes macrocephala* Koidz. and *A. lancea* (Thunb.) DC. These perennial herbal plants are distributed in China, Korea and Japan, and most of them, particularly *A. lancea* are used as traditional medicinal plants. They can be reproduced and cultivated by seeds, division propagation and rhizome. The dry atractylodes rhizome is used for the clinical indications: oedema; vomiting, diarrhoea, anorexia, abdominal distension; night blindness, blurring of vision; rheumatism; eczema, weeping skin lesions. The main components of the volatile oil in its rhizome are hinesol, eudesmol and atractylone. The medicinal plant is cultivated in many regions of China, including Hubei Province. *A. lancea* distributed in mountainous Luotian and Yingshan Counties of Hubei Province in the Mt. Dabieshan range was morphologically different from the types from other regions and was classified as a new subspecies, *Atractylodes lancea* (Thunb.) DC subsp. *Luotianensis* S. L. Hu et X. F. Feng (Hu *et al*., 2001). The atractylodes rhizome produced in Luotian and Yingshan is well recognized for its good quality and high content of volatile oil and was given geographical indication protection in 2011. This study firstly investigates the chromosomal karyotype of this plant from the area and the detection of 5S and 45S rDNA loci, which provides new data for its independent taxonomic position and for the identification of its chromosomes and genome structure.

Nucleolus organizer regions (NORs) are recognized as secondary constrictions in satellited (SAT) chromosomes. The 18S–5.8S–25S ribosomal RNA genes (45S rDNA) and intergenic spacer regions (18S–25S rDNA) exist as tandem repeats at the NORs and at other chromosomal sites where they may not be associated with an NOR. The 18S–5.8S–25S rRNA genes are present in several hundreds of tandemly repeated units of the three genes with intergenic spacers, organized in one or more clusters within the genome (Banaras *et al*., 2012; Aman *et al*., 2013). The genes that code for 5S rRNA (5S rDNA) make up an independent multilocus, multigene family and are organized into clusters of tandem repeats with up to thousands of copies of repeated units (Appels & Honeycut, 1986). In most higher eukaryotes, the 5S rRNA genes are arranged in tandem arrays at one or more chromosomal loci, mostly separated from the 45S rDNA (Fedoroff, 1979). Fluorescence *In situ* hybridization (FISH) analysis revealed that most of the diploid plants examined had two sites (*i.e.* a single locus) of both 5S and 18S–5.8S–26S rDNA, although some diploids had multiple sites (Fukui *et al*., 1994; Ansari *et al*., 1999; Raina & Mukai, 1999; Zhang & Sang, 1999). The rDNA genes (5S and 18S–5.8S–25S rDNA) and their chromosomal organization have been shown to be suitable for the karyological characterization of species with small and similarly sized chromosomes as well as for establishing taxonomic and phylogenetic analysis (Baum & Appels, 1992; Shibata & Hizume, 2002). In this study, a single locus of 5S rDNA and two locus of 45S rDNA were detected in *A. lancea* (Thunb.) DC subsp. *Luotianensis*, which were located on different chromosomes.

Materials and Methods

Plant material and chromosome preparation: The seeds of *Atractylodes lancea* (Thunb.) DC. subsp. *Luotianensis* S. L. Hu et X. F. Feng were collected on the mature plants cultivated in the fields at one typical site in

Yingshan County, Hubei Province, China. After the seeds were dried in the air, they were placed on wet filter paper in a petri dish to germinate in an incubator at 22°C in the dark. Root tips from 1–2 cm roots were harvested and pretreated in 2mM hydroxyquinoline solution for 2h at room temperature, and then fixed in ethanol: acetic acid solution (3:1). After fixation at room temperature (RT) overnight, they were stored in 70% ethanol at 4°C for cytological observations.

