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Determination of genetic relationships between evergreen azalea cultivars in China using AFLP markers*

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Abstract: Evergreen azaleas are among the most important ornamental shrubs in China. Today, there are probably over 300 cultivars preserved in different nurseries, but with little information available on the cultivar itself or relationships between cultivars. Amplified fragment length polymorphism (AFLP) markers were employed to determine the genetic relationships between evergreen azalea cultivars in China. One hundred and thirty genotypes collected from gardens and nurseries, including cultivars classified in the groups East, West, Hairy, and Summer, unknown cultivars, and close species, were analyzed using three primer pairs. A total of 408 polymorphic fragments were generated by AFLP reactions with an average of 136 fragments per primer pair. The average values of expected heterozygosity and Shannon's information index were 0.3395 and 0.5153, respectively. Genetic similarities were generated based on Dice coefficients, used to construct a neighbor joining tree, and bootstrapped for 100 replicates in Treecon V1.3b. Principal coordinate analysis (PCO) was performed based on Dice distances using NTSYS-pc software. The AFLP technique was useful for analyzing genetic diversity in evergreen azaleas. Cluster analysis revealed that cultivars in the West and Summer groups were quite distinct from other groups in the four-group classification system and that the East and Hairy groups should be redefined.

Key words: *Rhododendron* spp., *Tsutsusi*, Fluorescent label, Dice coefficient, Principal coordinate analysis (PCO), Neighbor joining tree

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1 Introduction

Evergreen azaleas, which belong to the genus *Rhododendron* subgenus *Tsutsusi* (Chamberlain and Rae, 1990), are among the most popular cultivated azaleas. Many species of *Tsutsusi* azaleas native to Asia were introduced to Europe by plant hunters from China and Japan in the 18th century and have since been used as parents to create new cultivars. Hybridisation activities reached remarkable levels in Europe, especially in Belgium and the United Kingdom (Scariot *et al*., 2007b).

Today, there are at least four or more well-defined groups of evergreen azalea cultivars, including Belgian or pot azaleas, Hirado azaleas, Kurume azaleas, and Satsuki azaleas (Scariot *et al*., 2007b). The latter three groups are often known as Japanese azaleas. Hirado azaleas are putative hybrids of *Rhododendron scabrum* (Scariot *et al*., 2007b). Kurume hybrids came from the natural hybridization between the species *Rhododendron kaempferi* and *Rhododendron kiusianum*, and spread mainly on the Kirishima Mountain in Japan. Human breeding activities probably started 200 years ago (Kobayashi *et al.*, 2003). Now the Kurume azaleas have dimorphic leaves, funel-shaped corolla, and sometimes with stamens and pistil petaloid (*R. kaempferi* var. *plenum* Nakai) or calyx petaloid (*R. kaempferi* var. *komatsui* Nakai) (Chamberlain and Rae, 1990). Satsuki azaleas

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were thought to be hybrids of *Rhododendron indicum* and *Rhododendron eriocarpum* (Scariot *et al*., 2007b), and have been cultivated since 17th century (Shen, 2004). This species has monomorphic leaves, broadly funnel-campanulate corolla, blooms from mid May until late July, and is often used for bonsai (Chamberlain and Rae, 1990).

In China, azaleas became popular as ornamental flowers in the Tang dynasty and are now recognised as one of the ten famous traditional flowers. Exotic cultivars arrived from Japan and Europe in the 19th century, and these cultivars were collected, hybridised, and maintained by gardeners from all over the country. There are now over 300 cultivars preserved in Dandong (Liaoning Province), Wuxi (Jiangsu Province), and Jiashan (Zhejiang Province), all of which are usually classified as belonging to four different groups according to their phenotype and origin (Huang and Qiang, 1984): (1) East azaleas, formed by cultivars with small leaves and hose-in-hose flowers; (2) West azaleas, including plants with large flowers and semi-double to double petals; (3) Hairy azaleas, characterised by large simple flowers and finely pubescent stems and leaves; and (4) Summer azaleas, cultivars blooming in the early summer. However, these definitions are vague and not particularly useful in practice. Thus, it is not surprising to see different cultivars sharing the same name or find that the same plant is called differently. As such, characterisation based on molecular data could help improve the classification and diversity of evergreen azaleas.

Over the last few decades, DNA-based markers have been proven to be very successful in assessing genetic diversity and classifying azalea cultivars both within and between species. These markers have been applied to a number of technologies, such as random amplified polymorphic DNA (RAPD) markers (Iqbal *et al*., 1995; Kobayashi *et al*., 1995), the amplified fragment length polymorphism (AFLP) technique (Escaravage *et al*., 1998; de Riek *et al*., 1999; Dendauw *et al*., 2002; Scariot *et al*., 2007b), *mat*K sequence (Kurashige *et al*., 1998), *trn*K sequence (Kurashige *et al*., 2001), internal transcribed spacer (ITS) sequence (Tsai *et al*., 2003), sequence tagged microsatellite site markers (Dendauw *et al*., 2002), and expressed sequence tag (EST) markers (Scariot *et al*., 2007a). Among these markers, AFLPs are the most appropriate for phylogenetic studies because of their wide genome coverage and high discrimination capacity (Scariot *et al*., 2007a).

In this work, we used AFLP markers to: (1) evaluate the efficiency of AFLP markers for genetic diversity assessment of azaleas; (2) determine the genetic similarity and genetic relationships among azalea cultivars; (3) contribute to the horticultural classification of cultivated azaleas in China.

2 Materials and methods

2.1 Plant materials

A total of 130 azalea genotypes were analyzed (Table 1), most of which were chosen from four local groups within the evergreen azaleas: East azaleas (63 genotypes), West azaleas (14 genotypes), Hairy azaleas (9 genotypes), and Summer azaleas (29 genotypes). Within the East and Hairy azaleas, several cultivars obtained from bud sporting were included (e.g., 'Yuhudie' with pink flowers and 'Baihudie' with pure white flowers are bud sports of 'Zihudie'; 'Fenshanhu' is a bud sport of 'Hongshanhu'). Three individuals of *R. simsii* with differently coloured flowers were collected from Lushan Botanical Garden. Aside from *R. simsii*, *R. denudatum*, which belong to the subgenus *Hymenanthes*, was included in this work. Eleven unknown genotypes were also used in the analysis. The three genotypes marked as C71, C72, and C73 were similar to 'Zihudie' but featured stickier surfaces and thicker hairs.

2.2 DNA extraction

Young healthy leaves (approximately 2 g fresh weight) were collected from potted plants maintained in the greenhouse. Several leaf materials were directly harvested from wild plants. All the leaves were dried in silica gel until DNA extraction. About 0.5 g dried leaf tissue and 0.05 g polyvinylpyrrolidone (PVP) were ground into a fine powder in liquid nitrogen, and incubated in 4 ml of preheated cetyltrimethylammonium bromide (CTAB) extraction buffer (100 mmol/L Tris-HCl pH 8.0, containing 2% (20 g/L) CTAB, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), 1.4 mol/L NaCl, and 0.22 g/L β-mercaptoethanol) for 40 min at 65 °C. After homogenisation with 4 ml chloroform/isoamylalcohol (24:1 v/v) and centrifugation (10000×*g*, 10 min, 4 °C), the supernatant was

Table 1 Names, group types, collected locations, and origins of evergreen azaleas included in AFLP analysis