Chromosome number and karyotype analysis: To determine the chromosome number and karyotype, the mitotic chromosome preparations from the fixed root-tips were stained with 1% carbol fuchsin solution according to the procedure of Li *et al*. (1995). Good chromosome samples at metaphase were captured with CCD camera, and five good chromosome plates at metaphase were used to measure the chromosome parameters and construct the ideogram according to the chromosome length, centromere position and number of satellites. Nomenclature for the centromeric positions of chromosome follows Levan *et al*. (1964) and the karyotype classification follows Stebbins (1971).

Dual-color in situ hybridization: Slide preparations of chromosomes for FISH were made mainly according to Zhong *et al*. (1996) and Ge *et al*. (2009) with some modifications; an enzyme mixture containing 0.6% cellulose Onozuka RS (Yakult, Tokyo, Japan), 0.2% pectinase (Merck, Darmstadt, Germany), and 0.5% Snailase (Beijing Baitai Biochem Co. Ltd., Beijing, China) was used.

The 5S and 45S rDNAs isolated from tomato provided by Prof. Lijia Li, College of Biology, Wuhan University, China, were labeled with biotin-11-dUTP (Sabc, China) and digoxigenin-11-dUTP (Roche, Basel, Switzerland), respectively, by nick translation and used as probes. The hybridization signals of 5S and 45S rDNAs probes were detected using Cy3-labeled streptavidin (Sigma, St. Louis, Missouri, USA) and FITC-conjugated sheep anti-digoxigenin antibody (Roche, Basel, Switzerland), respectively. Signals from 45S rDNAs were amplified one time by FITC-conjugated anti-sheep IgG antibody (Roche). Preparations were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) solution (Roche)(1 mg/mL), mounted in antifade solution (Vector Laboratories, Peterborough, UK), and viewed under a fluorescence microscope (Nikon Eclipse 80i, Japan) equipped with a CCD camera. Images were processed by Adobe Photoshop (8.0).

Results and Discussion

Chromosome number and karyotype of *Atractylodes lancea*: In the incubator at 22°C, the roots appeared on the seeds in 3 days and reached 1-2 cm length in about 7 days on wet filter paper in the petri dish. It was difficult to obtain the mitotic cells at metaphase with good chromosome morphology. Majority of cells observed had $2n$=24 (Fig. 1a, b), but a minority seemed to have $2n$=23, 25. The two sister chromatids of each chromosome were easily visible at prometaphase/ metaphase. These chromosomes showed even condensation at prometaphase and metaphase and had no obvious heterochromatic blocks in the centromeric and other regions (Fig. 1a, b).

The karyotype was constructed from the chromosome parameters of the cells with $2n$=24 (Fig. 1c, d). The chromosome lengths ranged from 1.93μm to 3.79μm with gradual increasing gradients (Table 1). The relative lengths were from 6.18% to 12.17%. The longest chromosome had nearly the doubled length of the shortest one. Based on the arm ratio, the chromosomes 1, 4, 7, 8, 10, 11 were classified as metacentric (m) chromosomes, the remaining six (2, 3, 5, 6, 9, 12) as submetacentric (sm) chromosomes. Only chromosome 5 carried the satellite, but the frequency of the satellited chromosomes was low in the prometaphase / metaphase cells. According to the classification standards of karyotype symmetry by Stebbins (1971), the karyotype of *A. lancea* belonged to 2A, the most symmetric one. Then the species maintained the primitive karyotype. The karyotype formula was $2n$=24=12m+12sm (2 SAT).

Detection of 5S and 45S rDNA loci by dual FISH: The double-target *in situ* hybridization with biotin-labeled 5S rDNA and digoxigenin-labeled 45S rDNA probes revealed two 5S rDNA signals and four 45S rDNA signals (Fig. 2a, b). These 5S and 45S rDNA signals were located on different chromosomes. Two 5S rDNA signals were localized at the interstitial parts of the short arms of two longest chromosomes, while the four 45S rDNA sites of different sizes appeared at terminal parts of four chromosomes, two certainly at satellites of the chromosome 5, the other two on the short arms of another chromosome with unknown identity. It was difficult to identify individual chromosomes from DAPI images, because the chromosome morphology was deformed to some extents after FISH process.

The karyotype analysis for other three species of *Atractylodes*, *A. chinensis* (DC.) Koidz. K, *A. japonica* Koidz. K and *A. koreana* (Nakai) Kitam. K collected in north-east China also showed that they had $2n$=24, and that the cells with other chromosome numbers ($2n$=20, 22) appeared at low percentages (Ge *et al.*, 1987; Ge, 1989; Wang *et al*., 1997). The other numbers ($2n$=20, 22) were given earlier for *A. japonica* and *A. koreana* (Li *et al.,* 1985; Li & You, 1987; Li & Li, 1988), which were thought to result from poor quality of cytological images (Wang *et al*., 1997). The karyotype of these three species expressed some same characteristics, the same karyotype symmetry, two pairs of the satellited chromosomes, the similar ratio between the longest and shortest chromosomes, but also obvious differences in chromosome types and percentages (mainly m, sm, rarely st). The satellites were also small and were difficult to detect on the condensed chromosomes. The impressive differentiation of the karyotype between the three species and our subspecies was the different satellited chromosomes, four for the former and two for the later, though its four chromosomes carried 45S rDNA loci (Fig. 2). So the karyotype of this subspecies diverged from those of other species by differential expression of 45S rDNA loci and other chromosomal rearrangements (Zhang and Chen, 1991; Ge, 1989; Wang *et al*., 1997), which likely occurred after it occupied the specific area.