ID	Name	Type	Collected location	Origin
Subgenus *Tsutsusi*				
B01	Baijiaren	West	Jiashan, Zhejiang	Unknown
B02	Shieryichong	West	Jiashan, Zhejiang	Unknown
B03	Hupo	West	Jiashan, Zhejiang	Unknown
B04	Yindujin	West	Jiashan, Zhejiang	Unknown
B05	Jinpao	West	Jiashan, Zhejiang	Unknown
B06	Guanshang	West	Jiashan, Zhejiang	Unknown
B07	Shuanghuahong	West	Jiashan, Zhejiang	Unknown
B08	Fuguiji	West	Jiashan, Zhejiang	Unknown
B09	Yuruijin	West	Jiashan, Zhejiang	Unknown
B10	Hengdi	West	Jiashan, Zhejiang	Unknown
B11	Huangguan	West	Jiashan, Zhejiang	Unknown
B12	Danding	West	Jiashan, Zhejiang	Unknown
B13	Fengmingjin	West	Jiashan, Zhejiang	Unknown
B14	Huamudan	West	Jiashan, Zhejiang	Unknown
C02	Dazhusha	East	Jiashan, Zhejiang	Unknown
C04	Dantaowusedaqiao	East	Jiashan, Zhejiang	Unknown
C05	Wusedaqiao	East	Jiashan, Zhejiang	Unknown
C08	Xiaoqinglian	East	Jiashan, Zhejiang	Unknown
C09	Daqinglian	East	Jiashan, Zhejiang	Unknown
C13	Hongshanhu	East	Jiashan, Zhejiang	Unknown
C14	Fenshanhu	East	Jiashan, Zhejiang	Unknown
C15	Baipingfu	East	Jiashan, Zhejiang	Unknown
C18	Fenzhuanglou	East	Jiashan, Zhejiang	Unknown
C19	Xitangxiaotaohon	East	Jiashan, Zhejiang	Unknown
C21	Dayuanyangjin	East	Jiashan, Zhejiang	Unknown
C24	Yunshang	East	Jiashan, Zhejiang	Unknown
C29	Duanyang	Summer	Jiashan, Zhejiang	Unknown
C31	Yushuang	East	Jiashan, Zhejiang	Unknown
C33	Hongzhuangsuguo	East	Jiashan, Zhejiang	Unknown
C34	Waiguohong	East	Jiashan, Zhejiang	Unknown
C35	Liuqiuhong	East	Jiashan, Zhejiang	Unknown
C36	Zhuangyuanhong	East	Jiashan, Zhejiang	Unknown
C38	Yinghong	East	Jiashan, Zhejiang	Unknown
C40	Ningbohong	East	Jiashan, Zhejiang	Unknown
C41	Caiwubao	East	Jiashan, Zhejiang	Unknown
C43	Taojuru	East	Jiashan, Zhejiang	Unknown
C45	Zuihaitang	East	Jiashan, Zhejiang	Unknown
C48	Zijuan	East	Jiashan, Zhejiang	Unknown
C49	Hongyue	East	Jiashan, Zhejiang	Unknown
C50	Lanying	East	Jiashan, Zhejiang	Unknown
C51	Lanlian	East	Jiashan, Zhejiang	Unknown
C54	Zhichun	East	Jiashan, Zhejiang	Unknown
C59	Huahudie	East	Jiashan, Zhejiang	Unknown
C62	Aidingbao	East	Jiashan, Zhejiang	Unknown
C64	Dahezhichun	East	Jiashan, Zhejiang	Unknown
C65	Shuizhishanchuan	East	Jiashan, Zhejiang	Unknown
C66	Shuangtaodaqiao	East	Jiashan, Zhejiang	Unknown
C67	Songjiangtaohong	East	Jiashan, Zhejiang	Unknown
C68	Yanzhi	East	Jiashan, Zhejiang	Unknown
C69	Bizhi	East	Jiashan, Zhejiang	Unknown
C70	Xiaomeigui	East	Jiashan, Zhejiang	Unknown
C71	Unknown	Unknown	Lushan, Jiangxi	Unknown

To be continued

Table 1

ID	Name	Type	Collected location	Origin
C72	Unknown	Unknown	Lushan, Jiangxi	Unknown
C73	Unknown	Unknown	Lushan, Jiangxi	Unknown
C74	Zihudie	Hairy	Wuxi, Jiangsu	China
C75	Yuhudie	Hairy	Wuxi, Jiangsu	China
C76	Baihudie	Hairy	Wuxi, Jiangsu	China
D01	Unknown	Unknown	Dandong, Liaoning	Unknown
D02	Linu	Unknown	Dandong, Liaoning	Unknown
D03	Shinu	Unknown	Dandong, Liaoning	Unknown
D04	Hongfushi	Unknown	Dandong, Liaoning	Unknown
D05	Xiquedengmei	Unknown	Dandong, Liaoning	Unknown
D06	Fenzhenzhu	Unknown	Dandong, Liaoning	Unknown
D07	Fentianhui	Unknown	Dandong, Liaoning	Unknown
G01	Bailing	Summer	Jiashan, Zhejiang	Japan
G02	Cuishan	Summer	Jiashan, Zhejiang	Japan
G03	Dahong	Summer	Jiashan, Zhejiang	Japan
G04	Shouguangguan	Summer	Jiashan, Zhejiang	Japan
G05	Shouguangzhiyu	Summer	Jiashan, Zhejiang	Japan
G06	Xiaotian	Summer	Jiashan, Zhejiang	Japan
G07	Qufangzhijing	Summer	Jiashan, Zhejiang	Japan
G08	Fushiying	Summer	Jiashan, Zhejiang	Japan
G09	Bikong	Summer	Jiashan, Zhejiang	Japan
G10	Riguang	Summer	Jiashan, Zhejiang	Japan
G11	Yingzhizhu	Summer	Jiashan, Zhejiang	Japan
G12	Rizhaozhiguang	Summer	Jiashan, Zhejiang	Japan
G13	Xinriguang	Summer	Jiashan, Zhejiang	Japan
M01	Yuling	Hairy	Wuxi, Jiangsu	China
M02	Meiguiliuqiuhong	Hairy	Wuxi, Jiangsu	China
M03	Wanlihong	Hairy	Wuxi, Jiangsu	China
M04	Guoqihong	Hairy	Wuxi, Jiangsu	China
M05	Taoyuan	Hairy	Wuxi, Jiangsu	China
M06	Qianchongdazi	Hairy	Wuxi, Jiangsu	China
PJ003	Turuimeigui	East	Wuxi, Jiangsu	Unknown
PJ006	Hongshanhe	East	Wuxi, Jiangsu	Unknown
PJ007	Zigaoyu	East	Wuxi, Jiangsu	Unknown
PJ010	Yanzhi	East	Wuxi, Jiangsu	Unknown
PJ012	Wucaibingfen	East	Wuxi, Jiangsu	Unknown
PJ013	Xiayijin	East	Wuxi, Jiangsu	Unknown
PJ014	Danxiuyu	East	Wuxi, Jiangsu	Unknown
PJ021	Caiwubao	East	Wuxi, Jiangsu	Unknown
PJ025	Lulaihong	East	Wuxi, Jiangsu	Unknown
PJ026	Liulangwenying	East	Wuxi, Jiangsu	Unknown
PJ034	Fendie	East	Wuxi, Jiangsu	Unknown
PJ044	Hongyi	East	Wuxi, Jiangsu	Unknown
PJ052	Yanyanghong	East	Wuxi, Jiangsu	Unknown
PJ058	Tianguinu	East	Wuxi, Jiangsu	Unknown
PJ060	Xueyafulun	East	Wuxi, Jiangsu	Unknown
PJ061	Hongzhuangsuguo	East	Wuxi, Jiangsu	Unknown
PJ065	Duanyang	Summer	Wuxi, Jiangsu	Unknown
PJ068	Diezhao	East	Wuxi, Jiangsu	Unknown
PJ069	Wuzi	East	Wuxi, Jiangsu	Unknown
PJ072	Huodeguo	East	Wuxi, Jiangsu	Unknown
PJ073	Xiaojiao	East	Wuxi, Jiangsu	Unknown
PJ076	Meihuarong	East	Wuxi, Jiangsu	Unknown
PJ077	Boyun	East	Wuxi, Jiangsu	Unknown

To be continued

Table 1

ID	Name	Type	Collected location	Origin
PJ086	Rizhichu	East	Wuxi, Jiangsu	Unknown
PJ111	Taobanzhusha	East	Wuxi, Jiangsu	Unknown
PJ113	Guoqihong	East	Wuxi, Jiangsu	Unknown
PJ135	Dantaobai	East	Wuxi, Jiangsu	Unknown
X01	Zhoubianyinhong	Summer	Wuxi, Jiangsu	Unknown
X02	Xueliqing	Summer	Wuxi, Jiangsu	Unknown
X03	Zhenruzhiyue	Summer	Wuxi, Jiangsu	Unknown
X05	Zijinlian	Summer	Wuxi, Jiangsu	Unknown
X06	Taobanshaoyao	Summer	Wuxi, Jiangsu	Unknown
X08	Yuling	Summer	Wuxi, Jiangsu	Unknown
X09	Aiguo	Summer	Wuxi, Jiangsu	Unknown
X10	Qiuyue	Summer	Wuxi, Jiangsu	Unknown
X11	Huabao	Summer	Wuxi, Jiangsu	Unknown
X12	Zichanghua	Summer	Wuxi, Jiangsu	Unknown
X13	Xiahong	Summer	Wuxi, Jiangsu	Unknown
X14	Chundexiang	Summer	Wuxi, Jiangsu	Unknown
X15	Xiangpanzhiyue	Summer	Wuxi, Jiangsu	Unknown
X16	Honglian	Summer	Wuxi, Jiangsu	Unknown
Y1	*R. simsii*	Species	Lushan, Jiangxi	China
Y2	*R. simsii*	Species	Lushan, Jiangxi	China
Y3	*R. simsii*	Species	Lushan, Jiangxi	China
Y6	Unknown	Unknown	Wuxi, Jiangsu	Unknown
ZX040	Baixue	East	Wuxi, Jiangsu	Unknown
Subgenus *Hymenanthes*				
Y5	*R. denudatum*	Species	Lushan, Jiangxi	China

transferred to a fresh tube. The DNA was precipitated with 4 ml of ice-cold isopropanol and 1.2 mol/L NaAc and then centrifuged (10000×*g*, 10 min, 4 °C). The pellet was washed with ethanol (70%), dried, and dissolved in 0.6 ml TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0). The solution was incubated for 1 h at 37 °C with the addition of 1.5 μl RNase (10 mg/ml, TaKaRa) and then extracted with phenol (saturated, pH 8.0)/chloroform/isoamylalcohol (25:24:1 v/v/v) of the same volume several times until the middle layer disappeared. DNA was precipitated by the addition of 1.2 mol/L NaAc and about 2.5 times the volume of the final supernatant of ice-cold (−20 °C) ethanol for half an hour and then centrifuged (10000×*g*, 10 min, 4 °C). The residue was washed with ethanol (70%) twice, dried, and dissolved in TE buffer. DNA concentration and quality were checked against to a standard series of lambda-DNA (TaKaRa) after electrophoresis on 1% (10 g/L) agarose gel.