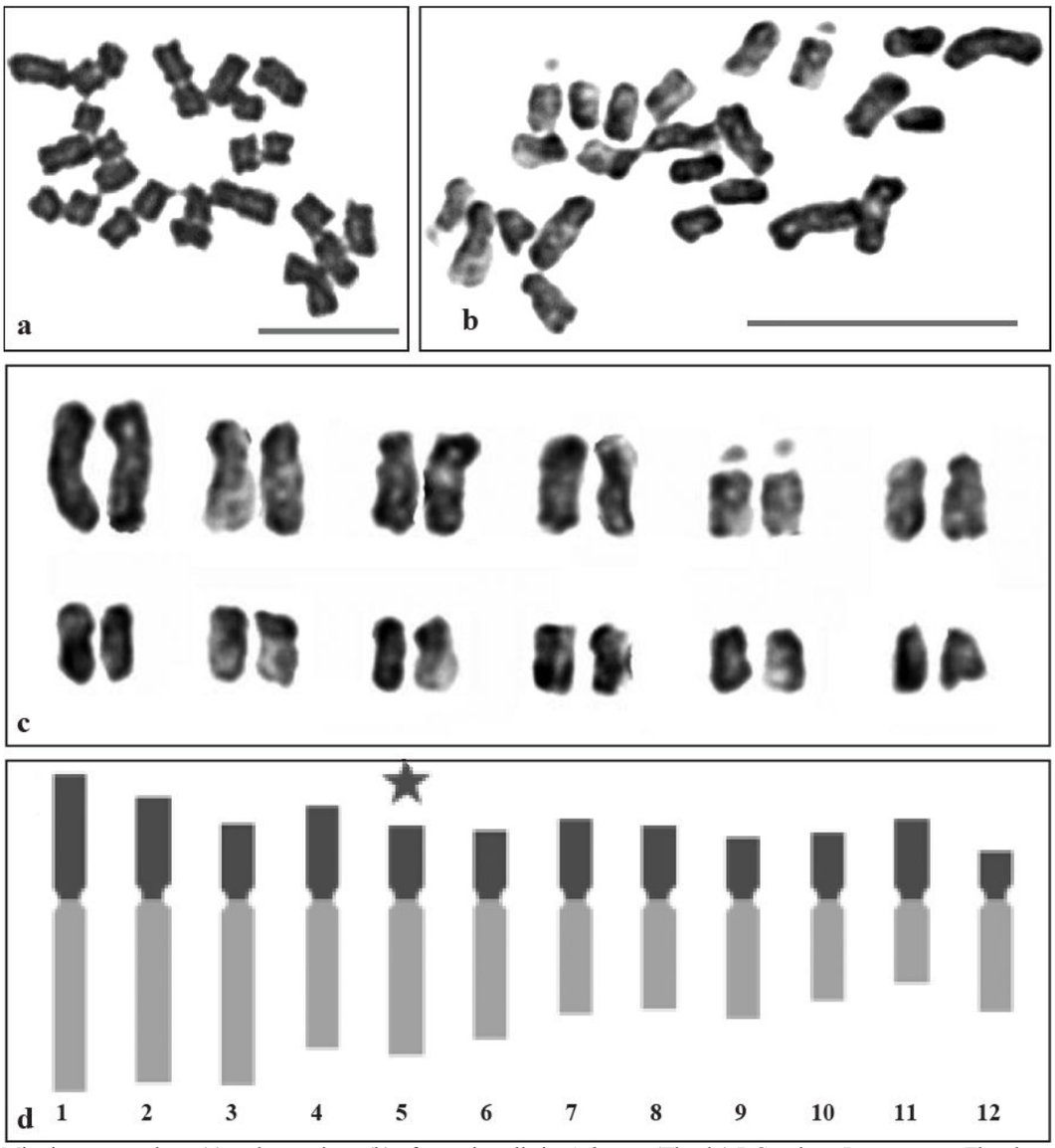

Fig. 1. Mitotic prometaphase (a) and metaphase (b) of root-tip cells in *A. lancea* (Thunb.) DC. subsp. *Luotianensis*. The chromosome karyotype (c) was constructed from the chromosomes in the cell (b). The ideogram (d) was based on the chromosome parameters in Table 1. The * on the short arm of the chromosome 5 represents the satellite determined from the chromosome morphology of the cell (b). Bar: 10μm.

Table 1. Chromosome parameters and classification for *A. lancea* (Thunb.) DC. subsp. *Luotianensis*.

No. of chromosomes	Length of short arm (μm)[a]	Length of long arm (μm)	Arm ratio	Absolute length (μm)	Relative length (%)	Type
1	1.47	2.32	1.58	3.79	12.17	m
2	1.2	2.20	1.83	3.40	10.93	sm
3	0.89	2.23	2.51	3.13	10.04	sm
4	1.1	1.81	1.65	2.91	9.34	m
5	0.85	1.89	2.22	2.74	8.79	sm(sat)
6	0.82	1.71	2.09	2.52	8.11	sm
7	0.95	1.38	1.45	2.34	7.50	m
8	0.87	1.34	1.54	2.21	7.11	m
9	0.72	1.46	2.03	2.18	7.02	sm
10	0.78	1.25	1.60	2.02	6.50	m
11	0.94	1.02	1.09	1.96	6.31	m
12	0.56	1.36	2.43	1.93	6.18	sm

[a] The satellites (sat) are excluded from the calculation of the length of short arms