2.3 AFLP reactions

The AFLP method was performed essentially as described by Vos *et al*. (1995) with minor modifications. Genomic DNA (generally 200 to 300 ng) was digested with 0.5 μl each *Eco*RI and *Mse*I (10 U/μl, New England Biolabs (NEB)) in a final volume of 20 μl containing 2 μl 10× NE buffer *Eco*RI (NEB) and 0.2 μl bovine serum albumin (BSA) (10 mg/ml, NEB). After mixing, DNA samples were incubated first at 37 °C for 3 h and then at 65 °C for 20 min to denature the enzymes. Ligation was performed by mixing 5 μl digested DNA, 5 μl ligation solution (5 pmol *Eco*RI-adapter, 50 pmol *Mse*I-adapter, 0.4 μl T4 DNA ligase (5 U/μl, Fermentas), and 1 μl 10× T4 DNA ligase buffer (Fermentas)). Afterwards, the mixture was incubated first at 22 °C for 5 h and then at 65 °C for 15 min.

A pre-selective polymerase chain reaction (PCR) reaction mixture contained 1 μl ligased DNA, 2.0 μl 10× PCR buffer (Mg^{2+} free, TaKaRa), 1.6 μl deoxyribonucleoside triphosphates (dNTPs) (2.5 mmol/L, TaKaRa), 1.6 μl $MgCl_2$ (25 mmol/L, TaKaRa), 0.2 μl Taq polymerase (5 U/μl, TaKaRa), 8 pmol each of *Eco*RI and *Mse*I adapter-directed primers with non-selective nucleotide (Invitrogen) to yield a total volume of 20 μl. Preamplification was performed with the following profile: 94 °C for 3 min, 20 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min,

followed by 72 °C for 8 min and holding at 10 °C. After checking for the presence of fragments around 100 to 1000 bp in length by agarose electrophoresis, the PCR product mixture was diluted 10-fold with distilled water and used as a template for selective PCR amplification. Three pairs of fluorescent-labelled *Eco*RI-*Mse*I primer combinations (PCs) were used: *Eco*RI-ACT/*Mse*I-CAT (PC1), *Eco*RI-AAG/*Mse*I-CAT (PC2), and *Eco*RI-AAG/*Mse*I-CTA (PC3). Selective amplification was performed with the following touchdown PCR profile: 94 °C for 3 min, 12 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, with the annealing temperature decreasing by 0.7 °C per cycle, followed by a further 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, an extension at 72 °C for 10 min, and holding at 10 °C. All amplifications were conducted using a C1000 Thermal Cycler (Bio-Rad). Five microlitres of each selective PCR product with different fluorescent labels and 20 μl distilled water were pooled together, and 3 μl of this mixture was run on an ABI 3130 capillary sequencer (Applied Biosystems Inc.) using GS-500 LIZ (Applied Biosystems Inc.) as an internal size standard, and POP-7 polymer (Applied Biosystems Inc.).

2.4 Band scoring and data analysis

Fragments were analyzed automatically in GeneMapper V4.0 (Applied Biosystems Inc.) and scored from 70 to 480 bp. Bin width was set to 0.8 (Meudt and Bayly, 2008); fragments were scored as identical if the difference in size was less than 0.8 bp. In order to minimise the effect of low-intensity background peaks (noise), filters for fragment selection were set (de Riek *et al*., 1999) towards average signal intensity (I >120) and fragment frequency (f >0.15). All profiles were exported from GeneMapper to Microsoft Excel and converted into a binary matrix indicating the presence and absence of fragments for all individuals ("1" for present, "0" for absent).

The effective number of alleles, expected heterozygosity and Shannon's information index of different primer combinations were calculated using the software package of POPGENE Version 1.32 (Yeh and Boyle, 1997). A genetic distance matrix was generated based on Dice (Nei and Li, 1979) coefficients calculated from the binary matrix, used to construct a neighbour joining (NJ) tree, and then bootstrapped for 100 replicates in Treecon V1.3b (van de Peer and de Wachter, 1994). Principal coordinate analysis (PCO) was performed essentially as described by Meudt and Bayly (2008) based on Dice distances using NTSYS-pc software (Rohlf, 1998).

3 Results

3.1 Effectiveness of the primer combinations

Two (i.e., PC1 and PC3) of the three primer combinations we used had been tested previously by de Riek *et al*. (1999) and Scariot *et al*. (2007a; 2007b). These primers generated almost the same number of AFLP fragments, with PC2 generating a little more than PC1 and PC3. Our analysis method yielded an average of 136 fragments per primer pair, adding up to a total of 408 polymorphic fragments (Table 2), all of which were used in the analysis. The average values of expected heterozygosity and Shannon's information index were 0.3395 and 0.5153, respectively. A visual check of the AFLP profiles showed the presence of a few polymorphic bands between the initial cultivar and its bud sports, such as 'Hongshanhu' and 'Fenshanhu', and 'Zihudie' and 'Baihudie'.

3.2 Cluster analysis

An NJ tree was reconstructed based on the 408 AFLP characters using *R. denudatum* as an outgroup

Table 2 Characteristics of different AFLP primer combinations

Primer combination	Code	Number of markers		N_e	H_e	I
		Total	Per genotype			
*Eco*RI-ACT/*Mse*I-CAT	PC 1	130	57 (22–78)	1.5246	0.3226	0.4952
*Eco*RI-AAG/*Mse*I-CAT	PC 2	148	65 (32–90)	1.5655	0.3422	0.5189
*Eco*RI-AAG/*Mse*I-CTA	PC 3	130	55 (42–85)	1.5949	0.3534	0.5312
Average		136	59	1.5619	0.3395	0.5153
Total		408	177			

N_e: effective number of alleles; H_e: expected heterozygosity; I: Shannon's information index

(Fig. 1), and ten main lineages were evident. Of the three individuals of *R. simsii* with different flower colours collected from Lushan Botanical Garden, two (Y1 and Y2) grouped with two unknown cultivars (D1 and D2) maintained in Dandong and one (Y3) grouped with an unknown genotype (Y6) found in Wuxi. Among the 14 West azaleas, 12 grouped into three lineages and two (B4 and B12) grouped with Summer azaleas. In the remaining six lineages, those marked with A, B, C, D, E, and F were found in two major branches. Lineage A was comprised of 40 East azaleas collected from both Wuxi and Jiashan. In this lineage, cultivar 'Hongshanhu' (C13) and its bud sport 'Fenshanhu' (C14) grouped with 'Hongshanhe' (PJ006), which as appears identical to 'Hongshanhu'. Lineage B was sister to lineage A and was comprised of three East azaleas, nine Hairy azaleas, and one unknown genotype (D4) from Dandong. Lineage C included seven East azaleas. Lineage D, which included three Hairy azaleas and one Summer azalea, had fewer genotypes compared with lineages A, B, E, and F. Lineage E, formed mainly by East azaleas, was