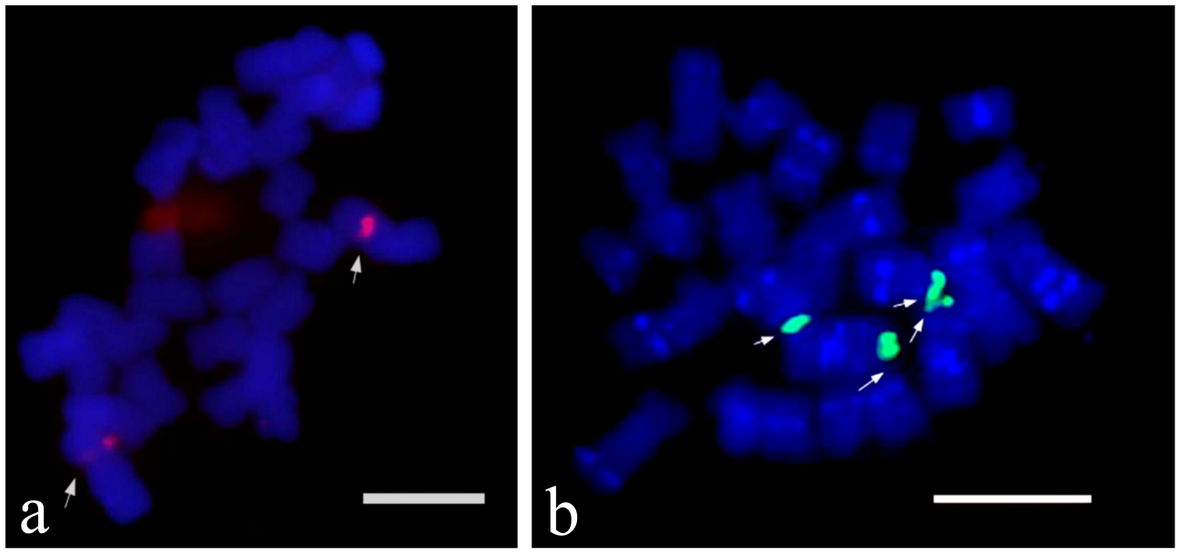

Fig. 2. FISH detection of 5S (a) and 45S (b) rDNAloci in *A. lancea* (Thunb.) DC. subsp. *Luotianensis*. Red and green signals (arrowheads) are from the labeled 5S and 45S rDNAs probes, respectively, and the blue color is from the DAPI staining of the chromosomes. Bar: 5μm.

Though the chromosome numbers of *Asteraceae* plants showed wide variations with x=2 to x=110～120, the most common number was x=9, even in polyploidy species. The number $2n$=24 in *A. lancea* was near to the most frequent one in this family, another species *A. macrocephala* in the same genus also had $2n$=24 (Ge *et al.,* 1987; Zhang & Chen, 1991). The two sites (a single locus) of 5S rDNA likely revealed the diploidy nature of this species, in spite of four sites (two loci) of 45S rDNA, as most of the diploid plants observed showed two sites of both 5S and 45S rDNA (Fukui *et al*., 1994; Ansari *et al*., 1999; Raina & Mukai, 1999; Zhang & Sang, 1999). The 5S and 45S rDNA loci provided the molecular markers for the identification of particular chromosomes, specifically for the non-satellited chromosomes.

Acknowledgements

This work was supported by a grant from Major National Science and Technology Programs in the "Twelfth Five-Year Period" of Ministry of Science and Technology, P. R. China (No. 2012ZX09304006).

References

Aman, S., M. Iqbal, S. Abbas, S. Banaras, M. Awais, I. Ahmad, Z.K. Shinwari and S.N. Shakeel. 2013. Molecular and comparative analysis of newly isolated beta-tubulin partial gene sequences from selected medicinal plants. *Pak. J. Bot*., 45(2): 507-512.

Ansari, H.A., N.W. Ellison, S.M. Reader, E.D. Badaeva, B. Friebe, T.E. Miller and W.M. Williams. 1999. Molecular cytogenetic organization of 5S and 18S–26S rDNA loci in white clover (*Trifolium repens* L.) and related species. *Ann. Bot*., 83: 199-206.

Appels, R. and R.L. Honeycut. 1986. rDNA: evolution over a billion years. In: *DNA Systematics: plants*. (Ed.): Dutta, S.K. CRC Press, Boca Raton, USA: 82-135.

Banaras, S., S. Aman, M. Zafar, M. Khan, S. Abbas, Z.K. Shinwari and S.N. Shakeel. 2012. Molecular identification and comparative analysis of novel *18s Ribosomal RNA* genomic sequences of wide range of medicinal plants. *Pak. J. Bot*., 44(6): 2021-2026.

Baum, B.R. and R. Appels. 1992. Evolutionary change at the 5S DNA loci of species in the *Triticeae*. *Plant Syst. Evol.*, 183: 195-208.

Fedoroff, N.V. 1979. On spacers. *Cell*, 16: 697-710.