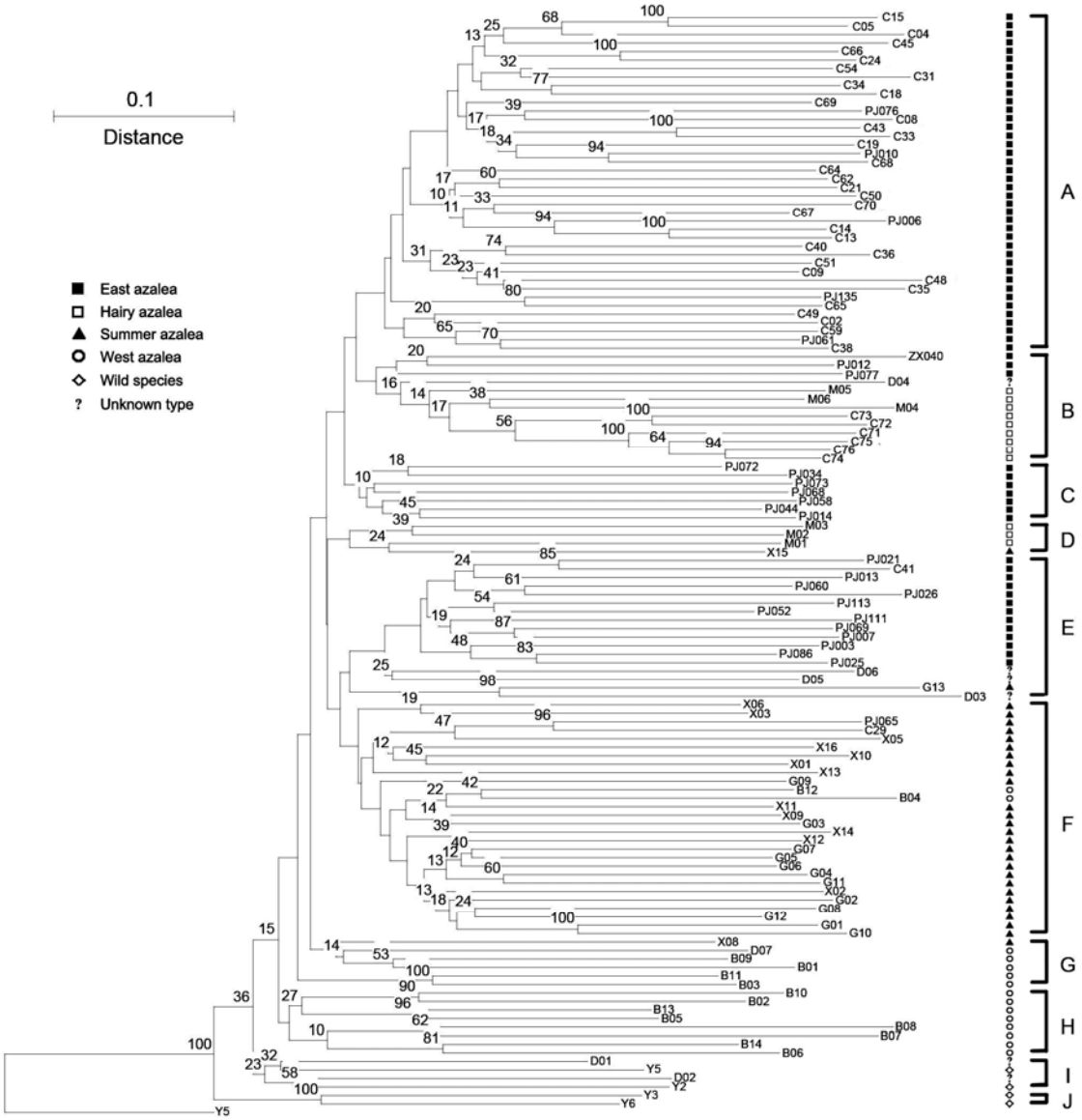

Fig. 1 AFLP phylogeny based on neighbour joining (NJ) tree analysis of Dice distance of a binary matrix of 408 characters and 130 genotypes in Treecon

Numbers above branches represent bootstrap support values based on 100 replicates

sister to lineage F, which contained 26 Summer azaleas and two West azaleas (B4 and B12).

3.3 Principal coordinate analysis

PCO of the AFLP data was performed using Nei and Li (1979) distances in NTSYS. The first three axes explained 8.6%, 7.0%, and 4.2% of the variation in the data. Consistent with the NJ tree, a two-dimensional plot of the first two axes (15.6% total variation explained) showed that lineages A, B, and E could be generally separated from each other (Fig. 2), except that a kind of continuum between lineages A and B and lineages A and E was observed. PCO findings were also highly congruent with phylogenetic analysis of the Summer azaleas, which formed a clear cluster away from the other genotypes. Cultivars mostly grown in Dandong (marked with "D") appeared in different areas in the PCO plot, just as the NJ tree showed. In most of the West azalea cultivars, as well as several East azaleas collected from Wuxi and wild species, however, NJ analysis suggested that they should group into five lineages; PCO analysis did not differentiate these cultivars clearly.

4 Discussion

Although the AFLP technique is highly polymorphic and reproducible, with the error rate per locus being estimated at only 2% to 5% (Bonin *et al*., 2007; Meudt and Clarke, 2007), several factors could bring about genotyping errors, including technical aspects and subjective errors in profile scorings. Therefore, to maximise the signal-to-error ratio from raw data, optimisation of parameters, such as peak height thresholds, bin widths, and the minimum fragment size that is scored, is very important (Meudt and Clarke, 2007). de Riek *et al*. (1999) established criteria for AFLP marker selection on evergreen azaleas by restriction of the marker frequency (f >0.15) and average signal intensity (I >120). These criteria proved to be very useful in the AFLP band scoring of azaleas by Scariot *et al*. (2007a; 2007b) and our own study.

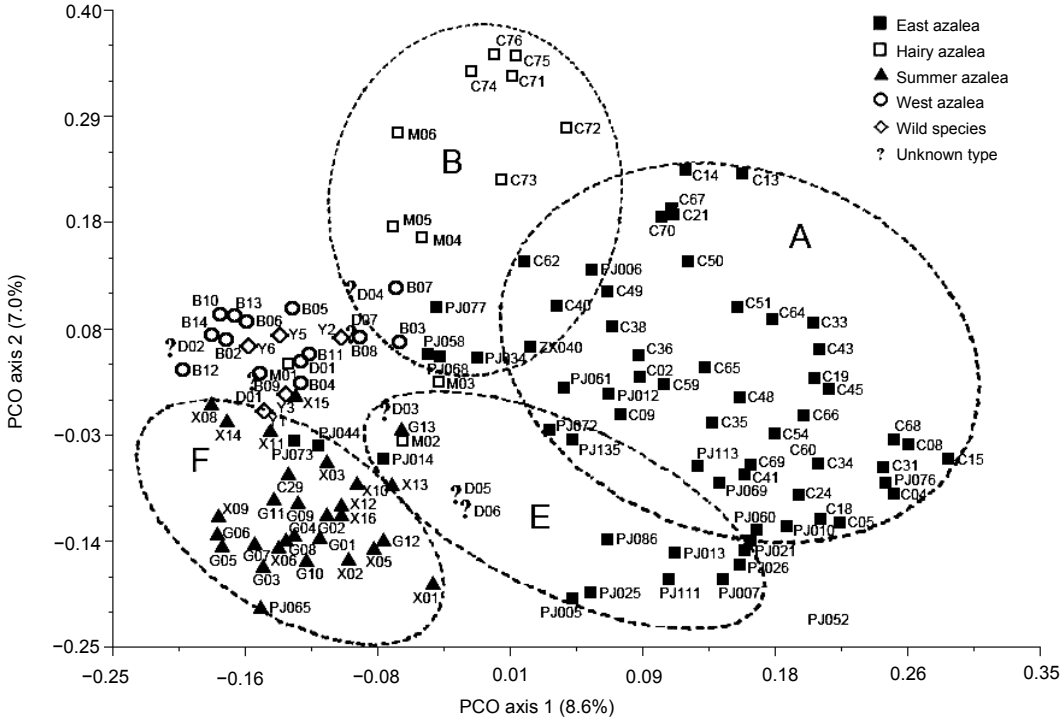

Fig. 2 Principal coordinate analyses of the Nei and Li distance matrix of the AFLP binary of 408 characters and 130 genotypes using NTSYS

Capitalised letters 'A', 'B', 'E', and 'F' refer to the main lineages marked on the NJ tree

The bootstrap values that support the main lineages were quite low, so the nodes these lineages derived from were not so effectively stable, especially in the big branch which was formed by lineages A to G. The smaller branches were supported by high bootstrap values. This phenomenon was also found in the research on genetic relationships in *Brassica rapa* (Zhao *et al*., 2005) and *Rosa* (Koopman *et al*., 2008). Both authors supposed that when a large number of complex cultivars or accessions were included in the research, the bootstrap values supporting main groups could be low. Interestingly in our research, the lineages with low bootstrap values often coincided with cultivar groups, just as Zhao *et al*. (2005) discovered in his research. It is revealed that most polymorphisms do not contribute to the phenotypic variation (Zhao *et al*., 2005).

The NJ tree reconstructed based on AFLP fragments supports the view that cultivars within the Summer group are quite distinct from other cultivars in the four-group classification system used in the present work. Even better, in this work, cultivars marked with "X" and reserved in Wuxi, grouped well with those cultivars (marked with "G") newly imported from Japan. Although the West azaleas we studied did not appear in one branch, they remained very similar to each other, in accordance with the four-group classification system.

The NJ tree tells a different story about the other cultivars we studied. The cultivars called 'East azaleas' did not group together but distributed over four branches, especially those collected from Wuxi, which usually have larger flowers and leaves than East azaleas collected in Jiashan. Thirteen East azalea cultivars (lineage E) were similar to the Summer group, seven cultivars (lineage C) out of the big branch formed by lineage A and B, and three cultivars in lineage B. Thus, the group 'East', which is the biggest group in the traditional classification system, is not defined appropriately and should be subdivided further.

Both the NJ tree and PCO reveal that reservation of locations is not related to the relationships of evergreen azaleas in China. Individuals with the same name but obtained from different places were always clustered together with high bootstrap values, such as 'Duanyang' (C29 and PJ065), 'Caiwubao' (C41 and PJ021), and 'Yanzhi' (C68 and PJ010). The seven cultivars obtained from Dandong (marked with "D") scattered over the entire dendrogram, or 2D plot, indicating that the relationships between cultivars in Dandong are probably more complicated than originally thought, likely due to the long cultivation and hybridisation history of azaleas in Dandong.

Of all the cultivars included in this study, we find out that the Summer group in China probably belongs to the Satsuki hybrids, for their coincidence on the special blooming time and leaf morphology. The West group most likely belongs to Belgian hybrids, according to the cultivars' origin. The plausible links between the East group and Kurume hybrids are a little complicated. Most of the East azaleas gathered in lineage A, which have small leaves and hose-in-hose flowers, are probably typical Kurume azaleas. The rest of the cultivars need further study and more reference accessions to compare with.

The confusing classification of azaleas in China appears to be the consequence of a more general and widespread problem of nomenclature also existent in Europe (Scariot *et al*., 2007b) that stems from confusion on primary accessions brought to China. Future studies may be carried out with addition of larger numbers of species, varieties, and cultivars assumed to be ancestors of evergreen azaleas to reveal the genetic conformity between reference accessions and the Chinese gene pool.

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Compliance with ethics guidelines

Hong ZHOU, Jin LIAO, Yi-ping XIA, and Yuan-wen TENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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