Fukui, K., Y.K. Kamisugi and F. Sakai. 1994. Physical mapping of 5S rDNA loci by direct-cloned biotinylated probes in barley chromosomes. *Genome*, 37: 105-111.

Ge, C.J. 1989. Cytological study of *Atratylodes chinesis*. *Guhaia*, 9: 105-109.

Ge, C.J., Y.K. Li and B.S. Xu. 1987. Karyotype analysis in *Atratylodes macrocephala*. *Acta Bot. Yunnan*., 9: 116-118.

Ge, X.H., J. Wang and Z.Y. Li. 2009. Different genome-specific chromosome stabilities in synthetic *Brassica* allohexaploids revealed by wide crosses with *Orychophragms*. *Ann. Bot.*, 104: 19-31.

Hu, S.L., X.F. Feng, J. Wang and X.G. Ge. 2001. A new subspecies of *Atractylodes* from China. *Acta Phytotaxonomica Sin*., 39: 84-86.

Levan, A., K. Fredga and A.A. Sandberg. 1964. Nomenclature for centromeric position on chromosomes. *Hereditas*, 52: 201-220.

Li, J.Y. and M.H. Li. 1988. Studies on three major nucleus types of Chinese *Atractylodes*. *J. Shengyang Agri. Coll.*, 19: 58-61.

Li, J.Y. and W.C. You 1987. Analysis of nucleus type of Chromosomes in Japanese *Atractylodes*. *J. Shengyang Agri Coll.*, 18: 75-77.

Li, J.Y., W.C. Yang and D.W. Ma. 1985. Analyses of chromosome nuclear patterns of Korean *Atractylodes*. *J. Shengyang Agri. Coll.*, 16: 70-72.

Li, Z., H.L. Liu and P. Luo. 1995. Production and cytogenetics of intergeneric hybrids between *Brassica napus* and *Orychophragmus violaceus*. *Theor. Appl. Genet*., 91: 131-136.

Raina, S.N. and Y. Mukai. 1999. Detection of a variable number of 18S–5.8S–26S and 5S ribosomal DNA loci by fluorescent *in situ* hybridization in diploid and tetraploid *Arachis* species. *Genome*, 42: 52-59.

Shibata, F. and M. Hizume 2002. Evolution of 5S rDNA units and their chromosomal localization in *Allium cepa* and *Allium schoenoprasum* revealed by microdissection and FISH. *Theor. Appl. Genet*., 105: 167-172.

Stebbins, G.L. 1971. *Chromosomal Evolution in Higher Plants*. Edward Arnold, London, UK.

Wang, C., X.F. Xing, Z.X. Wang and M.Y. Liu. 1997. Studies on the cytology of *Atractylodes* DC. in the northeast of China. *Bullet. Bot. Res*., 17: 79-84.

Wang, S.Q. 1023. Study on chromosomal structural heterozygosity in *Paeonia emodi*, an endangered species. *Pak. J. Bot*., 45(3): 713-718.

Young, H.A., G. Sarathand and C.M. Tobias. 2012. Karyotype variation is indicative of subgenomic and ecotypic differentiation in switchgrass. *BMC Plant Biol*., 12: 117.

Zhang, D. and T. Sang. 1999. Physical mapping of ribosomal RNA genes in Peonies (*Paeonia*, Paeoniaceae) by fluorescent *in situ* hybridization: implications for phylogeny and concerted evolution. *Am. J. Bot*., 86: 735-740.

Zhang, D.C. and A.Z. Chen. 1991. Studies on karyotype of two medicinal materials in *Atractylodes macrocephala*. *J. Anhui Normal Univ. (Nat. Sci.)*, (1): 33-38.

Zhong, X.B., J.J. de Hans and P. Zabel. 1996. Preparation of tomato meiotic pachytene and mitotic metaphase chromosomes suitable for fluorescence in situ hybridization (FISH). *Chromosome Res*., 4: 24-28.

(Received for publication 24 July 2013)